AIRWAY REMODELLING AND TRANSFORMING GROWTH FACTOR (TGF)-β SUPERFAMILY SIGNALLING IN ALLERGEN-INDUCED ASTHMA

A thesis submitted for the degree of

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Statement of contribution

My contribution to the work described in this thesis is stated below.
Under the supervision of Prof. D.S Robinson and Prof. A.B. Kay:

I undertook and established the study design and protocols (including adaptation of protocols for methacholine and allergen challenge) as well as monitoring of volunteer safety.

I was responsible for correspondence with the ethics committee in the three year study period.

I undertook or assisted in the recruitment of subjects for the clinical studies alongside the study research nurse, Mrs Maxine Aizen. I performed all methacholine and allergen challenges.

I performed bronchoscopy (70%) together with all sample collection and processing.

I performed all immunohistochemistry, *in-situ* hybridisation and confocal microscopy work. Assistance with tissue cutting and cell counting was provided by Miss Julia Barkans (as a blinded observer).

I was responsible for all data analysis and statistical evaluation (other than the mixed modelling data analysis performed by the NHLI Institutional statistician).

Harsha H Kariyawasam

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Abstract

Bronchial biopsies obtained at baseline, 24 hours and 7 days after allergen challenge from mild atopic asthmatics were evaluated to follow the initiation and resolution of allergen-induced inflammation and remodelling in relation to AHR. Further, the expression patterns of the TGF-β Superfamily ligands (TGF-β_{1-3}, activin-A, BMP-2, BMP-4 and BMP-7) and their respective signalling pathway Type II receptors, Type I receptors and signalling Smad proteins were defined in baseline asthma and after allergen provocation in comparision with normal volunteers. In the dual asthmatic response (DAR) group AHR was markedly increased 24 hours and 7 days after allergen challenge. Reticular basement membrane (RBM) tenascin expression was elevated at 24 hours and returned to baseline levels at 7 days. RBM procollagen III, cellular procollagen I, fibroblast HSP-47 and α-smooth muscle actin expression were all higher at 7 days when compared to baseline. At 24 hours eosinophils, macrophages, neutrophils and $CD3^{+}$ T cells were all significantly increased but returned to baseline levels by 7 days. The asthmatic airway demonstrated increased TGF-β_3 and activin-A expression. In asthma, allergen challenge was associated with sustained up-regulation of BMP-7 in inflammatory cells at 24 hours and 7 days and in epithelium at 7 days. Eosinophils were identified as a major source of BMP-7. Epithelial expression of BMPRII, ActRIIA and ActRIIB was significantly less in asthma than in normal volunteers. ActRIIA epithelial expression was significantly increased at 24 hours and 7 days after allergen challenge. Epithelial expression of ALK-1, ALK-2, ALK-5 and ALK-6 expression in asthma was significantly less when compared to the normal airway. Receptor down-regulation was demonstrated for ALK-5 24 hours post-allergen whilst up-regulation was seen for ALK-1, ALK-2 and ALK-6 at 24 hours and at 7 days post-allergen. Inflammatory-like cells expressing ALK-1 and ALK-4 were less in the asthmatic airway whilst ALK-3 expressing cells were increased in comparison to the normal airway. ALK-1 (24 hours and 7 days), ALK-3 (24 hours) and ALK-4 (24 hours) expressing submucosal inflammatory-like cells were increased post-allergen. The baseline asthmatic airway demonstrated decreased expression of Smad7. Increased expression of Smad7 was evident 24 hours post-allergen. Increased expression of pSmad2 at 24 hours and pSmad1/5 expression at 24 hours and 7 days was evident. Increased airway remodelling remains associated with AHR at a time point when cellular inflammation returns to baseline. In asthma there is markedly altered expression of the TGF-β Superfamily signalling pathways components compared to normal airways.

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Dedication

.....To my Mother, Mal, Pri, Nilmini and Kuimari
For leading me into intellectual pursuits....

Publications related to thesis

Peer review journals

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Safety and tolerability of three consecutive bronchoscopies in mild asthmatic volunteers after allergen challenge (in press Thorax, 2007)

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List of abbreviations

ADAM33-A disintegrin and metalloprotease33

AHR- Airway hyperresponsiveness

ALK-Activin-like kinase

AP-1: activator protein-1

APC-Antigen presenting cell

AM-Alveolar macrophages

α-SMA-Alpha smooth muscle actin

ASM-Airway smooth muscle

BAMBI-BMP and activin membrane bound inhibitor

BAL-Bronchoalveolar lavage

BM-Basement membrane

BMP-Bone morphogenetic protein

BU-Biologic Units

CBP-CREB-binding protein

CREB: cyclic AMP response element binding protein

CE-Contractile element

COPD-Chronic obstructive pulmonary disease

CTGF-Connective tissue growth factor

Cys-Cysteinyl

DC-Dendritic cell

EAR-Early asthmatic reaction

ECM-Extracellular matrix

EGF-Epidermal growth factor

EGFR-Epidermal growth factor receptor

EMTU-Epithelial mesenchymal trophic unit

ER-Endoplasmic reticulum

ERK- Extracellular signal-regulated kinase

FEV_1-Forced expiratory volume in 1 second

FFC-Fibril forming collagens

FGF-Fibroblast growth factor

FN-Fibronectin

GAG-Glycosaminoglycans

GDF-Growth differentiation factor

GM-CSF-Granulocyte macrophage colony stimulating factor

GR-Glucocorticoid receptor

HAC-Histone acetylase

HB-EGF-Heparin binding epidermal growth factor-like growth factor

HDAC-Histone deacetylase

HSP-Heat shock protein

IBD-Inflammatory bowel disease

IFN-Interferon

IgE –Immunoglobulin E

IL-Interleukin

ISH-In situ hybridisation

KO-Knock-out

LAP-Latency associated peptide

LAR-Late asthmatic reaction

LM-Light microscopy

LO-Lipooxygenase

LPR-Late phase response

LPS-Lipopolysaccharide

LTBP-Latent TGF-ß binding protein

LT-Leukotrienes

Mø-Macrophages

MAPK-Mitogen activated protein kinase

MBP-Major basic protein

MC-Mast cell

MHC-Major histocompatibilty complex

MIF-Müllerian inhibiting substance

MMP-Matrix metalloproteinase

mRNA-messenger ribonucleic acid

NES-Nuclear export signal

NF-κB: nuclear factor κB

NGF-Nerve growth factor

NLS-Nuclear localisation signal

PAR-Protease activated receptor

PC-Provocative concentration

PCNA-Proliferating cell nuclear antigen

PDGF-Platelet derived growth factor

PECs-Parallel elastic components

PEF-Peak expiratory flow

PGs-Proteoglycans

RANTES-Regulated upon activation normal T cell expressed and secreted

RAST-Radioallergoabsorbent test

Raw-Airway resistance

RBM-Reticular basement membrane

RER-Rough endoplasmic reticulum

Rh-recombinant

RTK-Receptor tyrosine kinase

SAD-Smad activation domain

SARA-Smad anchor for receptor protein

SBE-Smad binding element

SCF-Stem cell factor

SEC-Series elastic components

SER-Single early response

sGaw-Specific airways conductance

SM-Smooth muscle

Smurf-Smad ubiquitination regulatory factor

SPT-Skin prick test

STAT 6-Signal transducer and activator of transcription

TCR-T cell receptor

TLC-Total lung capacity

TGF-Transforming growth factor

TIMP-Tissue inhibitor of matrix metalloproteinases

TNF-Tumour necrosis factor

TSP-1-Thrombospondin

VEGF-Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Introduction to the thesis

Asthma is increasing in world-wide prevalence with the chronicity of the disease representing a considerable economic burden. At present the therapeutic focus is predominantly with anti-inflammatory strategies but less than 50% of asthmatics report adequate control with current therapy developed to target specific components of inflammation. Asthma frequently shows progression of airway obstruction and the role of structural change of the airway wall, termed remodelling, is rapidly gaining importance. The exact components of airway remodelling that contribute to the symptoms and chronicity of disease is not identified. The relationship between the inflammatory response, remodelling events and airway physiological changes remain to be defined.

There are increasing reports of the safety of bronchoscopic airway sampling in asthma and it is therefore possible to develop more longitudinal studies in human asthma whereby the disease is followed in a time-course dependent fashion, where inflammatory and remodelling changes are examined in relation to symptoms, lung function and airway hyperresponsiveness (AHR). Only by differentiating changes contributing to disease pathogenesis from those that are an appropriate healing response in an injured airway can remodelling become a therapeutic target.

Aberrant signalling by the Transforming Growth Factor (TGF)-β Superfamily is now increasingly recognised as driving the remodelling process, but the exact expression pattern and the functional consequences of TGF-β Superfamily signalling in asthma remains undefined. There is an urgent need to explore molecular and cellular pathways of remodelling as exploring such mechanisms may offer the prospect of developing alternative therapeutic strategies aiming to regulate or even reverse abnormal airway structural change.

In this thesis a time course bronchoscopic study of allergen-induced asthma is presented. The airway physiological response to allergen challenge and its relationship to inflammation and remodelling are examined. The pattern of expression and signalling via TGF-β Superfamily ligands in asthma is compared to normal volunteers. Modulation of TGF-β Superfamily signalling in response to allergen-induced airway injury is then examined. Finally, the safety and tolerability data of three consecutive bronchoscopies is presented with view to planning future longitudinal studies in asthma.

1.2 Asthma

1.2.1 Introduction and clinical significance

Asthma is defined as a chronic inflammatory disease of the airways characterised by variable airflow obstruction and associated airway symptoms (WHO/NHLBI Workshop Report, 1995). Airway hyperresponsiveness (AHR) is the term used to desribe this abnormal and exaggerated tendency of an airway to narrow (Cockcroft *et al.* 1977). AHR is a ubiquitous feature of asthma and can serve as an indicator of 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.

Currently eight million people in the United Kingdom (an estimated 13 % of the population) have a diagnosis of asthma and around 5.1 million are treatment dependent (Hallsworth *et al.* 2003). The prevalence of asthma has increased four-fold in the last two decades (Toelle *et al.* 2004) and is consistent with an epidemic. Despite the estimated therapeutic cost of £850 million a year to the National Health Service, 74% of these asthmatics still experience symptoms. Approximately 5-10% of these patients remain treatment refractory and experience severe debilitating disease accounting for a 30% of the therapeutic cost. In addition nearly 1300 asthma deaths a year still occur (Sidebotham & Roche 2003). Control of severe asthma is often not possible with the current treatments available as stated in the Global Initiative for Asthma (GINA) guidelines (2004, NIH publication no. 02-3659). There is therefore an urgent unmet clinical need in asthma.

1.2.2 Epidemiology of asthma

Asthma prevalence has increased in areas where its prevalence has previously been low whilst overall the prevalence has stabilised or even slightly decreased in areas where prevalence has been high as shown by the recent International Study of Asthma and Allergies in Childhood (ISAAC) report (Asher *et al.* 2006).Whilst it is appreciated that genetic susceptibility is an important disease determinant of asthma the marked increase in the prevalence of the disease in such a short time period cannot be explained on the basis of newly acquired genetic changes. It is more likely that new environmental influences have unmasked pre-existing individual genetic susceptibility in populations. Asthma prevalence rates are highest in affluent societies with a temperate climate whilst lowest rates are seen in rural, economically

underdeveloped states. Adoption of a more affluent lifestyle is associated with increased asthma prevalence and the current increasing worldwide trend of allergic sensitisation may be a reflection of such lifestyle changes (Weinberg 2000). Epidemiological studies provide evidence of the major role of environmental factors in asthma, although familial clustering of asthma confirms the importance of genetic predisposition. Indeed if one considers that the exposure of affluent communities to environmental determinants of asthma is broadly similar and widespread it is probable that it is the genetic factors that determine individual disease risk.

1.2.3 Asthma phenotypes

There are several asthma phenotypes that present with these characteristic disease features. Although exact disease pathogenic mechanisms may differ between such phenotypes, the airway pathology is nevertheless characteristic Th2-mediated airway inflammation (Bentley *et al.* 1992b; Bentley *et al.* 1992a). This inflammatory process is mostly confined to conducting airways and is multi-cellular involving predominantly $CD4^+$ T cells, eosinophils and mast cells.

The term 'atopy' was first proposed by Coca and Cooke in the early 1920s as a new classification of hypersensitivity (Coca & Cook 1923). Atopy is defined as the hereditary predisposition to produce IgE specific for proteins (allergens) encountered at mucosal surfaces. This can be associated with elevated generalised total IgE synthesis. In atopic or extrinsic asthma sensitisation is associated with airway recognition of allergen and the generation of a specific Th2 cytokine mediated inflammatory response. The majority of patients with asthma are atopic (Platts-Mills 2001).

The term intrinsic asthma is used to describe individuals who often present with late-onset asthma where no sensitisation to environmental allergens can be demonstrated and who have normal total serum IgE (Corrigan 2004). Some forms of late-onset asthma may have an occupational cause, secondary to work-place non-IgE sensitisation (Malo 2005). Asthma here often reverts if the offending exposure is abolished but prolonged exposure can lead to the establishment of a severe and unremitting disease phenotype even in the absence of any further occupational exposure. Whilst the IgE dependence of occupational asthma is not always shown, there is evidence that local airway IgE synthesis, possibly to self-antigen, may drive

the disease in intrinsic asthma (Ying *et al.* 2001). Importantly the immunopathlogy of both types of asthma are very similar to atopic asthma.

1.2.4 Airway hyperresponsiveness (AHR)

An individual with AHR demonstrates increased sensitivity of the airway, with narrowing occuring with low concentrations of stimulants when compared to normal individuals. The airway in asthma will narrow more than the normal airway can be induced to narrow even at the highest amounts of the stimulus. This tendency for excessive airway contraction is termed maximal airway narrowing. Whilst the increased airway sensitivity leads to troublesome everyday symptoms such as cough and chest tightness to what may be sometimes trivial stimuli such as tobacco smoke and perfumes, it is the tendency to excessive airway narrowing that will lead to severe life threatening deterioration and sometimes asthma death

The exact mechanisms that lead to AHR remain undefined (Cockcroft & Davis 2006). Most asthmatics demonstrate baseline AHR that is relatively constant. Superimposed on this is a more variable or episodic component of AHR, that can be induced in response to triggers such as allergen or infection that is associated with an influx of inflammatory cells. It is therefore possible that there are probably two separate AHR components, each with different mechanisms of development, which summate for the development of the asthma phenotype. Currently, much of the evidence relating to the pathogenesis of AHR remains circumstantial. It is suggested that the episodic and variable component of AHR is related to acute airway inflammatory events in response to disease triggers. It is likely that the symptoms in patients with mild and episodic asthma are a result of this variable component of AHR. More severe disease is associated with more persistent AHR that is increasingly refractory to anti-inflammatory therapy, suggesting non-inflammatory pathways may be implicated, possibly pathways related to airway remodelling. Such a line of thinking may explain why corticosteroid therapy is effective in reducing inflammation and controlling symptoms in the majority of mild to moderate asthmatics, yet AHR is not completely abolished despite reductions in cellular inflammation (Lundgren *et al.* 1988; Duddridge *et al.* 1993; Adelroth *et al.* 1990).

1.2.5 Airway obstruction (FEV_1)

Variation in the forced expiratory volume in 1 second (FEV_1) is routinely used in the clinical setting to diagnose and monitor asthma, and to subsequently follow asthma control. Measurement is made by taking a deep breath to maximally fill the lungs (total lung capacity or TLC) and exhaling as forcefully as possible into a spirometer. The total volume of air expired in the 1st second of expiration is taken as measure of FEV_1 and is an important measure of any airway obstruction. Reversibility in FEV_1 in response to short-acting β_2 agonists is often used in the diagnosis of the asthma phenotype.

1.3 Model of provoked asthma

1.3.1 Introduction

Allergen exposure in atopic asthma is associated with an inflammatory cell influx alongside an increase in AHR and bronchial obstruction (Cockcroft *et al.* 1977). The ability to provoke the disease with allergen inhalation in a controlled setting has been used as a model of the disease and as a result the IgE-mediated allergic asthma phenotype is the most frequently studied. Inhaled allergen challenge of sensitized atopic asthmatics can mimic the exacerbation of natural disease with its associated and sustained increases in AHR. This model is a valuable means by which inflammation and repair processes associated with AHR at baseline and further increases in AHR that can occur with disease provocation can be studied.

1.3.2 Principles of smooth muscle contraction

From first principles, the load that the muscle can overcome will determine the degree of shortening it can undergo. Tissue components account for the loads that limit airway smooth muscle (ASM) shortening. The contractile elements of the muscle cell must pull against the tethering loads (termed series elastic components or SECs) at the end of the muscle in order for it to shorten. The SEC can be depicted as a spring. If the spring is stiff (i.e tissue with high elasticity), then the degree of shortening in the ASM cell is limited. If the elasticity is low then the muscle will easily shorten to an exaggerated degree. In the airways the SECs is primarily the cartilage and in small airways the parenchymal tissue interaction. Importantly there is evidence for increased elastin degradation in asthma (Bousquet *et al.* 1992). However the contractile elements of the muscle cell are prevented from contraction by the presence of ECM elements that run parallel to the smooth muscle cells (termed parallel elastic

components or PECs) and prevent excessive ASM shortening. Alterations in PECs may allow excessive ASM shortening in asthma.

1.3.3 Quantification of AHR

The principle involved in measuring AHR is to administer a stimulus at precisely determined successive doses measuring the response at each step. Thus a dose-response relationship is obtained and the response can be quantified. A wide range of both pharmacological and physical stimuli are available and can be divided into direct and indirectly acting agents. Direct acting agents (i.e methacholine and histamine) induce bronchoconstriction by affecting the smooth muscle cells in contrast to indirect agents such as adenosine affect inflammatory mediator release and neural pathways which then act on smooth muscle secondarily inducing bronchoconstriction. Exercise is a useful physical stimulus but here only a single stimulus can be administered on one occasion. Hyperventilation, cold air and osmotic hypertonic saline are other examples of physical stimulants. Given that 'naturally occurring' asthma is associated with indirectly acting triggers measuring AHR via indirect agent challenges may be more reflective of inflammatory cell induced AHR (van den *et al.* 2001). Measurement of AHR by direct acting agents such as methacholine or histamine may better relate to AHR generated as a result of ASM abnormalities, suggesting direct measures of AHR reflect components of AHR related to airway structural change (Cockcroft & Davis 2006).

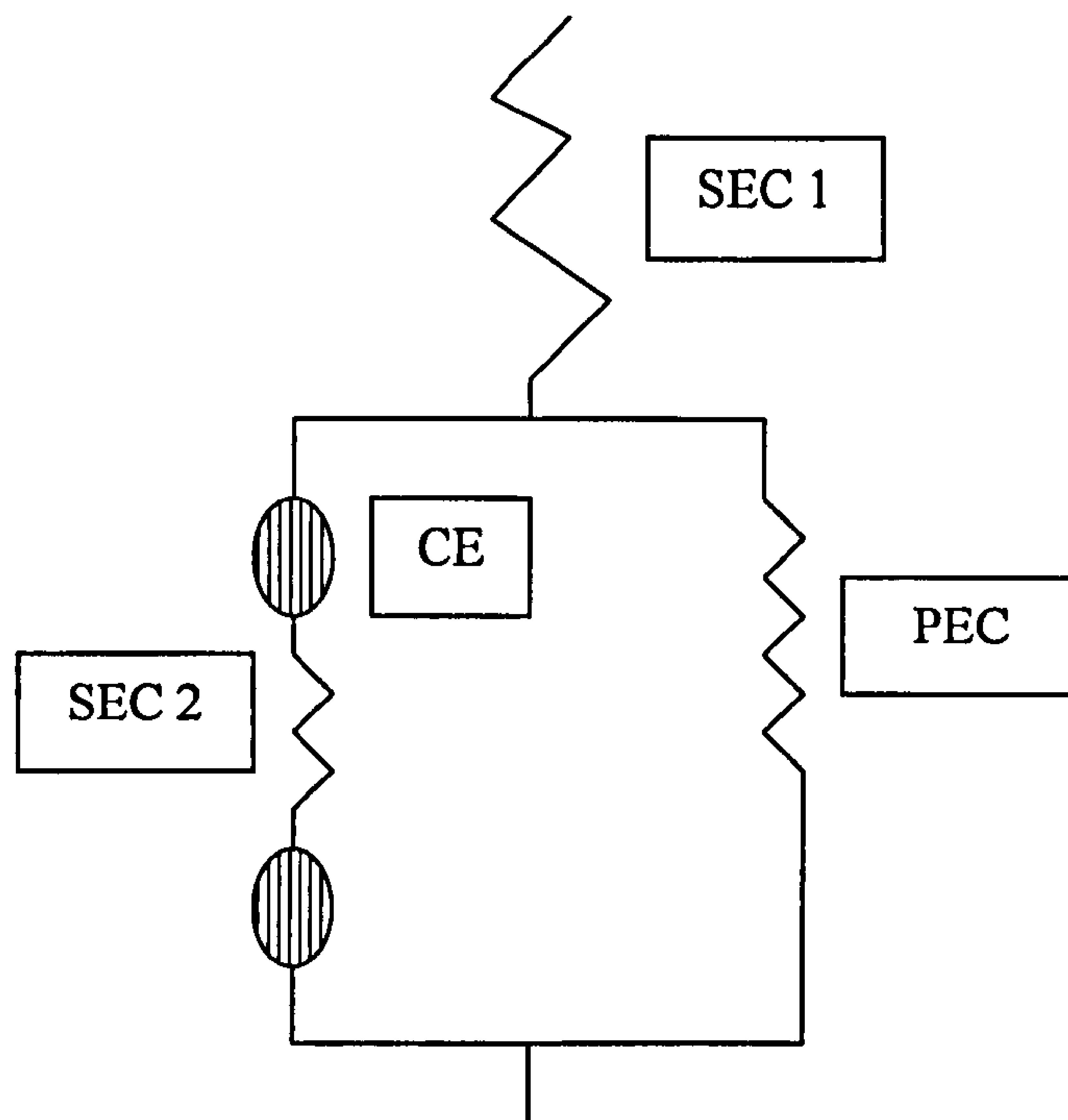


Figure 1.1 Illustration of factors that influence airway smooth muscle contraction (adapted from Schellenberg R, Asthma and Rhinitis, Eds. Busse-Holgate)

The figure illustrates a mechanical model of the factors that effect airway smooth muscle contraction. The contractile element (CE) will shorten only when it overcomes the different elastic loads conferred by the series elastic component (SEC1) located outside the smooth muscle fibre. The parallel elastic elements (PECs) lie within the muscle tissue. PECs include both intracellular smooth muscle elements and extracellular matrix components.

1.3.4 Measurement of response

Any measurement of the airway calibre such as the forced expiratory volume in one second (FEV_1), peak expiratory flow (PEF), airway resistance (Raw) or specific airway conductance (sGaw) may be used. Change in FEV_1 to an inhaled substance is the most reproducible measurement of AHR and is thus the most commonly used. It is not as sensitive for example as measuring the sGaw (specific airways conductance) and in studies where a weak stimulus is used measurements of AHR using more sensitive parameters may be used.

1.3.5 Quantification of results

The degree of AHR can be expressed in a number of ways. With agents where dose-response curves can be constructed AHR is quantified according to different properties of the curve: threshold, slope at one point or overall slope. The amount (dose or concentration) of the stimulus that causes a pre-set change in the airway parameter is most commonly used. This is most frequently expressed as the provocative concentration (PC) which produces a fall in the FEV_1 of 20% from the baseline FEV_1 value (known as the PC_{20}). The PC_{20} represents a measure of airway responsiveness. PC_{20} should be repeatable within one doubling concentration of the bronchoconstriction inducing agent.

1.3.6 Specific airway allergen challenges

Histamine and methacholine are the archetypal direct agents and are the two most common constrictor stimuli that have been studied on a population basis. Although the responses to equivalent concentrations of drugs are similar, methacholine has the advantage in that it can be increased to higher concentrations (256 mg/ml) without encountering such marked systemic side-effects. The methacholine challenge is probably the method of choice for population studies, though there are practical problems obtaining the pure agent for human administration. Methacholine is a synthetic cholinergic agonist and bronchoconstriction occurs as a direct result of agonist activity on the M_3 receptors of airway smooth muscle (Roffel *et al.* 1990).

1.3.7 Safety of allergen challenge and methacholine sensitivity testing

Before any commencement the subject must be adequately informed and the testing conducted in suitable quiet surroundings with trained personal, medical supervision and resuscitation facilities available. Adequate facilities for treatment of acute severe

asthma should be available and the subject should be physically and mentally capable of co-operating

1.3.8 Inhaled allergen challenge

Whole allergen challenge in sensitized asthmatics leads to the immediate or early asthmatic response (EAR) which is maximal within 30 minutes and resolves between 1 and 3 hours. Up to 50% of these individuals will experience a second delayed phase of bronchoconstriction termed the late asthmatic reaction (LAR) (Cockcroft *et al.* 1977). This response is defined as a fall in the FEV_1 of 15% from the baseline value and is maximal 6-12 hours after allergen challenge (Dorman *et al.* 2004) The development of a either a single early response (SER) alone or both SER and a LAR (dual asthmatic response or DAR) is generally consistent although it is believed that the development of the LAR is related to the dose of allergen given (Ihre *et al.* 1988) and possibly baseline AHR in an individual. Traditionally the LAR has been considered an all or nothing response using an arbitrarily defined definition of a fall in FEV_1 by 15% from the baseline value 3-6 hours post-allergen. In the DAR group, allergen-induced increased AHR is sustained for days and weeks (Cockcroft *et al.* 1977).

The EAR is thought be IgE and mast cell (MC) dependent, with allergen cross-linking IgE antibodies bound to the MC leading to MC degranulation with the release of histamine and cysteinyl leukotrienes that have direct stimulatory effect on airway smooth muscle. Leukotrienes are derived from arachidonic acid as a result of the action of phospholipase A_2 (PLA_2) on membrane phospholipids. Arachidonic acid is metabolised either via the cyclooxygenase pathway to produce prostaglandins, prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) or via the lipooxygenase pathway to produce leukotriene LTB_4 or the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 (Dahlen *et al.* 1980). LTC_4 and LTD_4 are potent constrictors of airway smooth muscle and lead to increased microvascular permeability. At a mucosal level, the fall in FEV_1 is associated with vascular dilatation and leakage, with activation of cough neural pathways and smooth muscle constriction. In the skin the early reaction is seen as the classical wheal and flare reaction. The cause and significance of the LAR and associated increased AHR is less certain but is associated with an influx of inflammatory cells, particularly eosinophils and Th2 T cells (Robinson *et al.* 1993). The IgE-mast cell axis may still have a role in that the skin late phase response (LPR)

can be induced by the injection of either IgE or anti-IgE (Dolovich *et al.* 1973; Solley *et al.* 1976) and more recent clinical studies with therapeutic doses of anti-IgE was associated with decreased LPR (Ong *et al.* 2005).

1.3.9 A general model of allergic inflammation

A schematic representation of the immunological events that underlie the allergen-challenge model of atopic asthma is illustrated in Figure 1.2. In this model of disease allergen specific IgE binds to cells expressing both the high affinity (mast cells, basophils) and low affinity IgE receptor (B cells, monocytes and T cells). With such IgE-receptor association these cells are now sensitised for activation on encountering specific allergen. Inflammation can be viewed as the response of tissue to injury. The aim of this inflammatory response is to repair the injury. Acute inflammatory reactions are characterised by an influx of leukocytes and changes in the calibre and permeability of the vasculature leading to tissue oedema. Allergic inflammation is initiated by allergen binding to the IgE on the surface of mast cells or basophils (and possibly other cell types) as well as allergen trapping and focussing by antigen presenting cells leading to $CD4^+$ T cell activation generating a Th2 pattern of cytokines (IL-4, IL-5, IL-9 and IL-13). A complex inflammatory cascade leading to eosinophilic and other inflammatory cell infiltration, elevated serum IgE levels and mucus hypersecretion now occurs.

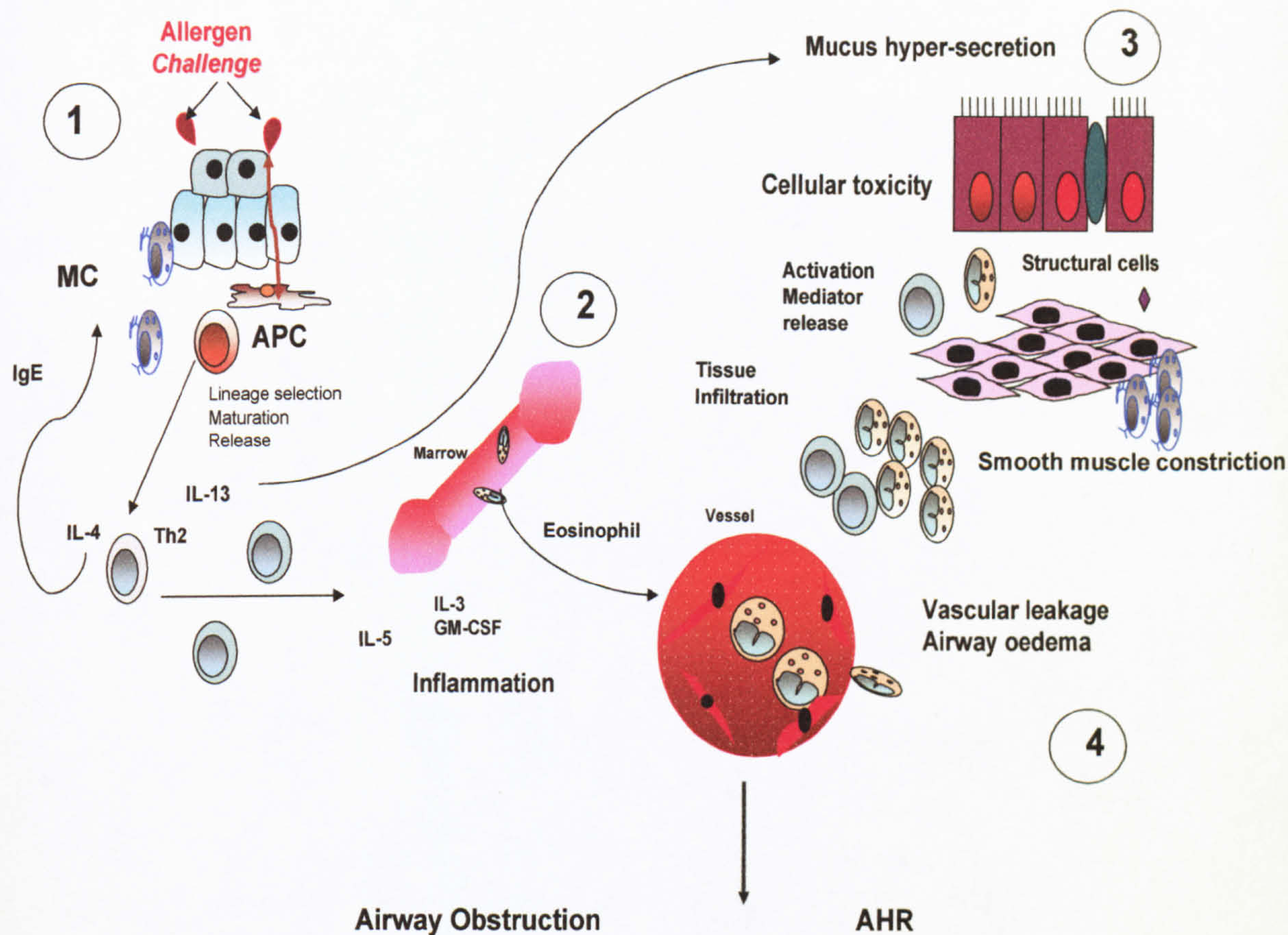


Figure 1.2: Illustration of the immunological events that underly the allergen challenge model of allergic inflammation.

1. Allergen impaction of the mucosal surface leads to its solubilisation and diffusion to sites of mast cell (MC) residence where cross-linking of two or more high affinity (FcεRI) IgE receptors lead to MC activation, subsequent degranulation and release of mediators such as histamine and leukotrienes that induce bronchoconstriction that characterises the immediate early asthmatic response.
2. Antigen presenting cells (APC) also take up allergen and present processed allergen peptide in the context of MHC Class II to naïve T cells which undergo activation leading to the release of Th2 cytokines. IL-5 in particular is essential for the maturation, release and trafficking of eosinophils from the bone marrow.
3. Intense cellular infiltration characterises the late asthmatic response where inflammatory mediator release is associated with mucus hypersecretion (IL-13), vascular oedema and leakage and airway smooth muscle constriction.
4. Airway obstruction (as defined by a fall in the FEV_1) and increased AHR are considered a physiological consequence of this cellular recruitment and activation.

1.4 Inflammation and airway hyperresponsiveness

1.4.1 Introduction

Atopy is one of the strongest risk factors for the development of asthma. Nearly 50% of the population are atopic as shown by positive skin prick tests (SPT) to common environmental allergens. Only 10% of these will develop asthma as evidenced by the development of AHR (Lau *et al.* 2000). The inheritance of AHR is independent of atopy (Townley *et al.* 1986) and intrinsic asthmatics are by definition non-atopic. The development of the asthma phenotype appears to require a genetic susceptibility to AHR and an inappropriate response to environmental allergens.

Much of the relationship between AHR and inflammation has arisen out of observations in allergen-induced IgE-mediated asthma. Initial research was focused on the measurement of AHR with immunological studies restricted to cells obtained from blood or post-mortem specimens. Allergen challenge was shown to be associated with the observation of increased AHR (Cockcroft *et al.* 1977), supporting the earlier observations of natural disease exacerbations by environmental triggers (ALTOUNYAN 1964). The first report of fibreoptic bronchoscopy (FOB) with bronchoalveolar lavage (BAL) in human volunteers was in 1974 (Reynolds & Newball 1974) and the first study of volunteers with asthma involving bronchial biopsy (BB) was in 1977 (Molina *et al.* 1977) The establishment of bronchoscopy thus brought about the capacity to sample directly the airways of patients with asthma and therefore the systematic investigation of the pathological basis of asthma. Several subsequent studies have demonstrated the coexistence of allergen-induced AHR with increases in cellular inflammation (De Monchy *et al.* 1985; Metzger *et al.* 1986; Gauvreau *et al.* 1999). The focus of much of asthma research has been to explain in causal terms how the presence and functional activation of inflammatory cells lead to AHR that manifests as sensitivity to non-specific irritants and variable airflow obstruction leading to episodic breathlessness. Causality can be implied from the co-existence of AHR alongside inflammation. Theoretically at least inflammation induced swelling of the airway wall internal to the ASM, as a result of vascular distension from increased blood flow and inflammatory oedema, will amplify the effect of a given amount of ASM contraction. Also any peri-airway inflammation may increase the external airway diameter leading leading to less tension in the surrounding airway attachements. This will impair the parenchymal tissue with

attachments to the small airways in particular from restraining excessive airway contraction.

Many studies have failed to show a close correlation between indices of inflammation such as cell counts and AHR (Crimi *et al.* 1998). This maybe a reflection of the phenotypic complexity of patients in terms of clinical and physiological features reflecting a need to relate specific phenotypes to specific inflammatory patterns. Most studies have been cross-sectional and there is a need for longitudinal studies where inflammatory events can be related to symptoms, lung function and AHR over time. At present, despite detailed insight into cellular recruitment, the precise mechanisms by which cellular inflammation leads to allergen-induced AHR is not known.

1.4.2 Mast cells

Mast cells (MC) are critical effector cells that underlie the immediate hypersensitivity reaction in allergic inflammation. The mast cell surface is sensitised with allergen specific IgE via the high affinity FcεRI receptor. Cross-linking of IgE, bound to mast cells via FcεRI, leads to non-cytotoxic energy-dependent degranulation. This leads to release of preformed granule mediators such as histamine as well as membrane-derived lipid mediators. Cytokines, chemokines and growth factors are also secreted. Mast cells are classified into two principal subgroups on the basis of distinct protease expression patterns. The MC_{TC} group express both tryptase and chymase (as well as carboxypeptidase and cathepsin G-like protease); the MC_T group express only tryptase. Although MC_T group predominate in the lung, mast cells that specifically infiltrate ASM are of the connective tissue MC_{TC} type (Brightling *et al.* 2002). Tryptase is an important ASM mitogen and may contribute to the increased ASM mass via both hypertrophy and hyperplasia. MCs are a significant source of Th2 cytokines and growth factors with important contributions to tissue eosinophilia (IL-5), fibrosis (selected TGF-β superfamily ligands) and B cell IgE synthesis via IL-4 and IL-13 (Levi-Schaffer & Weg 1997; Smith & Levi-Schaffer 2000). Whilst normal MC_{TC} do not express IL-5, they are an important source of IL-4 and IL-13 (Brightling *et al.* 2003b).

The contribution of MCs to AHR is suggested by the ability of either IgE or anti-IgE injection to induce the skin LPR (Dolovich *et al.* 1973; Solley *et al.* 1976) and inhaled polyclonal IgE, a non-specific MC activator, to induce the LAR (Kirby *et al.* 1986).

Recent studies targeting a neutralising anti-IgE antibody in atopic asthma demonstrate a reduction in AHR as measured by the indirect agent adenosine-5-monophosphate which leads to bronchoconstriction via A_{2B} receptor activation on MCs. This reduction in AHR was in association with reduction of cellular airway inflammation. No reduction in AHR measured in terms of methacholine direct challenge testing was seen supporting the concept that basal AHR may be unrelated to cellular inflammation (Djukanovic *et al.* 2004). The most convincing support for MCs in the genereation of AHR is provided by the finding of MC infiltration of ASM in asthmatic airways as the only distinguishing feature from eosinophilic bronchitis (Brightling *et al.* 2002).

1.4.3 Dendritic cells

The microenvironment of the lung is ideally adapted to deal with the high quantities of exogenous material that is deposited on the mucosal surface during respiration. In normal airways the epithelium is a tightly sealed layer. The mucosal dendritic cell (DC), a key cell for antigen uptake, is located immediately beneath the basement membrane. The DCs have long arm-like processes that can reach across the tight junctions and into the airway lumen to reach antigen. These DCs now traffic into the draining lymph nodes where naïve and memory T cell populations are located. Activated antigen-specific T cells leave the lymph node to re-circulate in the periphery or remain in the lymph node to interact with antigen-specific B cells, thereby regulating antibody production (Lambrecht & Hammad 2003). Inhaled allergen challenge in asthma is associated with a rapid fall in circulating myeloid DCs (Upham *et al.* 2002) suggesting tissue recruitment. In a murine model of allergen induced AHR, depletion of $CD11C^{+}$ DCs resulted in failure of $CD4^{+}$ Th2 production of IL-4, IL-5 and IL-13 alongside diminished eosinophilic inflammation, goblet cell hypertrophy and AHR (van Rijt *et al.* 2005).

1.4.4 Macrophages

In both stable and clinically active asthma macrophages (Mø) are the most abundant cells in both the distal alveolar spaces and the conducting airways. Allergen challenge is associated with an increased number of Mø suggesting that there is increased recruitment of Mø into the airway during the late asthmatic response (Chanez *et al.* 1991). Mø here demonstrate an activated phenotype (Gosset *et al.* 1991). Mø display surface low affinity IgE receptors (FcεRII or CD23) which can bind antigen via IgE and become activated for pro-inflammatory function (Melewicz *et al.* 1982). The

expression of the low affinity IgE receptor is around 80% on Mø from asthmatics versus 8-20% in normals (Melewicz *et al.* 1981). Expression is further enhanced after allergen challenge (Carroll *et al.* 1985). In response to activation Mø are known to secrete a wide variety of mediators such as LTC4 (Rankin *et al.* 1982) and LTB4 (Damon *et al.* 1989), growth factors such as transforming growth factor-beta (TGF-β) (Vignola *et al.* 1996) and the important cytokines IL-1 (lymphocyte activation) (Borish *et al.* 1992), IL-4 (Pouliot *et al.* 2005), GM-CSF (Howell *et al.* 1989) as well as IL-6 and TNF-α (Gosset *et al.* 1991). In particular IL-4 and GM-CSF will contribute to IgE production and eosinophilia whilst growth factors will play an essential role in Mø driven tissue repair and fibrosis.

1.4.5 T cells

The T cell hypothesis in asthma, particularly chronic asthma, arises from the concept that the disease is both sustained and propagated by the persistence of a specialised subset of chronically activated T memory cells sensistised against an array of antigens (allergens, microbes, occupational agents) which traffic into the lung in response appropriate antigen exposure. Immunohistochemical evaluation of bronchial biopsy studies from asthmatics have shown increased numbers of activated ($CD25^+$) T cells in the mucosa (Azzawi *et al.* 1990) that correlate with the numbers of activated eosinophils and AHR (Bradley *et al.* 1991). Interestingly allergen-peptide challenge studies confirm that T cell responses can orchestrate both the initiation and propagation of the LPR in asthma (Larche *et al.* 2003). Such responses are in the absence of IgE mediated MC activation and degranulation (Ali *et al.* 2004).

Originating in the thymus, T cells migrate into lymphoid tissue before returning into the blood steam, unless encountering antigen. Specific antigen recognition is via the surface T cell receptor (TCR). An antigen must first be processed by an antigen presenting cell and presented in the context of a major histocompatibility molecule (MHC). T cells fall into two classes with different functional characteristics on the basis of expression of co-receptors that interact with MHC molecules. $CD8^+$ cells recognise MHC Class I molecules whilst CD4 cells bind MHC Class II molecules. $CD4^+$ cells are key cells that drive the inflammatory component of asthma. $CD4^+$ cells can be further subdivided according to their cytokine profiles and chemokine receptors into Th1 and Th2 subsets. Th1 cells are characterised by γ-interferon (IFN) expression whilst Th2 cells express IL-4, IL-5, IL-9 and IL-13 (Larche *et al.* 2003).

Asthmatics have increased Th2 cytokine profiles compared to normal individuals and approximately 90% of the inflammatory cells expressing the Th2 cytokines IL-4 and IL-5 mRNA were activated T cells (Robinson *et al.* 1992). AHR correlated with the numbers of BAL cells expressing IL-4 and IL-5 but not γ-IFN. Allergen challenge in asthma is associated with activated T cell recruitment with induction of IL-5 expression (Bentley *et al.* 1993).

The working hypothesis of how T cells contribute to at least acute increases in AHR, for example during exacerbations, has been based on T cell regulated (via IL-4) IgE synthesis and the recruitment and activation of inflammatory cells. The contribution of T cells to more persistent or basal AHR may be through direct interaction with structural cells. Although direct T cell-airway smooth muscle interaction has not been demonstrated (Brightling *et al.* 2002) the Th2 cytokines IL-5 and IL-13 can enhance ASM contractility (Hakonarson *et al.* 1999; Laporte *et al.* 2001).

1.4.6 Th2 cytokines

Th2 cytokine dominated inflammation is a consistent finding of the asthma phenotype and there is continued debate on the role of Th2-mediated inflammation, particularly eosinophil infiltration, in relation to the pathogenesis of AHR. A single dose of a chimeric monoclonal anti-CD4 antibody showed significant clinical efficacy in severe chronic steroid-dependent asthma, supporting a role for T cell mediated pathways in asthma (Kon *et al.* 1998). Knocking out Th2 mediated inflammation via either blocking antibodies or knockout mice to Th2 cytokines followed by allergen challenge effectively abrogates inflammation and AHR (Gavett *et al.* 1994) , whereas transfer of Th2 cells directly induces airway inflammation in naïve mice after allergen challenge (Cohn *et al.* 1997). The contribution of Th2 cytokines to AHR remain an important focus of research.

IL-4 is important in the polarisation of naïve T cells into a Th2 phenotype and is a key cytokine responsible for B cell immunoglobulin switching for IgE synthesis. Clinical studies using inhaled rhIL-4 have been associated with increased sputum eosinophilia alongside increases in AHR at both 24 hours and 48 hours after inhalation (Shi *et al.* 1998a). Furthermore IL-4 knockout mice demonstrate reduced IgE, IL-5 production and cellular inflammation (Coyle *et al.* 1995). However IL-4 is not apparently obligatory for the development of the asthma phenotype. Absence of IL-4 in murine

models of allergen challenge confirmed the induction and persistence of AHR in the absence of increased airway inflammation (Hogan *et al.* 1998; Cohn *et al.* 1998). The finding that the IL-4 receptor IL-4R (Gavett *et al.* 1997) and its signalling transcription factor STAT-6 (signal transducer and activator of transcription 6) (Kuperman *et al.* 1998) were essential for the development of the asthma phenotype suggested that another ligand that can signal through the same receptor and signalling cascade must exist. The structural similarity of IL-13 to IL-4 suggested that IL-13 may be important in asthma pathogenesis. The blockade of IL-13 by administration of a soluble form of the IL-13R chain that binds only IL-13 was able to reverse AHR and mucus production (Wills-Karp *et al.* 1998; Grunig *et al.* 1998) whilst IL-13 administration recapitulates AHR, eosinophilic inflammation, mucus cell hyperplasia and mucus secretion (Zhu *et al.* 1999). IL-13 deficient mice do not develop AHR despite sustained cellular inflammation (Walter *et al.* 2001). IL-13 blockade also inhibited the development of AHR without any effect on inflammatory cell recruitment (Wills-Karp *et al.* 1998).

The exact mechanism by which IL-13 induces AHR is not certain. IL-13 can induce an array of chemokines related to T cell and eosinophil recruitment. Chemokines are chemoattractant cytokines selectively produced at sites of inflammation and are subdivided into families based on the position of cysteine residues such as C-C, C-X-C and CX_3C, where X is any amino-acid. Eotaxin (a C-C chemokine) and the homologues eotaxin 2 and 3 display very high selectivity for eosinophils exclusively through the chemokine receptor CCR3 (Jose *et al.* 1994). Chemokines are produced by a range of cells in response to inflammatory stimuli and they primarily serve as chemoattractants. IL-13 induces eotaxin production from airway epithelial cells and smooth muscle (Lilly *et al.* 1997; Wenzel *et al.* 2002). Such structural cell activation may be relevant to the mechanism of sustained AHR in asthma and may provide an explanation as to how IL-13 can induce AHR in the absence of traditional effector cells such as B cells, mast cells and eosinophils (Yang *et al.* 2001). The role of IL-13 in relation to airway remodelling and AHR is discussed in detail below.

IL-5 is an essential cytokine for the terminal differentiation of committed eosinophil bone marrow precursors. In conjunction with eotaxin IL-5 supports eosinophil chemotaxis and together with IL-3 and GM-CSF supports eosinophil survival (Kariyawasam & Robinson 2006). IL-5 knock out mice (Kopf *et al.* 1996) fail to

develop eosinophilia in response to allergen and helminth infection (Takamoto *et al.* 1997) whilst IL-5 over expression is associated with marked eosinophilia (Dent *et al.* 1990). Airway allergen challenge is associated with increased production of IL-5 by T cells and eosinophils (Bentley *et al.* 2003). Time-course studies on the kinetics of IL-5 secreting T cells and mobilisation of eosinophils demonstrate a convincing relationship to increases in AHR (Gibson *et al.* 1991). Inhaled IL-5 in a small study of eight asthmatics was associated with concomitant increases in sputum eosinophilia and AHR (Shi *et al.* 1998b). In animal studies anti-IL-5 administration was associated with attenuation of airway eosinophilia alongside AHR (Foster *et al.*1996).

IL-9 can directly induce airway inflammation and AHR as evidenced by IL-9 over-expression in the lung, leading to eosinophilic infiltration together with the induction of AHR and collagen production (Temann *et al.* 1998) whereas IL-9 blockade leads to attenuation of eosinophilia and AHR (Kung *et al.* 2001). IL-25, a more recently discovered cytokine, is believed to augment Th2 responses through the amplification of Th2 cytokine production (Fort *et al.* 2001).

1.4.7 CD8 T cells

Most cytotoxic T cells (Tc) express CD8 and destroy virus-infected cells. CD8 cells are also present in increased numbers in asthmatics (Robinson *et al.* 1992). CD8 cells are an important source of IL-5 and CD8 depletion is associated with the absence of eosinophilia and AHR in sensitised mice undergoing allergen challenge (Hamelmann *et al.* 1996). However, CD8 depletion prior to the sensitisation phase lead to enhanced Th2 responses suggesting that CD8 cells may actually have a protective role during allergen sensitisation (Stock *et al.* 2004).

1.4.8 Regulatory T cells

There two major categories of T regulatory cells (T Regs). $CD4^+CD25^+$ are naturally occurring cells that are hypo-responsive to *in-vitro* activation and can suppress proliferation and cytokine production from $CD25^-$ T cells. Suppression is probably through cell-cell contact with $CD25^-$ cells but may also involve IL-10 and TGF-β_1 secretion. The other set are IL-10 producing T cells, which must be induced, rather than occurring naturally. The exact role in asthma is uncertain but recent evidence suggest that their function maybe impaired in allergic disease (Ling *et al.* 2004).

1.4.9 Eosinophils

The precise role of the eosinophil in asthma pathogenesis remains uncertain. Eosinophils circulate in an activated or primed state in asthma compared to eosinophils derived from healthy normal individuals (Hakansson *et al.* 1990). It has been shown in animal studies that the products released by eosinophils can promote some of the pathophysiological hallmarks of asthma such as AHR. Major basic protein (MBP) comprises 50% of the granule protein content of the cell and is localised to the electron dense core. Cytotoxicity is a characteristic of MBP (Gleich *et al.* 1979). Several pathophysiological features of asthma can be evaluated in the context of MBP. The *in-vitro* effects of MBP include epithelial detachment and ciliary dysfunction. The cytostimulatory properties of MBP on basophils, mast cells and neutrophils (O'Donnell *et al.* 1983; Moy *et al.* 1990), result in the propagation of the inflammatory response. MBP may also be associated with the development of AHR (Uchida *et al.* 1993; Coyle *et al.* 1994). Post mortem analysis of asthma death patients consistently show eosinophils and extracellular MBP associated with nerve fibres in the airway smooth muscle. Eosinophils undergo activation and degranulation in response to endogenous tachykinins released from sensory nerves. MBP binds to the M_2 inhibitory muscarinic receptor on parasympathetic nerves leading to increased acetylcholine release with subsequent M_3 receptor activation and hence bronchoconstriction (Fryer *et al.* 1997).

Primed eosinophils are a major source of cysteinyl leukotrienes (Cys LTs) and have been identified as the predominant cellular source of LTC_4 in bronchial biopsies from asthmatic airway as shown by the co-localisation of LTC_4 and eosinophil cell markers (Cowburn *et al.* 1998). LTC_4 undergoes extracellular sequential enzymatic cleavage of glutamic acid and glycine from its sulphide chains to form the metabolites LTD_4 and LTE_4 respectively. These products can elicit paracrine effects that result in mucus hypersecretion (Marom *et al.* 1982), AHR, oedema (Joris *et al.* 1987) and potent bronchoconstriction (Adelroth *et al.* 1986). Collectively these findings suggest that eosinophils may be the major pro-inflammatory effector cells in the pathogensesis of asthma.

1.4.10 Neutrophils

Neutrophils account for 50-70% of the total white blood cell population.They originate from bone marrow myeloid precursor cells with a primary role in defence

against bacterial infection. They are one of the first cells recruited to the site of injured tissue, often in response to IL-8, tumour necrosis factor (TNF)-α and IL-1.

The finding that markedly increased numbers of BAL neutrophils were present in individuals with nocturnal asthma (Martin *et al.* 1991) and the observation of high numbers of neutrophils in rapidly fatal asthma (Sur *et al.* 1993) suggested an important role for the cell in this disease. There is often airway neutrophil recruitment following allergen challenge (Metzger *et al.* 1986). The mechanism by which neutrophilic inflammation may contribute to AHR is not currently known. Activated neutrophils have the capacity to release an array of inflammatory mediators (platelet activating factor, LTB_4 and IL-8), free oxygen radicals and proteases that contribute to propagating and sustaining inflammation (Bousquet *et al.* 2000).

1.4.11 Heterogeneity of cellular inflammation in asthma

All cellular components of the airway contribute to the complex inflammatory profile in asthma. At present the contribution of different cell types to the clinical asthma phenotype remains an important area of research but there is increasing recognition of at least four distinct inflammatory phenotypes that is either predominantly eosinophilic, neutrophilic, mixed cellular or paucigranulocytic (Wenzel 2006; Green et al. 2007).

Eosinophilic inflammation is the most characteristic cellular finding in the less severe forms of asthma. Although tissue eosinophilia correlates with the degree of AHR and is related to the late asthmatic response the relationship to asthma severity is weak (Wardlaw *et al.* 1988) (Green *et al.* 2002). It may be that it is the level of eosinophil activation that is important and this has not often been measured. However, studies so far with anti-IL-5 therapy in asthma have failed to demonstrate any reductions in AHR (Leckie *et al.* 2000; Flood-Page *et al.* 2003b) and are therefore further support for the apparent dissociation between AHR and eosinophlic inflammation. For example, treatment with anti-IL-5 monoclonal antibody failed to demonstrate any effect on AHR in asthmatic volunteers despite 55 % reduction in bronchial eosinophils (Flood-Page *et al.* 2003b). Until studies that can completely abolish airway eosinophilia are performed, the exact contribution of the eosinophil to AHR remains in doubt. Moreover eosinophils may be of importance in asthma exacerbation since asthmatics treated with glucocorticoids on the basis of airway eosinophilia

rather than lung function demonstrated reduced numbers of asthma exacerbations over the course of 12 months (Green *et al.* 2002).

Several mouse models of allergen induced airway inflammation demonstrate dissociation of AHR from inflammatory cells. For example in a chronic airway challenge model eosinophilic inflammation and airway remodelling persisted despite a fall in AHR to baseline values, whereas AHR was unaffected by eosinophil lineage ablation of eosinophils using GATA-1 knockout animals (McMillan & Lloyd 2004; Humbles *et al.* 2004).

Approximately 10-20% of asthmatics demonstrate increased airway neutrophils in the absence of increased eosinophils. Several studies confirmed that in more severe asthma, importantly steroid dependent asthma (Green *et al.* 2003), high numbers of neutrophils are present in sputum, BAL and bronchial tissue (Wenzel *et al.* 1997). In general terms neutrophilic asthma is considered to demonstrate a more severe asthma phenotype that is often steroid refractory (Holgate & Polosa 2006). It is not certain to what extent steroid therapy may have contributed to the airway neutrophilia and until recently it was argued that the neutrophil may be more of a bystander cell. However findings from a recent study using an anti-TNF-α antibody (etanercept) in 15 subjects with severe neutrophlic asthma showed reduction in neutrophils alongside statistically significant improvements in AHR (Howarth *et al.* 2005) suggesting that neutrophils may have important contributions to this more severe asthma phenotype.

The asthma phenotype characterised by absence of both eosinophils and neutrophils is termed paucigranulocytic (Simpson et al. 2006) and these patients can have marked AHR. Studies in this particular group are lacking but would be important to evaluate the contribution of airway structural change to this phenotype.

1.5 Airway remodelling

1.5.1 Introduction

Asthma is also characterised by airway remodelling or structural changes. These include marked goblet cell, smooth muscle and fibroblast hyperplasia together with the recruitment and activation of myofibroblasts (Brewster *et al.* 1990). Increased deposition of collagens and other extracellular matrix proteins occur both in the lamina reticularis of the basement membrane (RBM) and throughout the bronchial

mucosa together with prominent airway angiogenesis. Major goals of research are the determination of not only the mechanisms that drive structural change but also the relationship of such change to altered airway function in asthma, particularly in relation to severe steroid-refractory asthma phenotypes. Whilst it is accepted that abnormal structure will contribute by the way of altered geometric effects and changes in tissue biomechanics to airway physiology, the exact clinical consequences of remodelling remain uncertain. There is heterogeneity of disease such that in a proportion of individuals remodelling processes contribute to excessive ECM deposition leading to airway stiffness that can counteract ASM contractility and support a more fixed obstruction phenotype. In another proportion ASM hypertrophy and hyperplasia will dominate contributing to marked increases in AHR. Fibroblast accumulation and airway smooth muscle hypertrophy have been identified as the only changes associated with asthma severity in humans (Benayoun *et al.* 2003).

1.5.2 Epithelium

Probably the most important structural cell of the airway is the epithelial cell. The epithelium acts as a physical barrier between the external environment and the lung. The lung epithelium is continuously exposed to outside pollutants, allergen and microbes. It is able to sense environmental signals and respond directly by modulating both the innate and adaptive immune system to recruit further defence mechanisms.

Epithelial damage is a consistent feature of asthma and is present even in mild disease (Laitinen *et al.* 1985). Injury is visible as the selective loss of the columnar pseudo-stratified layer and clusters of such shed epithelium (termed creola bodies) is often observed in BAL fluid from asthmatics (Beasley *et al.* 1989). Airways biopsies may show denuded areas with only basal cells attached to the basement membrane. These changes can be related to the severity of asthma (Jeffery *et al.* 1989) and are not observed in chronic obstructive pulmonary disease (COPD). There are arguments that the injury is artefactual and maybe induced as a result of bronchoscopic sampling methods (Ordonez *et al.* 2000). The adhesion molecule CD44 (3v isoform) (Lackie *et al.* 1997) and epidermal growth factor receptor (EGFR) *c-erb* B1 (Puddicombe *et al.* 2000) is over-expressed in asthmatic epithelium. CD44 participates in epithelial repair by allowing epidermal growth factor (EGF) to be more effectively presented to its receptor. Tissue sampling injury would not induce the expression of CD44 and EGFR

suggesting that the epithelial injury process may have occurred *in-vivo* (Lackie *et al.* 1997). The consensus is that the airway epithelium in asthma exists in a fragile state with easy detachment of columnar cells from their basal attachments. Loss of epithelial integrity has obvious airway functional consequences such as loss of the mucociliary escalator clearance of inhaled debris and disruption of the barrier function allowing environmental agents to penetrate the luminal surface.

Equally and possibly more importantly, the epithelial phenotype changes to an immunologically active state that can actively participate and even direct inflammatory and remodelling events in the airway. In asthma the epithelium shows widespread evidence of continuous activation and epithelial stress as evidenced by markedly increased expression of transcription factors such as NFκβ and heat shock protein-27 (a marker of oxidant stress) (Merendino *et al.* 2002). The elevated expression of the cyclin dependent kinase $p21^{waf}$ (an inhibitor of the cell cycle), (Puddicombe *et al.* 2003) suggests that the epithlium is attempting to repair itself.

A damaged epithelium may lead to heightened AHR as suggested by correlation with histamine PC_{20} (Jeffery *et al.* 1989). The mechanism is possibly related to the release of bronchoactive mediators such as products of the cyclooxygenase and lipooxygenase pathway (Knight *et al.* 1995), the proinflammatory cytokines IL-6, IL-8, GM-CSF, and RANTES (regulated upon activation, normal T-cell expressed and secreted) (Cromwell *et al.* 1992; Davies *et al.* 1995) and the chemokine eotaxin (Lilly *et al.* 1997) leading to neutrophil, T cell and eosinophil recruitment. Epithelium is also an important source of growth factors (Zhang *et al.* 1999). Thus epithelial activation can lead to amplification of the inflammatory and remodelling processes that may contribute to the generation and maintenance of AHR.

Increased expression of the EGFR with subsequent pro-fibrotic growth factor release typically occurs in response to injury and is a marker of active epithelial repair (Davies *et al.* 1999). In normal epithelium elevated EGFR expression is only observed in areas of structural damage. In asthma the expression is disease related and is seen in both damaged and morphologically undamaged epithelium and is indicative of diffuse epithelial injury. Under normal circumstances the epithelium is able to repair itself quickly but in asthma there is increasing evidence that this process is defective with prolonged activation and therefore signalling to the underlying mesenchymal

cells leading to myofibroblast transformation and activation. Evidence for defective epithelial repair exists in that markers of epithelial proliferation such as Ki67 and PCNA are markedly reduced from an early age (Demoly *et al.* 1994) whilst over-expression of $p21^{waf}$ occurs (Puddicombe *et al.* 2003). Defective apoptosis has been shown by the increased expression of the anti-apoptotic proteins Bcl-2 and HSP-27 (Druilhe *et al.* 1998).

1.5.3 Mucus

Electron micrographs demonstrate distinct electron-lucent acidic-mucin rich confluent granules that characterise goblet (mucus) cells in the epithelium. In the normal human trachea it is estimated that the mean density of surface mucus cells is between 6000-7000 cells/mm^2. In response to environmental assault, infections and in disease states, including asthma, the mucus cells can undergo both hyperplasia and hypertrophy. Mucosal gland hypertrophy is an important feature of the remodelled airway (Ordonez *et al.* 2001). Mucus hypersecretion is an important characteristic of the asthma phenotype and contributes to the excessive mucus production associated with asthma death (Rubin *et al.* 2001).

1.5.4 Basement membrane

Basement membrane thickening is a consistent finding in the asthmatic airway (Dunnill *et al.* 1969). The basement membrane (BM) is composed of an upper layer called the basal lamina (true basement membrane) consisting of predominantly Type IV collagen and Type V laminin (Paulsson 1992). These components are predominantly epithelial derived. The epithelium is attached to the lamina densa. Extending internally from the lamina densa is the lamina reticularis or reticular basement membrane (RBM), and it is predominantly derived from the layer of fibroblasts immediately below termed the attenuated fibroblast sheath (Paulsson 1992).Thickening of the RBM is a characteristic early feature of asthma and has been demonstrated in children with difficult asthma between 6-16 years of age (Payne *et al.* 2003). The lamina densa in asthma does not differ from that in normal airways. The lamina reticularis, however, is increased two to three fold in asthmatics from the 3-4 µm thickness reported in normals (Roche *et al.* 1989). RBM thickening correlates with the number of subepithelial fibroblasts (Brewster *et al.* 1990), AHR (Boulet *et al.* 1997) and may reflect the degree of airway thickening below the RBM (Kasahara *et al.* 2002).

The major composition of the RBM is Type III and Type V collagen together with fibronectin and tenascin (Roche *et al.* 1989). The width of the lamina densa (approximately 80nm) is below the resolution of the light microscope and cannot be visualised by light microscopy (LM) without immunohistochemical staining. In contrast, the thicker RBM (approximately 4μm in normal adults) is visualised by LM. In addition to its role as anchorage for the epithelium, the BM provides an essential platform to allow epithelial and inflammatory cell migration via interaction of ECM components such as fibronectin and tenascin with cell surface integrins. The BM also provides an essential barrier that compartmentalises the epithelium from the underlying mesenchymal components. However, inflammatory cells can traffic via the BM into epithelium due to presence of pores that traverse the course of the BM. The pores are estimated to have a mean diameter of 1.76μ, large enough for inflammatory cell traffic, and is represented with an average density of 737-863 mm^2 density (Howat *et al.* 2001).

1.5.5 Fibroblasts

Fibroblasts are ubiquitous stromal cells with an important role in providing mechanical support for tissues via ECM production. Increased airway fibroblast numbers is a distinguishing feature of severe asthma (Benayoun *et al.* 2003) and the biology of recruitment, survival and activation into the myofibroblast phenotype are important areas of focus.

In the adult lung fibroblasts comprise one third of the total cell population and upon activation is a predominant source of ECM production. Fibroblasts are one of the largest cells in the body with individual cells estimated to be up to 100μm in length. The cells are morphologically distinct with an elongated spindle-like (stellate) shape with distinct cytoplasmic protrusions and invaginations that allow interaction with the surrounding ECM, vasculature and neural structures. The most important activator of fibroblasts is the transforming growth factor (TGF)-superfamily (Zhang *et al.* 1999; Zhang & Phan 1999) although insulin-like growth factor II, IL-4 and IL-13 also demonstrate potent stimulation. Platelet derived growth factor (PDGF), connective tissue growth factor (CTGF, a member of the PDGF family) and TNF-α all stimulate fibroblast activation and proliferation (Sasaki *et al.* 2000).

Fibroblasts display phenotypic plasticity and functional versatility depending on the anatomical region of residence (Schmitt-Graff *et al.* 1994). In the lung, the parenchymal population of fibroblasts reside predominantly in the interstitial space whilst the airway fibroblasts are seen beneath the basement membrane. The latter population is collectively termed the attenuated fibroblast sheath (see below) and is present in all animal species that have been examined (Brewster *et al.* 1990)

Detailed review of the biology of myofibroblasts is provided by Desmouliere *et al.* 2003. The myofibroblast cell phenotype is best characterised by the expression of the contractile protein typically found on vascular smooth muscle cells, alpha smooth muscle actin isoform (α-SMA). The transformation of fibroblasts to the active myofibroblast phenotype is an acute event in response to allergen in asthma and is characterised by expression of the isoform typical of smooth muscle in fibroblasts (Darby *et al.* 1990). Thus α-SMA expression is a valuable marker of remodelling events.

Myofibroblasts were first identified by electron microscopy on the basis of morphology in wound granulation tissue (Gabbiani *et al.* 1971) and continue to be observed in tissue undergoing repair and remodelling, as well as normal tissues where mechanical force development is required. The elongated cells are considered as intermediate between the fibroblast and smooth muscle cell phenotype, as it retains the capacity to express smooth muscle contractile proteins such as desmin. TGF-β_1 is a potent inducer of myofibroblastic differentiation (Desmouliere *et al.* 1993) in the presence of the fibronectin ED-A splice variant (Serini *et al.* 1998). TGF-β_1 is also a potent stimulus for myofibroblast collagen production (McAnulty *et al.* 1991). Actin is one of the most conserved eukaryotic proteins in cells and expressed as six specific isoforms. Although the exact function of the different isoforms remain uncertain their expression can change within the same population of smooth muscle cells during embryogenesis, injury and disease states. In the process of myofibroblast differentiation fibroblasts initially adapt the proto-myofibroblast phenotype that only expresses the β-and γ-cytoplasmic actins. Further differentiation is associated with α-SMA expression and is considered the most reliable marker of the myofibroblast phenotype (Darby *et al.*1990).

Mechanical stress can also induce α-SMA expression (Hinz *et al.* 2001). α-SMA isoform expression in the myofibroblast phenotype is associated with upregulation of the cells synthetic capacity and migratory potential (Hinz *et al.* 2001). The expression of α-SM actin is associated with increased contractile activity of cultured fibroblasts. α-SMA is fundamental for force generation within the myofibroblast and subsequent transmission of this force to the ECM leading to tissue remodelling (Hinz *et al.* 2001). Mechanical tissue stress can lead to conformational changes in fibronectin. Fibroblasts can attach to fibronectin via the integrin $\alpha_5\beta_1$ and syndecan–4 in a synergistic manner leading to actin stess fibre assembly and cell spreading (Midwood *et al.* 2006). The ends of actin stress fibres bind to actin-binding proteins such as vinculin and clustered integrins. Such focal adhesion domains are termed fibronexus junctions. The fibronexus is a distinctive feature for identifying myofibroblasts. In wound healing models wound closure is associated with the removal of myofibroblasts by apoptosis (Desmouliere *et al.* 1995). Reversal of myofibroblast phenotype in fibrotic disease states offers an important therapeutic target.

1.5.6 The attenuated fibroblast sheath

In 1990 a population of fibroblasts and myofibroblasts in the airways of normal and asthmatic individuals were described that resided in a subepithelial distribution below the basement membrane (Brewster *et al.* 1990). This fibroblast sheath is found approximately 2μm below the basal lamina closely opposed to the lamina reticularis of the basement membrane (RBM). The cells are flat and stellate in shape with a thick nuclear region rich in endoplasmic reticulum (ER). The fibroblast population in the lamina propria differ mainly in that they are fusiform in shape. The cells are seen to form a mesh-like network with multiple protrusions that interact with the lamina densa of the basement membrane. In the inactivated state the cells do not appear to display actin microfilaments. This attenuated fibroblast sheath extends from the larger divisions of the airway to the terminal divisions into the alveolar regional wall as the interstitial fibroblast population.

1.5.7 Airway smooth muscle in asthma

Airway smooth muscle (ASM) mass is increased in asthma and correlates with asthma severity (Benayoun *et al.* 2003). When the width or area of ASM is measured in transverse section of airways from post-mortem cases of fatal asthma, an average increase ranging from 50-100% is seen whilst in non-fatal cases the increase is 25-

55% compared with normal controls (Carroll *et al.* 1993). Whilst the exact pathways and mechanisms that lead to AHR remain uncertain the end result is the direct or indirect stimulation of ASM contraction.

In biological systems smooth muscle (SM) lines hollow organs such as blood vessels and the airway. Morphologically smooth muscle cells appear as spindle-shaped cells 2-5μm wide and 50-200μm in length. ASM is found at all levels of the bronchial tree from the trachea to the terminal bronchioles, arranged circumferentially around the airway to give the appearance of a descending spiral. The filament structure, in contrast to striated muscle, is less organised with no obvious sarcomeric structure (hence the name smooth). The contractile apparatus is composed of actin-containing thin filaments that project out and interact with myosin-containing thick filaments. Dense bodies contain the protein actinin and are functionally analogous to z-lines in striated muscle. They serve as anchors for the thin-filament actin. Intermediate filaments such as desmin and vimentin are cytoskeletal elements which provide a structural backbone against which contraction occurs. The sarcoplasmic reticulum is poorly developed in SM making SM more dependent on intra-cellular sources of calcium (in contrast to cardiac and skeletal muscle). Signalling cascades are initiated leading to force generation and cell shortening due to actin and myosin cross-bridge cycling. During contraction the smooth muscle cell shortens in length. Muscle force is generated by the interaction between thick and thin filaments which are free to interdigitate and slide past each other. The isometric force generated is proportional to the extent of overlap between the filaments.

Many of the symptoms and disability in asthma can be explained on the basis of ASM shortening. Increased amounts of ASM will encroach upon luminal space with alteration of mucosal folding. The increased area of ASM seen in transverse section is probably a result of more smooth muscle cells (hyperplasia) that are increased in size (hypertrophy) which are pushed further apart as a result of the accumulation of excessive ECM. At present the mechanisms of excessive smooth muscle accumulation in asthma remains unknown although the commonly held theory at present is that cytokines and growth factors released as part of inflammatory processes will drive ASM accumulation and growth. *In-vivo* experiments confirm that ASM from patients with severe asthma demonstrate altered responses to contractile

and relaxant agents compared to normal ASM suggesting an intrinsic propensity to exaggerated AHR is present (An *et al.* 2006).

Although there is some controversy as to the degree of ASM hypertrophy in asthma (Woodruff *et al.* 2004; Benayoun *et al.* 2003) it is agreed that hyperplasia accounts for the 50-83% increase in ASM in mild to moderate asthma. It has been demonstrated that asthmatic ASM has an increased propensity for proliferation *in vitro* versus ASM from normals. Again *in-vitro* studies have shown that altered proportions of ECM components can lead to an enhanced capacity of ASM to proliferate via an autocrine mechanism (Johnson *et al.* 2004) and ASM may have enhanced sensitivity to growth factors (Burgess *et al.* 2003). TGF-β Superfamily of ligands in particular have an important role in ASM proliferation and synthetic capacity (Black *et al.* 1996). *In-vitro* work suggests that inflammatory and ECM components can lead to marked functional plasticity of ASM in asthma leading to modulation of the cell into a contractile phenotype that can evolve further into supercontractile (Ma *et al.* 2002) and synthetic forms (Moir *et al.* 2003; Hirst *et al.* 2000). ASM cells have the synthetic capacity to contribute a significant proportion of pro-inflammatory cytokines and remodelling growth factors (Howarth *et al.* 2004). ASM cell adhesion receptor and costimulatory molecule expression allows the cell to directly response to the surrounding inflammatory milieu; the release of multiple cytokines, chemokines and growth factors, reviewed in detail in (Hirst 2003), in turn allow the cell to participate and maybe even direct several inflammatory and remodelling processes. Such contribution will be particularly relevant in asthma where the ASM mass is markedly increased to that of a normal airway (Carroll *et al.* 1993). ASM derived factors will act in both an autocrine and paracrine manner. Such findings have changed the impression of ASM as a passive bystander in asthma to an active immunomodulatory tissue that can both propagate and regulate mucosal inflammatory processes.

1.5.8 Angiogenesis

The airway vasculature, derived from the aorta, is a dense plexus of interconnecting vessels with branches that penetrate further into the deeper submucosa forming a secondary vascular complex with frequent anastomoses to the pulmonary circulation. In asthma, changes to the airway vasculature include increased numbers of vessels per unit area with increased vessel size, and associated vasodilation and leakage leading to mucosal oedema. Increased vascularity is present even in mild asymptomatic

asthma (Li & Wilson 1997). Capillary engorgement and oedema in animal models have shown to double the microvascular volume fraction of tissue leading to narrowed airway lumen and subsequent airflow resistance. The model by Moreno et al of airway wall remodelling (Moreno *et al.* 1986) suggests that even small increases in airway thickness as a result of vascular engorgement and leakage will significantly contribute to the airway obstruction observed after allergen challenge in asthma. Inflammatory cell infiltration, particularly eosinophils, is associated with increased vascularity (Salvato 2001). Vascularity correlates with asthma severity (Vrugt *et al.* 2000) whilst inversely correlating with the degree of airway obstruction and AHR (Hoshino *et al.* 2001). Increased vascularity is a marked observation in airways of people who died from asthma (Dunnill 1960). The most important inducer of airway angiogenesis is vascular endothelial growth factor (VEGF) (Lee *et al.* 2004a) (discussed further under growth factors).

1.5.9 Extracellular matrix

The extracellular matrix (ECM) is a gel-like substance composed of a variety of polysaccharides, collagens and water that confer tensile strength yet with elasticity and compressibility. It is the relative proportions of these components that contribute to the physical properties of the airway. Further, the ECM has a pivotal role in regulating cellular function by acting as a substrate for cellular adhesion, migration, differentiation, proliferation and survival (Ingber *et al.* 1994) as well as a scaffold or structural support for cells. The ECM contributes to approximately one quarter of the dry weight of the lung. Both the quantity and composition of the airway wall ECM is altered in asthma. There is an excessive deposition of ECM components such as collagens and proteoglycans not only in the RBM and throughout the airway wall but also between the smooth muscle cells (Huang *et al.* 1999).

Collagens

The collagens are the most abundant proteins in the lung and their specific expression and location critically determine structure and function in the respiratory tract and any abnormal or dysregulated expression may contribute to abnormal biomechanics and function of the airway.

Collagen biology is reviewed in detail by Gelse *et al.* 2003. The characteristic structural feature of the collagen molecule is the right handed triple helix composed of

three α-chains. Transcription of the gene in the nucleus is followed by translation into pre-pro-α-chains which protrude into the lumen of the rough endoplasmic reticulum (RER). The procollagen α-chains now undergo several post-translational modification stages that include hydroxylation of specific proline and lysine residues and glycosylation of hydroxylysine residues. The α–chains can now associate through the short non-helical regions of the collagen monomer (C-telopetides) which is rich in sulphydryl groups and allow covalent cross-linking and association into the triple helix which is propagated from the C-terminus to the N-terminus in a zipper-like fashion.

The post-translational modifications allow intra-molecular hydrogen bond formation between the polypeptide chains that is essential for maintaining the stability of the triple helix structure. These procollagen molecules are now packaged within the Golgi apparatus into secretory vesicles and secreted out into the extracellular space. Here the procollagen is converted into collagen by removing the N- and C-propeptides by C- and N-proteinases respectively. The triple-helical collagen molecule demonstrates a tendency to self-assemble into cross-banded fibrils.

HSP-47 is a collagen–specific chaperone that binds to the procollagen α-chain. HSP-47 is essential for the translocation of the procollagen into the RER and importantly directs the correct folding of the α-chains into triple helices (Satoh *et al.* 1996; Nagata 1998). HSP-47 is heat-inducible and resident in the endoplasmic reticulum (ER). HSP-47 is rapidly up-regulated in response to TGF-β_1 (Yamamura *et al.* 1998). Both collagen and HSP-47 are always co-expressed and there is a marked up-regulation of HSP-47 in pulmonary, renal and liver fibrotic diseases. Cells that fail to express collagen do not express HSP-47. The association of HSP-47 to nascent Pro α1(I) collagen occurs in the ER and dissociation in the cis-Golgi compartment. HSP-47 serves as a molecular chaperone for collagen in order to prevent nascent collagen chains from forming aggregates in the ER and undergoing intracellular degradation (Gelse *et al.* 2003).

Fibril-forming collagens (FFCs) represent 90% of the total collagen. Type I collagen is the predominant collagen in tissue ECM and accounts for around 85% of the collagen produced by fibroblasts although epithelial, endothelial and smooth muscle cells and alveolar type II pneumocytes also contribute to collagen synthesis (Gelse *et*

al. 2003). Type I collagen is the major collagen of skin, tendons, interstitial connective tissue of viscera excluding the brain, hyaline cartilage and the vitreous body. 90% of bone mass is accounted for by Type I collagen. In the lung Type I collagen contributes to 60-70% of the collagen content and is the predominant collagen of airway walls, blood vessels, lung interstitium and alveolar septa. The structure of Type I collagen is considered unusual in that it is an asymmetric heterotrimer of two $\alpha1(I)$ chains and a single $\alpha2(I)$ chain that following secretion aggregate into large rod-like, semi-rigid fibrils that confer high tensile strength or rigidity to the airway. As with other fibrillar collagens, in its mature form Type I collagen consists of three domains: a short amino-terminal non-triple helical domain (N-telopeptide), the central triple helical collagenous domain, and the short carboxy-terminal non-triple helical domain (C-telopeptide). Type III collagen accounts for 30% of the lung collagen and the relative proportion of Type III is relatively constant (Kirk *et al.* 1984; Kirk *et al.* 1984). Type III collagen is a more flexible molecule compared to Type I collagen.

The precise location and proportions of different collagen types in tissues implies cell specific regulated gene expression. Regulation occurs at the both the level of transcription and translation. Given that Type I collagen has been the most extensively studied collagen the mechanisms of collagen synthesis have focussed on its synthesis. The principles of processing, triple helix formation and secretion will most likely apply to other known collagens.

Early studies confirmed that the thickened RBM in asthma is partially a result of excessive Type III and Type V collagen deposition with lesser amounts of Type I collagen (Wison & Li 1997). Thickening of the RBM is present in children with difficult asthma to the same extent as adults (Payne *et al.* 2003) and there is increasing evidence that remodelling occurs early in childhood and that it may even predate the onset of symptoms in some cases (Baldwin & Roche 2002). The clinical significance of this thickening remains controversial. Firstly, similar thickening is seen in EB suggesting no significant role for RBM thickening in AHR (Brightling *et al.* 2003a). It has been argued also that a thickened stiff RBM will counteract excessive airway bronchoconstriction (Wiggs *et al.* 1997). It may be argued that the exact proportions of ECM may differ between the two leading to AHR in one and not the other. RBM thickness has been correlated overall airway thickness (Kasahara *et al.* 2002) as well

as to the amount of ASM in central airways (James *et al.* 2002). Several studies have however correlated the degree of thickening in the RBM to reduced airway distensibilty and increased airflow obstruction and thus asthma severity (Bento & Hershenson 1998).

Proteoglycans

Proteoglycans (PGs) such as decorin, perlecan, dermatan sulphate, heparin sulphate and hyaluronan, are a diverse population of molecules which are present in abundance and their biology is reviewed in the stated review (Kresse & Schonherr 2001). They are large molecules consisting of multiple glycosaminoglycan (GAG) chains branching out from a linear protein core. PGs have the capacity to bind with water and thus contribute a gel-like viscosity to the ECM that has a cushion-like effect for cells. They also in participate in regulation of cell signalling through their ability to bind growth factors as well as chemokines. Growth factors typically bind to the GAG chain of proteoglycans. Decorin especially binds to TGF-β_1 preventing TGF ligand activation (Redington *et al.* 1998).

Both small proteoglycans such as lumican and biglycan as well as large proteoglycans such as versican have been demonstrated to have increased deposition in asthma with significant correlation to AHR (Huang *et al.* 1999). Biglycan, versican, decorin and hyaluronan have all been shown to be prominent around ASM in lungs from post-mortem asthma deaths (Roberts 1995). Both hyaluronan and versican are localised around and between ASM cells (Roberts 1995) whilst decorin (important for binding and 'trapping' TGF) is found in abundance in areas with Type I collagen (Roberts 1995). The osmotic properties of proteoglycans may alter airway fluid mechanics and by binding water lead to increased tissue swelling and hence increased airway and may also effect tissue compressibility as has been demonstrated in the mechanics of hyaline cartilage in joints.

Tenascin

Tenascins are a family of modular ECM proteins with complex interactions with cells leading to modulation of cell adhesion, migration and growth. Tenascin-C is the most important member of this group and its expression is highly regulated. Transient expression of tenascin during organogenesis is seen but little expression is seen in fully developed organs. However in pathological states such as inflammation,

infection and carcinogenesis rapid induction of expression occurs (Chiquet-Ehrismann & Chiquet 2003). Asthmatics at baseline express significantly more tenascin in the RBM of the airway compared to the normal airway where there is minimal or no expression of tenascin. Expression is associated with acute cellular inflammatory events (Karjalainen *et al.* 2003). Allergen-induced airway injury is associated with further up-regulation (Phipps *et al.* 2004a). Thus tenascin expression is an excellent ECM marker to study the relationship to inflammatory events.

1.5.10 Matrix homeostasis

A balance between production and degradation achieves the regulation of ECM turnover. ECM degradation is dependent on the family of proteinases termed matrix metalloproteinases (MMPs). These zinc-dependent proteinases were initially identified in the involuting tails of tadpoles by their ability to degrade collagen (GROSS & LAPIERE 1962; GROSS 2004). On the basis of substrate specificity MMPs are broadly classified as collagenases (MMP-1, MMP-8 and MMP-13) which digest collagen, gelatinases (MMP-2 and MMP-9) which digest partially denatured collagen (gelatin), stromelysins (MMP-3, MMP-10 and MMP-11) which can degrade multiple ECM protein substrates and the elastases (MMP-7 and MMP-12). Other MMPs such as MMP-8 (neutrophil collagenase) and MMP-14 (membrane surface anchored) also exist. The potential of MMPs to cause significant host pathology necessitates their strict regulation. Rather than storing MMPs in cells and the ECM, active synthesis is undertaken in response to tissue injury (as after allergen challenge in asthma) and secreted as pro-enzymes that undergo proteolytic cleavage. The tissue inhibitors of metalloproteinases (TIMPs) serve to inhibit MMP activity by binding to MMPs in a 1:1 manner, this exact balance being critical in determining the normal matrix turnover. An imbalance can lead to either excess degradation or accumulation of ECM. In asthma, both an excess of MMPS to TIMPs (Tanaka *et al.* 2000) and vice versa (Mautino *et al.* 1999) has been reported and such imbalance will contribute to dysregulated ECM turnover.

An altered ECM has several functional implications for cellular inflammation such as alterations in cell-matrix binding affinity with subsequent effects on cell migration in addition to modulation of cytokine and growth factor storage and activation. It is therefore possible that ECM remodelling can contribute to the sustenance of the chronic inflammatory state that so far has defined the asthmatic phenotype.

Understanding the synthesis and regulation of ECM production in normal lung homeostasis and its alteration in disease states may allow more effective therapeutic intervention for fibrotic diseases

1.6 Airway remodelling and clinical consequences

1.6.1 Introduction

The realisation that a purely inflammatory model does not explain all the features of asthma and steroid ineffectiveness in the severe end of the disease lead to consideration that airway remodelling may contribute significantly to symptoms and disease severity. Despite the change in research focus towards airway remodelling, currently there are several questions that remain unanswered. One of the most pressing questions is the clinical consequence of airway remodelling for the patients, particularly the effects on AHR and airway obstruction. In mild asthma, AHR and reversible lung function are usually highly responsive to inhaled corticosteroids. In contrast, severity and chronicity of asthma is associated with a considerable spectrum of the disease phenotype. Here some patients demonstrate marked airway inflammation whilst in others inflammation is less prominent but there are increases in airway smooth muscle associated with increased severity of AHR. Another proportion of patients demonstrate progressive and irreversible airway obstruction.

1.6.2 Airway hyperresponsiveness (AHR)

It is possible that airway structural change can contribute to AHR associated with asthma chronicity and severity. All three layers of the airway wall (adventitia, the inner wall comprising the lamina propria including the basement membrane and the smooth muscle layer) are thickened in asthma. In fatal asthma there is loss of elastin in the airway wall and adjacent parenchyma leading to a loss of airway alveolar attachments (Mauad *et al.* 2004). It can be predicted that adventitial thickening and loss of airway-alveolar attachments can potentially uncouple the ASM from the surrounding lung parenchyma so that the parenchymal tethering that prevents the ASM from excessive shortening is lost and the airway is more liable to collapse. An increase in the airway wall thickness internal to the smooth muscle layer will amplify the airway narrowing at the time of ASM contraction. Mathematical modelling predicts that airways with increased smooth muscle narrow to a much greater extent than airways with less smooth muscle volume for a given degree of circumferential smooth muscle shortening (Moreno *et al.* 1986). This would be in keeping with

clinical findings that ASM mass is the only structural feature that distinguishes severe asthma from moderate disease (Benayoun *et al.* 2003). Greater airway smooth muscle mass will not only lead to an excessive degree of muscle shortening but also greater force generation leading to a disproportionate reduction in airway patency for a given degree of ASM contraction. ASM has been found to encroach onto the RBM and epithelium in severe asthma (Madison 2003), so that even minor contraction will affect airway narrowing. Such findings may explain the persistent AHR seen in asthma under basal conditions. It is predicted on basis of *in-vitro* studies that *in-vivo* dynamic changes in ECM will also affect basal AHR in asthma through alterations in ASM phenotype and contractility (Black *et al.* 2003; Black *et al.* 2001). The acute increases in AHR seen in response to allergen accompanied by eosinophil, T cell, macrophage and neutrophil infiltration together with the up-regulation of Th2 cytokines thereby implies that AHR in this setting is at least partially dependant on inflammatory events. It is probable therefore that there are two different components to AHR each with a distinctly different mechanism of activation and sustenance. It is possible that each component of AHR will require a separate therapeutic strategy.

1.6.3 Airway obstruction

By definition one of the clinical features of asthma is reversible airway obstruction. Unlike COPD, asthma is not usually associated with a rapid decline in FEV_1. However, a significant proportion of asthmatics demonstrate rapid rate of decline in lung function and can proceed to fixed airway obstruction (Ulrik & Backer 1999; Vonk *et al.* 2003; Bumbacea *et al.* 2004; Lange *et al.* 1998) which may result from progressive airway remodelling. Traditionally airway remodelling has been considered to represent a chronic repair process initiated in response to inflammatory processes with clinical consequences related to the time course of the disease. With the establishment of longitudinal studies of asthma much of these views are now changing.

In an Australian cohort, AHR in newborn asymptomatic children was an independent risk-factor for the development of asthma and this was not related to atopic status (Palmer *et al.* 2001). In a proportion of this group AHR was fully established by the age of 6 months. The presence of this early AHR identified a subgroup with increased risk of asthma and lower FEV_1 at 6 years. Such studies suggest an inherited trait for airway dysfunction and susceptibility to tissue injury and abnormal tissue repair. This

concept of tissue susceptibility and abnormal repair may explain the association of early AHR with abnormal lung function and airway structural change. RBM thickening is present in childhood asthma to the same degree in adult asthma (Payne *et al.* 2003), and there no convincing relationship of asthma duration to disease severity or lung function loss (Jenkins *et al.* 2003).The finding of RBM thickening in children before disease manifestation maybe an indication that tissue remodelling is present in the deeper submucosa and may suggest that structural change contributes even at this early stage to AHR. Tissue repair will occur in response tissue injury, as in response to environmental insults such as pollution and infection.

It appears that much of the loss of lung function occurs early on in the disease. In the Tuscon study, it was the children who were persistent wheezers during the first 3 years of life that demonstrated the lowest levels of lung function in subsequent years suggesting that structural change is established early on in the disease (Taussig *et al.* 2003). In a landmark longitudinal study of self-declared asthmatics, the rate of decline in FEV_1 were two-fold greater in the asthma cohort (even more so in the group that smoked) compared to the normal group (Lange *et al.* 1998). Data from the first five years of the study confirmed that the decline in FEV_1 was more marked in people who acquired asthma in that period rather than in individuals with long-term asthma (Ulrik & Lange 1994). If the loss in pulmonary capacity seen in adult asthmatics with long-term asthma is established in childhood then it is important to establish what factors initiate and perpetuate the remodelling process. In particular the relationship of inflammation to remodelling needs to be defined and the exact clinical consequences determined.

Progressive and accelerated decline in lung function over time in asthmatics can be explained in terms of excessive and altered airway ECM deposition, leading to altered mechanical properties of the airway. Whilst this is detrimental in terms of increasing airflow obstruction with increasing air-trapping and airways resistance, what effect such changes contribute to basal AHR in the chronic setting remain controversial. What is not clear is to what degree excessive smooth muscle contraction is a manifestation of fundamental changes in the structure and function of the smooth muscle cell itself and what effect alterations in the non-contractile ECM components contribute towards counteracting any such exaggerated airway contraction. As discussed earlier, interstitial fibrillar Type I collagen with inherent high tensile

strength deposited around and between ASM as well proteoglycans which by binding water increase tissue turgor will contribute to increased resistance of the airway wall to deformation by opposing ASM shortening under loading. Airway conductance studies in stable asthmatics at baseline (Colebatch *et al.* 1973) and after methacholine (Fish *et al.* 1981) have shown that asthmatic airways with increasing AHR dilate less than normal airways during lung inflation. This indicates that increased stiffness (or decreased compliance) in asthmatic airways associated with AHR may lead to a functional effect of non-contractile ECM effects on ASM phenotype and function. This concept would be in keeping with the observation of altered ECM production and deposition throughout the bronchial wall in asthma and the correlation of fibroblast proteoglycan production with AHR (Westergren-Thorsson *et al.* 2002) and the *in-vitro* observations of ECM components differentially modulating ASM phenotype and contractility (Johnson *et al.* 2004b).

Increased airway stiffness can however be considered as a regulatory process initiated by the airway as a mechanism by which to counteract the airway narrowing induced by excessive and abnormal ASM contraction (Niimi *et al.* 2003). ECM restriction of exaggerated ASM contraction maybe beneficial at first, but overtime, excessive ECM deposition around ASM may explain the progression to fixed airway obstruction. Asthmatics with a greater tendency to activate this ECM pathway may then be the group that shows progressive loss of airway patency and eventually fixed obstruction.

1.7 Mechanisms of airway remodelling

1.7.1 Introduction

At present our understanding of the mechanisms of airway remodelling are limited. There is clearly a need to appreciate the exact relationship of airway inflammation to airway remodelling. In particular it is important to understand what components of AHR are related to inflammation and remodelling respectively. At present there are no non-invasive methods of assessing airway remodelling and furthermore human airway research is limited mainly to morphological and *in-vitro* experimental work which is constrained further by ethical implications. Therefore animal models of airway remodelling have been instrumental in defining the dynamic and complex mechanisms involved.

1.7.2 Human models of airway remodelling

Time course studies with intradermal allergen challenge in skin thus confirmed that myofibroblasts (identified on the basis of morphology and expressing α-smooth muscle actin) were increased at 24 hours and further increased at 48 hours (Phipps *et al.* 2002). HSP-47 and procollagen I positive fibroblast-like cells were similarly prominent at 48 hours.Tenascin expression, a highly regulated member of the ECM family that is expressed during development and in response to injury, was evident as early on as 6 hours after allergen injection with a peak of expression at 24 hours and persistence even at 72 hours. Such remodelling changes may have been due to the availability of eosinophil-derived TGF-β_1. After only a single airway allergen challenge in asthmatics upregulation of tenascin in the RBM was also evident. Active collagen synthesis (HSP-47 expression) and TGF-β signalling (pSmad2) was co-localised to both epithelium and fibroblasts 24 hours following allergen (Phipps *et al.* 2004). Others have confirmed myofibroblast accumulation in the airway within 24 hours of allergen challenge as reported by Gizycki (Gizycki *et al.* 1997). The suggestion is that allergen provocation of asthma leads to acute activation of the epithelium and fibroblasts (the epithelial mesenchymal trophic unit or EMTU) together with up-regulation of markers of remodelling and activation of TGF-β signalling. It would important to know whether the activation of airway remodelling remains associated with inflammation and the relationship to AHR.

The lack of non-invasive methods of assessing remodelling in human asthma has meant that most of our current knowledge into human airway remodelling is on the basis of morphological and *in-vitro* experimental work. However, the airway represents a unique opportunity to understand the dynamic process of airway remodelling in that it can be easily and safely sampled by experienced operators through bronchoscopy. By using allergen challenge, one can provoke the disease and sample the airway in a time-course manner to in order to study the dynamic process of events in airway remodelling and the association of these events with changes in airway physiology.

1.7.3 Animal models of airway remodelling

Much of our identification and improved understanding of basic immunological mechanisms in allergic asthma has arisen in the last decade using animal models, predominantly mice. The popularity of murine models has mainly arisen because

many hypotheses involving molecular and one-gene mediated diseases can be effectively studied in whole animals. By performing chronic airway inhalational allergen challenge using appropriately selected strains that have been systemically sensitised to allergen it is possible to induce certain inflammatory and remodelling features in murine airways that mimic changes associated with human asthma (Blyth *et al.* 1996). The phenotypic expression of asthma is highly dependent on the genetic constitution of the murine strain (Brewer *et al.* 1999). Strains can be classified as to whether they are high or low responders in terms of specific IgE and inflammatory airway changes in response to repeated airway allergen challenges. In particular A/J and BALB/c respond to allergen with a high specific IgE production and an inflammatory response comparable to some aspects of the human airway response. It appears that the manifestation of AHR is strain dependent (Takeda *et al.* 2001) with A/J strain mice displaying the greatest degree of AHR and remodelling changes (Shinagawa & Kojima 2003). Strain heterogeneity effects cellular localisation, in particular of eosinophils (Takeda *et al.* 2001), and the propensity for angiogenesis (Rohan *et al.* 2000). Thus the protocol used to sensitise and subsequently challenge the animal must be borne in mind when developing animal models. The most successful results are obtained using a combination of systemic sensitisation with either subcutaneous or intraperitoneal injection of allergen adsorbed onto adjuvants such as aluminium hydroxide. There is a strong induction of Th2 responses. Most protocols now deliver multiple airway challenges with allergen through either aerosol delivery or intranasal or intratracheal installation. The route of allergen challenge may determine the longevity of the model as the induction of tolerance can occur with aerosol challenges (Sakai *et al.* 2001). The best models in terms of longevity have been established using either low mass concentrations of aerolised antigen (Temelkovski *et al.* 1998) or intranasal challenges (Shinagawa & Kojima 2003). Indeed the variability in strains and protocols used in murine models of airway remodelling may explain the sometimes conflicting results that have been obtained (Humbles *et al.* 2004; Lee *et al.* 2004b).

The mechanisms of airway remodelling have also been studied using transgenic mice that could be made to over or under express specific cytokines in the lung. For example over-expression of IL-5 (Lee *et al.* 1997), IL-11 (Tang et al. 1996) and IL-13 (Zhu et al. 1999) mimics some aspects of airway remodelling seen in asthma. Such transgenic models clearly provide valuable insights into dissecting the inflammatory

cascades that propagate remodelling. It must be remembered, however, that transgenic models mimic isolated aspects of a complex process clearly representing an artificial disease environment and process. Inducing the remodelling process in a situation that more closely mimics the induction and propagation in humans will provide a more relevant insight into the disease process that can be directly transferred into therapeutic outcomes. Models of disease in large animals such as sheep and monkeys, on the presumption that such models may provide greater homology to human disease, have also been developed.

The salient features of selected studies using animal models of airway remodelling are summarised in Table 1.1 in order to appreciate the different allergen sensitisation routes and protocols used by groups (Hogaboam *et al.* 2000; Blyth *et al.* 2000; Leigh *et al.* 2002; McMillan & Lloyd 2004; Kumar *et al.* 2004; Cho *et al.* 2004; Johnson *et al.* 2004a; Snibson *et al.* 2005; Tran *et al.* 2004). These have provided us with essential information on inflammatory and remodelling processes but there is considerable controversy as to the exact relevance to human disease (Gelfand 2002; Persson 2002). For cellular and molecular findings in an animal model to be translated into effective therapeutic outcomes in human disease the model must display similar physiological and immunological characteristics to humans and disease events must have a temporal relationship from initiation and disease progression to end stage. Whilst murine models essentially induce airway inflammation and repair response using the similar principle of allergen exposure, such models provide insight into mechanisms that are induced in response to airway injury rather than chronic asthma. Such models do not display background chronic inflammation or baseline AHR in the airway as in the human disease. Such fundamental differences between animal models and human disease may explain to some extent why in animal models of airway injury several aspects of remodelling can be prevented by corticosteroids. However the studies in human asthma are also fundamentally different in that many have addressed only the reversal of remodelling using corticosteroids rather than preventative strategies by obtaining baseline bronchial biopsies and following remodelling changes when on steroid therapy (Beckett & Howarth 2003). It is therefore important to develop preventative studies of remodelling in human asthma if possible.

Also there is convincing evidence that TGF-β_{1-3}, activin and BMP (i.e TGF-Superfamily ligand) signalling is activated after airway allergen challenge in a mouse model of allergen induced airway injury (Rosendahl *et al.* 2001)(Rosendahl et al. 2002) and administration of an anti-TGF-β_1 antibody abrogates several aspects of remodelling (McMillan *et al.* 2005). These models are discussed later under the relevant sections.

Table 1.1: Summary of animal models of airway remodelling (overleaf).

OVA=Ovalbumin
Alum= aluminium hydroxide (a Th2-promoting adjuvant used in most mouse models of allergic airway inflammation)
HDM=House Dust Mite
IP=Intraperitoneal
IN= Intranasal
IT=Intratracheal
AW=Airway
FOB=Fibreoptic bronchoscopy
N/A=Not Available
SC=Subcutaneous
IM=Intramuscular

Table 1.1: Summary of animal models of airway remodelling

Author (ref)	Species	Strain	Sensitisation Route Allergen	Challenge Protocol	AHR	Eosinophils	TGF	Remodelling
Hogabaum *et al* 1999	Mouse	CBA/J	IP/SC A.Fumigatus	IN weekly for 3 weeks IT 4th week	Increased	Increased	Increased	Increased
Blyth et al 2000	Mouse	BALB/c (anti-IL-5)	IP OVA	IT 3 days	N/A	Eliminated	N/A	Decreased
Leigh *et al* 2002	Mouse	BALB/c	IP(2x)/IN(1x) OVA(alum)	IN Acute 2days	Increased	Not increased after 24 hours	N/A	ECM Not increased
				IN Chronic six 2 day periods	Increased	Not increased after 24 hours		ECM Increased
McMillan & Lloyd 2004	Mouse	BALB/c	IP OVA (alum)	AW				
				Acute daily for 6 days	Increased	Increased	Negative	Increased
				Chronic subsequently 3 times weekly	Increased	Increased	Increased	Increased
Kumar *et al* 2004	Mouse	BALB/c	IP OVA (alum)	AW 3 days week for 6 weeks.	Increased	Increased	N/A	Increased
				Day29,34,38,42 given anti-IL-5	No effect	Decreased		Decreased
				anti-IL-13	Decreased	Decreased		Decreased
Cho *et al* 2004	Mouse	C57BL (IL-5 deficient)	IP OVA (alum)	IN 2 days per week for 3 months	N/A	Decreased	Decreased	Decreased
Johnson *et al* 2004	Mouse	BALB/c	HDM	IN 5 days per week for 7 weeks	Increased	Increased	N/A	Increased
Snibson *et al* 2004	Sheep	N/A	SC A/W HDM	FOB segmental lung 2 days a week for 3 months Weekly for 3 months	N/A	Increased at week 26	N/A	Increased
Tran *et al* 2004	Monkey	Rhesus	SC HDM (alum) IM Pertussis toxin	AW Days 1-3 every 2 weeks (total 11 episodes) periodically for 5 months	N/A	N/A	N/A	Increased

1.8 The pathogenesis of airway remodelling

1.8.1 Introduction

Much of the research so far has focused on defining complex changes of airway remodelling that occur in asthma. An urgent research priority that remains is to define the precise biological mechanisms that lead to such structural change; to what extent different remodelling aspects must interact to define the various asthma phenotypes seen in clinical practice and what aspects contribute to disease severity. Increasing severity and chronicity is associated with different disease characteristics that may be associated with various degrees of either fixed airway obstruction, increasing AHR with or without a degree of corticosteroid refractoriness. Individual genetic susceptibility factors predisposing to airway injury, the inflammatory response to tissue injury, the ability to regulate this inflammatory response and the degree of structural cell activation leading to tissue repair all contribute to different aspects of disease pathology and functional consequences.

1.8.2 Early origins of asthma and genetic susceptibility

A linear model of disease that is currently established is that environmental sensitisation leads to establishment of Th2 allergic inflammation subsequently leading to airway remodelling over time. The predominant focus of asthma research into the mechanisms of airway inflammation and how this may causally relate to AHR and the asthma phenotype lead to the basic concept that airway remodelling is a result of airway inflammation and must therefore be a chronic slow process developing over time and contributes to the disease phenotype late on in the disease process. This concept has now being challenged by bronchoscopic studies in paediatric asthma. Bronchial biopsies from children with asthma show that marked remodelling is present very early on in the disease suggesting remodelling may be an early disease event. (Cutz *et al.* 1978) (Payne *et al.* 2003) and may even predate the onset of symptoms by up to four years (Pohunek *et al.* 2005). In established childhood asthma collagen deposition and fibroblast proliferation have greater diagnostic significance than eosinophilic inflammation (Cokugras *et al.* 2001). Other studies have gone onto confirm that airway remodelling markers such as RBM thickening and abnormal structural cell activation is consistently present in childhood asthma and may occur even in the absence of increased eosinophilic inflammation (Fedorov *et al.* 2005). Whilst RBM thickening is considered to reflect remodelling events deeper in the submucosa (James *et al.* 2002), there is little or no correlation of RBM thickness with

the duration of asthma (Payne *et al.* 2003) or cellular inflammation (Cokugras *et al.* 2001; Payne *et al.* 2003; Payne *et al.* 2004), suggesting that RBM thickening, at least, may be independent of inflammation. It is therefore possible that airway remodelling may precede inflammation, suggesting the airway in asthma may have an intrinsic propensity for injury and abnormal repair.

Genetic susceptibility is a significant disease risk factor and such susceptibility is a result of several genes which interact at different stages of disease pathogenesis that can result and may explain the complex disease phenotypes with variable therapeutic responses observed in clinical practice. The discovery of ADAM33 (a disintegrin and metalloprotease33), an asthma susceptibility gene that shows strong linkage to AHR, maybe relevant and supports the concept of tissue susceptibility and impaired repair in asthma AHR (Van Eerdewegh *et al.* 2002). ADAM33 is selectively expressed in fibroblasts and ASM. The exact role is uncertain but ADAM33 is able to release growth factors, modulate the expression of cell-surface receptors and may therefore play an important role in differentiation and proliferation of airway mesenchymal cells (Holgate *et al.* 2006). This is important in that fibroblast accumulation and ASM hypertrophy, not airway inflammation, are the only selective determinants of severe persistent symptoms (Benayoun *et al.* 2003). Polymorphisms in ADAM33 are associated with a more rapid annual decline in post-bronchodilator FEV_1 (Jongepier *et al.* 2004). Such genetic associations may explain why outcomes in adult asthma can to a certain extent be predicted in childhood (Simpson *et al.* 2005).

1.8.3 Inflammation

Most fibrotic diseases share a common paradigm of a persistent inflammatory stimulus with lymphocyte-monocyte interactions that generate fibrogenic cytokines that can initiate and even propagate fibrotic processes. Inflammatory cells are a significant source of growth factors such as TGF-β_1 (Minshall *et al.* 1997) and other TGF-Superfamily ligands, lipid mediators (Wenzel 2003) and the important cytokine IL-13 that can directly activate structural cells of the airway. It is therefore not unexpected that some aspects of airway structural cell activation and remodelling will be related to inflammatory consequences.

T cells

There is increasing recognition of the contribution of $CD4^+$ Th2 released cytokines in fibrotic diseases, particularly IL-13. In contrast Th1 dominated reactions are associated with the attenuation of the fibrotic process, related to the dominant anti-fibrotic effect of γ-interferon (IFN). Whilst some of the pro-fibrotic effects of Th2 cytokines will be related to the up-regulation of pro-fibrotic genes includings the collagens and MMPs (Sandler *et al.* 2003), the recruitment and activation of inflammatory cells, particularly IL-5-dependent recruitment of eosinophils, that are subsequently an important source of fibrogenic factors such as TGF-β_1 will also drive fibrosis. Anti-IL-5 therapy was associated with decreased expression of several remodelling components (Flood-Page *et al.* 2003a).

The mechanisms of airway remodelling were initially studied using transgenic mice that could be made to over-express specific cytokines in the lung. IL-4, IL-5 and IL-9 over-expression was associated with marked mucus metaplasia whilst IL-9 and IL-5 over-expression lead to thickening of the BM and AHR (Temann *et al.* 1998; Lee *et al.* 1997). IL-11 over expression was associated with ASM hyperplasia (Tang *et al.* 1996). IL-4 and IL-13 induced fibroblast differentiation into myofibroblasts and induction of Type III collagen even in the absence of cellular inflammation. The blockade of IL-13 by administration of a soluble form of the IL-13R chain that binds IL-13 was able to reverse AHR and mucus production (Wills-Karp *et al.* 1998) whilst IL-13 administration leads to AHR, eosinophilic inflammation, mucus cell hyperplasia and secretion. Importantly IL-13 over-expression lead to a dramatic fibrotic response in the lung with subepthelial fibrosis (Zhu *et al.* 1999). Given that both IL-4 and IL-13 signal via the same receptor and the STAT-6 signalling pathway, it is surprising that it is only IL-13 that demonstrates marked fibrogenic properties. This maybe related to the ability of IL-13 to induce the production of TGF-β_1 and also activate stored latent TGF-β_1 (Lee *et al.* 2001).

IL-13 induction of TGF-β_1 and other profibrotic molecules in the lung may be predominantly through activation of epithelium (Wen *et al.* 2002). In addition IL-13 demonstrates the ability to activate the fibrotic process independent of TGF-β_1 (Kaviratne *et al.* 2004) by stimulating myofibroblast proliferation via platelet derived growth factor AA (PDGF-AA) (Ingram *et al.* 2003).

$CD8^+$ T cells may also contribute to airway remodelling. In a prospective study of asthmatics over 7.5 years the loss of post-bronchodilator FEV_1 correlated to the increasing number of $CD8^+$ T cells rather than the degree of eosinophilic inflammation (van Rensen *et al.* 2005).

Lipid mediators

The lipoxygenase pathway is particularly active in eosinophils, mast cells and neutrophils.The cysteinyl-leukotrienes (CysLTs) act at their cell-surface receptors CysLT1R and CysLT2R on target cells to contract bronchial smooth muscle and to increase permeability of small blood vessels. Several lines of evidence suggest a role for CysLTs in airway remodelling. *In-vitro* studies confirm increased structural cell activation with marked proliferation of airway epithelium and as well fibroblasts in response to LTC_4 (Leikauf *et al.* 1990) whilst LTD_4 participates with growth facotors in induction of ASM proliferation (Panettieri *et al.* 1998). The clinical consequences of such findings remain to be determined.

Eosinophils

Recent studies in animal models and humans have firmly established the role of eosinophils in lung fibrosis. IL-5 deficient mice displayed markedly less total lung collagen, peribronchial collagen Type III and IV deposition, and α-smooth muscle actin expression compared to wild type mice in response to chronic ova challenge (Cho *et al.* 2004). Importantly the reduction in cells that stained positively for MBP paralleled the reduction in the total number of cells that expressed TGF-β_1 together with the expression of integrin $\alpha_v\beta_6$, an activator of latent TGF-β_1.This was in contrast to wild type mice. In a more recent study, mice with targeted eosinophil depletion were protected from peribronchial collagen deposition and increased airway smooth muscle mass in response to chronic allergen exposure (Humbles *et al.* 2004). AHR and mucus production did not decrease in this model. This maybe a reflection of the fact that IL-13, a key cytokine implicated in the pathogenesis of AHR and mucus production, was not affected. Significantly, studies in humans have been in agreement with animal models. The use of a monoclonal antibody against IL-5 in humans has demonstrated a reduction in airway eosinophils associated with decreased BAL TGF-β_1 levels and expression of the ECM components tenascin, lumican and procollagen III (Flood-Page *et al.* 2003a). Eosinophils are an important source of several fibrogenic cytokines and modulators of remodelling such as the fibroblast and smooth

muscle cell mitogen heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Powell *et al.* 1993), TGF-α (Wong *et al.* 1990), nerve growth factor (NGF) (Solomon *et al.* 1998), TGF-β_1 (Wong *et al.* 1991) and TGF-β_2 (Balzar *et al.* 2005). IL-4 and IL-13 are also produced by eosinophils (Moqbel *et al.* 1995; Schmid-Grendelmeier *et al.* 2002).

Macrophages

Investigation of the exact role of macrophages (Mø) in airway remodelling has been neglected. This is despite Mø being important cells in the process of wound repair and as a cellular source of TGF-β_1 (Rappolee *et al.* 1988; Wahl *et al.* 1990). In the bleomycin model of pulmonary fibrosis Mø derived TGF-β_1 drives the fibrotic process (Khalil *et al.* 1996). Mø produce IL-13 (Hancock *et al.* 1998) and given the high numbers of airway macrophages present, will represent a significant inflammatory cell source of IL-13.

Mast cells

There is increasing recognition that mast cells (MC) play an important contribution to the fibrotic process. MC derived mediators can modulate both fibroblasts and ASM function and are listed below. Of particular interest is tryptase which signals through the G-protein coupled protease activated receptor type-2 (PAR-2) present on inflammatory cells as well as epithelium, fibroblasts and ASM (Hallgren & Pejler 2006) activation of which can lead to structural cell activation and proliferation (Akers *et al.* 2000) (Berger *et al.* 2001). Tryptase can lead to activation (Cairns & Walls 1996) and proliferation of epithelium (Cairns & Walls 1997). Tryptase also induces fibroblast proliferation and ECM production (Cairns & Walls 1997).

Mast cells are the only cells that are known to lie in the ASM layer in asthma (Brightling *et al.* 2002) and given the close relationship of ASM mass to asthma severity and AHR, it is possible that mast-cell induced ASM remodelling represents an important though not yet defined mechanism of airway remodelling. Mast cells secrete the chemokine CCL19 (Kaur *et al.* 2006) which can lead to ASM migration and hence contribute to the increases in ASM mass and hence possibly AHR in asthma. Tryptase can stimulate ASM proliferation (Berger *et al.* 2001). Mast cell derived growth factors also have significant potential to contribute to remodelling by mesenchymal cell activation and proliferation (Cho *et al.* 2003) leading to increased

ECM production. Both tryptase and chymase have the potential to activate MMPs leading to ECM degradation (Johnson *et al.* 1998) (Tchougounova *et al.* 2001). In fact mast cells can secrete MMP-9 (Baram *et al.* 2001). Mast cells are also sources of LTC_4 (Gulliksson *et al.* 2006). Mast cell derived IL-4 and IL-13 may also modulate remodelling (Brightling *et al.* 2003).

Neutrophils

The neutrophil mediators LTB_4, elastase and proteinase are potent inducers of airway glandular mucus production (Cardell *et al.* 1999; Witko-Sarsat *et al.* 1999). Neutrophil elastase and cathepsin G, both major neutrophil proteases, can degrade ECM components such as elastin, collagen and proteoglycans. Neutrophils also secrete MMP-9 (Wenzel *et al.* 2003).

In asthma the inflammatory process can extend into the smaller airways and alveolar compartments (Kraft *et al.* 1999). It is therefore possible the neutrophil derived elastase contributes to the elastin degradation documented in asthma (Bousquet *et al.* 1992). In severe asthma remodelling of cartilage in airways only 1-5mm in diameter has also been reported (Roberts 1995). Remodelling as a result of degradation of cartilage and elastin may contribute to AHR and airway obstruction by decreasing ASM load and thus the force required for the ASM to constrict the airway (Bramley *et al.* 1995). Neutrophils may also be an important source of TGF-β_1 (Chu *et al.* 2000) in asthma further implicating a role for these cells airway remodelling. In the bleomycin model of pulmonary fibrosis a lack of neutrophil elastase is associated with marked attenuation of fibrosis. Neutrophil elastase is demonstrated to be a potent activator of TGF-β_1 (Chua *et al.* 2007).

1.8.4 Epithelial-mesenchymal trophic unit (EMTU) activation

Airway injury is associated with release of cytotoxic mediators, free oxygen radicals and collagenases from both inflammatory cells and epithelium. Epithelial and RBM integrity will be impaired in consequence. The resultant activation of the epithelium allows participation in the immune system through the expression of adhesion and signalling receptors, the secretion of cytokines and growth factors (Kelly *et al.* 2005) with the aim of tissue repair and restoration. The vast surface area of the airway epithelium suggests that the epithelium is a significant source of growth factors. Epithelial and RBM restitution must occur rapidly and in a co-ordinated way. An

important group of factors involved in this process is the epidermal growth factor (EGF) family and the EGF (R) receptors, the expression of which are markedly up-regulated in the asthmatic airway epithelium in response to injury (Puddicombe *et al.* 2000). The airway repair process begins with the deposition of ECM proteins over the surface of the denuded epithelium. This provisional matrix acts as a substrate over which the basal epithelial cells at the 'wound edge' can attach to, proliferate, flatten out and subsequently migrate over. The provisional ECM is composed of the remnant components of the basement membrane such as Type IV collagen and laminin as well as blood derived factors such as fibrin and fibronectin. Epithelial cells serve too as a source of fibronectin and collagens, in particular Type I and Type III collagen. Activation of the attenuated fibroblast sheath now occurs, particularly in response to secretion of TGF-β Superfamily of growth factors. The marked increase in the myofibroblast cell numbers is associated with an increased capacity for synthesis of collagen as well as other ECM components. Myofibroblast migration can now occur in response to chemotactic gradients into the submucosa along ECM fibrils. Using cell surface integrins attachments to collagen and fibronectin occurs. While one end of the fibroblast remains attached to the ECM, the cell can extend a cytoplasmic projection and attach to another ECM area. The site of original attachment is broken via proteases such as MMPs secreted by the fibroblasts. The cytoskeletal network of actin fibres now contract causing the cell to move itself forward.

The repair process in asthmatic epithelium may be impaired. The normal response to EGFR signalling is epithelial proliferation with view to tissue repair. In asthma there is impaired proliferation as evidenced by low expression of PCNA (Fedorov *et al.* 2005; Demoly *et al.* 1994) and evidence of ongoing injury (as evidenced by high expression of the caspase cleavage product p85). This impaired repair phenotype can be likened to a chronic wound scenario with continuous activated and dysregulated epithelium that drives inflammation and remodelling (Boxall *et al.* 2006). An important and possibly fundamental pathway is the epithelium interacting with the underlying attenuated fibroblast sheath through which communication and translation of environmental signals into the deeper submucosal compartment where the major remodelling changes associated with asthma occurs (Holgate *et al.* 2000).

The human lung is an outgrowth of the embryonic endodermal foregut called the laryngotracheal groove, seen as a diverticulum of the primitive pharynx ventral wall

around 4-5 weeks of gestation (Warburton & Lee 1999). The proximal portion gives rise to the larynx and trachea whilst the distal portion now undergoes a process of dichotomous branching onto the surrounding splanchnic mesenchyme with determined precision of repeated bud outgrowth and division of the terminal units. This process is termed branching morphogenesis and is complete by 25 weeks gestation (Warburton & Lee 1999). The stereotypic nature of this process implies that a 'hard-wired' genetic process must exist. Most organ systems, including the lung, are composed of the two primary tissue layers of epithelium and mesenchyme, termed the epithelial mesenchymal trophic unit (EMTU). During organogenesis, epithelium and mesenchymal interaction must occur for morphogenesis and cell differentiation (Warburton & Lee 1999). There is increasing evidence that such co-operation together with signalling programmes and growth factors that are fundamental to organogenesis is reactivated in the process of tissue repair in disease. Therefore in asthma activation of epithelium and fibroblast signalling is reminiscent of the process of epithelial-mesenchymal signalling in the process of branching morphogenesis in the embryonic lung. In the developing lung it is the mesoderm surrounding the endoderm (termed the splanchnic mesoderm) that controls the extent of branching in the respiratory tract. Budding endoderm exerts a high rate of epithelial cell proliferation but such proliferation is inhibited at points of branching. Growth factors, particularly of the fibroblast growth factor (FGF) and TGF-β Superfamily (including TGF-β_1, activins and bone morphogenic proteins or BMPs) are important. FGF-10 produced by the mesoderem primarily stimulates initial bud growth (i.e epithelial cell proliferation). Tips of the epithelial buds produce BMP-4 which serves to repress FGF-10. The proto-oncogene *n*-myc acts to stimulate branching but the expression of *n*-myc is inhibited by TGF-β_1 that is itself expressed at high concentrations along the formed airway walls (Warburton *et al.* 2005). Deposition of Type I and III collagen, fibronectin and proteoglycans serve to stabilise the branch point. In the distal branching points the basal lamina underlying the lung epithelial layer is porous and therefore allows direct communication between epithelial and mesenchymal cell processes. Whilst in embryogenesis the coordinated activity of epithelium and mesenchymal tissue leads to the regulated and organised process of organogenesis, any prolonged activation or dysregulation of this process in disease settings may lead to excessive and abnormal tissue deposition (Warburton & Bellusci 2004).

In asthma the continuous activated state of the airway epithelium is evident even in children taking inhaled steroids and where there was no significant eosinophilic inflammation (Fedorov *et al.* 2005). Activated epithelium will synthesise and secrete growth factors, particularly of the TGF-β Superfamily and IL-13, angiogenic factors such as vascular endothelial growth factor (VEGF) and other Th2 cytokines all of which leads to constant signalling to the underlying mesenchymal cellular pathway (Zhang *et al.* 1999). Such a chronic wound model will not only further establish and propagate inflammation but lead to chronic structural cell activation associated with progressive structural changes associated with severity of disease. The role of IL-13, in particular, is of importance to EMTU signalling (Richter *et al.* 2001). It has been shown that STAT6 signalling alone in airway epithelium leads to leads to AHR (Kuperman *et al.* 1998; Kuperman *et al.* 2002).

Thus it is possible that the development of Th2 inflammation and tissue repair in response to airway injury may occur in parallel rather than sequential events, the interaction of which may lead to chronic epithelial activation and fibroblast and ASM signalling that can drive the disease process.

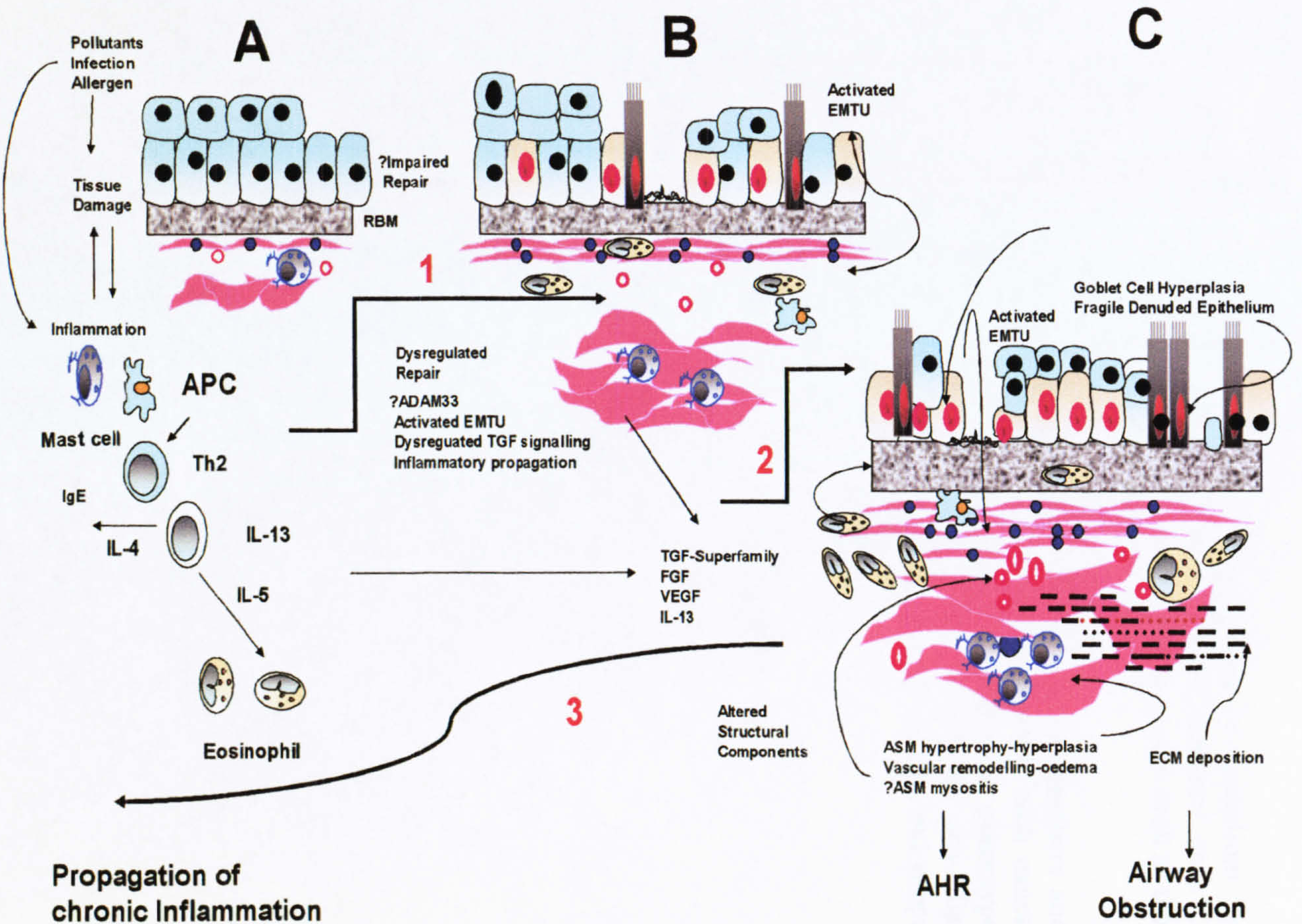


Figure 1.3: Summary of some current concepts in the pathogenesis of airway remodelling in allergen-induced asthma.

Figure 1.3: Summary of some current concepts in the pathogenesis of airway remodelling in allergen-induced asthma.

Figure 1.3A demonstrates an early asthmatic airway with intact airway epithelium, and increased thickening of the reticular basement membrane (RBM). Exposure to environmental insults may lead to airway damage and individuals with a genetic predisposition to atopy and/or dysregulated or impaired airway repair may subsequently develop sustained inflammation and tissue repair.

Figure 1.3B demonstrates increased and sustained activation of epithelium leading to signalling to inflammatory cells and also the underlying mesenchymal cells (activated EMTU). Both structural and inflammatory cell sources of growth factors such as those of the TGF-β Superfamily as well as VEGF and IL-13 are important.

Figure 1.3C illustrates progressive structural changes with increased numbers and size of fibroblasts and airway smooth muscle, vascular remodelling together with excessive and dysregulated ECM deposition, the balance of which may lead to a phenotype that is characterised by increases in AHR or airway obstruction. In turn the pro-inflammatory environment generated by chronic structural cell activation will sustain and propagate the inflammatory response to ongoing environmental insults.

1.9 Growth Factors

Introduction

Growth factors are a heterogeneous group of signalling factors which share a common feature of regulating cell proliferation. Although there is a large list of growth factor molecules with diverse ranging biochemical properties described, it is possible to identify subgroups of growth factors within this group on the basis of amino acid sequences and structural conformation or shared biological activation or function. Once the three-dimensional structures of these molecules were defined it became apparent that specific tertiary levels of structural organisation existed and was a shared feature amongst certain growth factors. It was this realisation that introduced the concept of growth factor 'superfamily' as a means of classifying these factors. However such shared structural features do not necessarily predict shared biological functions within such superfamilies but indicate that these signalling factors will have evolved from a more restricted precursor in parallel to diversification in cellular function and maybe a means that allows nature to achieve target cell specificity. An important characteristic of growth factors is that they act locally within the tissue compartment produced with both autocrine and paracrine effects.

Given the complexity of cellular tissue, it is obvious that cellular response to a specific growth factor has the potential to be modulated by the prior or concurrent action of other growth factors in the cellular microenvironment. Such a conceptual framework allows us to understand how the effect of a growth factor will be altered depending on the tissue and cellular setting present.

1.10 Vascular endothelial growth factor (VEGF)

Angiogenesis is a prominent feature of chronic inflammatory conditions and tumours (McDonald 2001). It is driven by the overproduction of multiple growth factors that include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiogenin. VEGF is a potent multifunctional cytokine which can drive not only inflammation and vascular and tissue remodelling but also enhance antigen presentation and Th_2 inflammation in asthma (Lee *et al.* 2004).

The mechanisms that lead to altered vasculature in asthma are not fully understood. Whist several inducers of angiogenesis can be identified in asthma such as FGF

family, TGF-α and PDGF, it is VEGF that is the most potent inducer of angiogenesis and the expression in asthma is markedly elevated compared to normals (Hoshino *et al.* 2001). VEGF was originally identified on the basis of its ability to induce tissue oedema. VEGF exists as six different isoforms generated as a result of alternate splicing. The major isoforms differ in terms of their bioavailability which is determined to a significant extent by ability to bind to heparin and heparin sulphate of the ECM. The ECM can therefore form a VEGF reservoir that is mobilised by proteolysis.

The lung is an organ with one of the highest levels of VEGF expression. Much of the work on VEGF has been in relation to its essential role in angiogenesis and endothelial cell survival (Gerber *et al.* 1998). Transgenic animal model of VEGF overexpression, as expected, generated neo-angiogenesis and vascular leakage with mucosal oedema. Interestingly airway inflammation with an exaggerated immune response to allergen challenge and remodelling induction was observed. VEGF directly contributes to the entire asthma phenotype (Lee *et al.* 2004).

1.11 Transforming growth factor (TGF)-β superfamily

1.11.1 Introduction

In mammals the transforming growth factor (TGF)-β superfamily is comprised of more than 35 members. They include the TGF-$\beta_{1\text{-}3}$ isoforms, activins, inhibins and bone morphogenetic proteins (BMPs) as well as the growth differentiation factors (GDFs), müllerian inhibiting substance (MIF), nodal and leftys (Piek *et al.* 1999). These factors demonstrate a remarkable diversity of biological function, from key roles in embryogenesis to regulation of cell growth, differentiation and apoptosis. The ligands are translated as pre-propeptide precursors with an N-terminal signal peptide followed by a prodomain and the mature domain. The typical structural features of this family of proteins are two separate anti-parallel β sheets that are perpendicular to a four-turn α-helix. The structural hallmark of the TGF-β Superfamily is the conserved six to nine cysteine residues in the mature domain, termed the cysteine knot (Sun & Davies 1995). The rigid cysteine knot scaffold serves to stabilise the entire structure and is the region required for dimerisation of the ligands via intermolecular disulphide bond formation. Such striking structural conservation of the family members leads to their characteristic physiochemical properties. The diverse functions

of individual ligands are cell and tissue specific and must always be considered in the context of what other cellular and environmental signals are present.

1.11.2 TGF-β isoforms

There are three different isoforms in mammals termed TGF-β_1, TGF-β_2 and TGF-β_3 which are the product of three separate genes located on chromosomes 19, 1 (de Martin *et al.* 1987) and 14 (ten Dijke *et al.* 1988) (Lander *et al.* 2001) respectively. It is TGF-β_1 that was first discovered as a 'factor' with ability to 'transform' function of cells (Assoian *et al.* 1983) and much research has focused on this prototypic isoform. There is very high sequence homology across the isoforms with 74% homology between TGF-β_1 and TGF-β_2, 78% between TGF-β_1 and TGF-β_3 and 82% between TGF-β_2 and TGF-β_3.

(Adapted from *Scheufler C et al,* 1999)

Figure 1.4: Structural homology of TGF-β Superfamily members

The superposition of TGF-Superfamily protein members illustrates the conformational structural homology present. Typically there are two separated anti-parallel β-sheets and a four-turn α-helix that runs approximately perpendicular to the strands. The overall folding topology can be viewed as a hand with the α-helix representing the wrist, the cysteine knot the wrist and the β-sheets the fingers. BMP-2 is represented in blue, BMP-7 in red, TGF-β_2 in orange and TGF-β_3 in yellow. For clarity only monomers of each protein are presented.

The biologically active mature 112 amino acid C-terminal domains of the TGF-β isoforms are conserved more than 97% across species. The C-terminal region encodes the mature 25 kDa active portion. Such sequence homology explains the similar biological activities of these isoforms observed *in-vitro*. Valuable insight into isoform function has been gleaned from knock-out (KO) murine models. TGF-β_1 KO mice succumb to overwhelming widespread systemic inflammation in the perinatal period at 3-4 weeks (Kulkarni *et al.* 1995) confirming the essential anti-inflammatory role of TGF-β_1. The KO mouse for TGF-β_2 (perinatal mortaility) and TGF-β_3 (death within 20 hours of birth) are not viable as a result of gross embryonic malformation (Sanford *et al.* 1997; Kaartinen *et al.* 1995) and may therefore suggest that these isoforms maybe more functional during the process of epithelial-mesenchymal interaction and tissue repair. Whilst the exact roles (overlapping and non-overlapping) remain to be defined all three isoforms are potent inhibitors of epithelial cell proliferation but can stimulate cells of mesenchymal origin (Fynan & Reiss 1993).

1.11.3 TGF-β isoform expression in mild asthma

TGF-β proteins are regulated by synthesis but are also bound to ECM proteins as inactive forms. The early studies with asthma reported similar distribution and expression levels of TGF-β_1 protein or mRNA in airway biopsies obtained from asthmatics compared to normal volunteers (Aubert *et al.* 1994) and studies since have confirmed that there is no difference in the expression of the TGF-β_1 isoform in the airway between normal and mild asthmatic airway (Hoshino *et al.* 1998). One study reported even weaker staining of airway asthmatic epithelium for TGF-$\beta_{1\text{-}3}$ compared to normals (Magnan *et al.* 1997). Inflammatory sources, particularly eosinophils (Vignola *et al.* 1997) and macrophages (Aubert *et al.* 1994; Vignola *et al.* 1996), together with fibroblasts (Vignola *et al.* 1997) have been identified as important sources of TGF-β in asthma with further increased recruitment and TGF-β_1 expression in response to allergen provocation. Such cell types are present in increased numbers in asthma and together with increased ECM components that function as an important reservoir of TGF (Redington *et al.* 1998) may explain the findings of increased TGF-β_1 levels in BAL between normal and asthmatic airways at baseline with further increases in asthma in response to allergen challenge (Redington *et al.* 1997). Given that TGF-β_1 KO mice display normal embryological development unlike the TGF-β_2 and TGF-β_3 KO mice, it is probable that the latter isoforms have significant roles in epithelial-mesenchymal signalling. At least in models of skin

injury the speed of healing and the degree of scar tissue is related to the amount of wound TGF-β_1, TGF-β_2 and TGF-β_3, with paucity correlated with scar-free healing with reduced expression of collagen I and collagen III (Shah *et al.* 1995). Exogenous TGF-β_1 and TGF-β_2 addition was associated with marked scarring whilst the converse was found with the addition of TGF-β_3 (Shah *et al.* 1995). Further detailed studies to determine the differential expression and functional significance of the TGF-$\beta_{1\text{-}3}$ isoforms in asthma are urgently required.

1.11.4 Synthesis and activation

There is very little sequence conservation between the promoter regions of the TGF-β isoforms indicating that isoform gene transcription is differentially regulated. The exact transcriptional programmes involved are probably context dependent and remain to be fully identified. Additionally all three TGF isoforms can up regulate their own expression (Obberghen-Schilling *et al.* 1988).

Each TGF-β isoform is encoded as a 390-442 amino acid precursor protein that contains a signal sequence, essential for the proper folding and secretion of the isoform. The most critical post-translational modification is the proteolytic cleavage of this signal sequence by an endoprotease furin with the release of the 112 amino acid mature TGF-β ligand (C-terminal domain). The translational protein product common to the TGF-β Superfamily is a 29 amino-acid signal sequence, a pro-peptide region termed the latency associated peptide (LAP) which now non-covalently associates with the C-terminal domain. The TGF-LAP product now dimerises by the formation of intermolecular disulphide bonds with the LAP region and another TGF-β mature region. For TGF-β and activin (presumably in other TGF-β Superfamily members also) the LAP region is essential for this folding and dimerisation process. Without dimer formation the TGF-β cannot be exported out of the cell. The dimeric molecule covalently bonds with a 135 kDa latent TGF-β binding protein (LTBP) which serves to anchor the latent TGF-β ligand to the ECM. The latency conferred to TGF-β by the LTBP prevents binding of secreted TGF-β to ubiquitously expressed receptors and assures a readily accessible extracellular reservoir of TGF-β that can be activated on demand.

Thrombospondin (TSP-1) can activate latent TGF-β by binding to a specific site on LAP leading to a conformational change in the latent complex leading to an active state (Schultz-Cherry *et al.* 1995). The epithelial integrins $\alpha_5\beta_6$ and $\alpha_4\beta_8$, up-regulated in response to lung injury, are important activators of TGF-β ligands (Araya *et al.* 2006). The $\alpha_5\beta_6$ knock out mouse fails to develop pulmonary fibrosis in response to bleomycin confirming the critical role of the epithelium and the TGF-β Superfamily in initiating and regulating mesenchymal cell activation and function (Munger *et al.* 1999).

1.11.5 TGF-β_1 and inflammation

The inflammatory response to tissue injury is the initiation of a complex cascade of events leading to recruitment, migration and activation of inflammatory cells to the site of injury or antigen deposition. Resolution of the response is characterised by regulated apoptosis and matrix deposition. The ultimate goal of this repair response is the elimination of the inciting agent, resolution of inflammation and the restoration of normal tissue architecture. TGF-β_1 has an essential role in all these processes.

TGF-β_1 was initially viewed as an anti-inflammatory cytokine because TGF-β_1 KO mice died of an overwhelming MHC Class II mediated autoimmune-type inflammatory response (Kulkarni *et al.* 1995; Letterio *et al.* 1996) characterised by circulating autoantibodies, marked tissue leukocyte infiltration and increased IFN-γ expression (McCartney-Francis et al. 1996). Since then important roles for TGF-β_1 in both innate and adaptive immunity have been defined.

TGF-β_1 is released from the α-granules of activated platelets, structural cells, inflammatory cells and ECM stores. Activated TGF-β_1 is involved early on in the inflammatory response as it is a chemotactic factor for monocytes (Wahl *et al.* 1987, lymphocytes (Adams *et al.* 1991), mast cells (Gruber *et al.* 1994) and neutrophils (Postlethwaite & Seyer 1995; Allen *et al.* 1990). TGF-β_1 may also influence eosinophil chemotaxis as suggested by *in-vitro* experiments (Luttmann *et al.* 1998). Cells can transmigrate out of the vessel wall at inter-endothelial cell junctions into tissues along a concentration dependent TGF-β_1 gradient (Wahl *et al.* 1987). By promoting integrin expression such as $\alpha_5\beta_1$ (the functional receptor for fibronectin) and $\alpha_3\beta_1$ (the ligand for laminin and Type IV collagen), TGF-β_1 also enables cell adhesion which is critical for regulated and selective cell trafficking to inflammatory

sites (Wahl *et al.* 1993). TGF-β_1 mediated induction of gelatinases (MMP-2 and MMP-9) which then degrade ECM in the path of the cell allows cell migration via tissues to occur (Wahl *et al.* 1993).

TGF-β_1 initially potentiates the inflammatory cascade by the up-regulation of interleukin-1β, TNF-α and IL-6 from activated monocytes and macrophages which act downstream on other inflammatory cells (McCartney-Francis & Wahl 1994). For example IL-1 is an important lymphocyte proliferation cytokine. TGF-β_1 primes T lymphocytes such that the cells are more readily activated with an increased rate of proliferation and IL-2 production in response to subsequent stimulation (Cerwenka *et al.* 1994). TGF-β_1 may also contribute to T cell survival via delayed apoptosis (Cerwenka *et al.* 1996). It may have a critical role in shaping the T cell functional repertoire. TGF-β_1 modulates the differentiation and activation of DCs (Strobl & Knapp 1999) that may subsequently determine the polarisation direction of naïve T cells into either the Th1 or Th2 type (Foucras *et al.* 2000) (Van Weyenbergh *et al.* 2001). In the thymus of the TGF-β_1 KO mouse thymocytes are markedly sensitive to TCR-enforced lethality such that both positive and negative thymocyte selection is disrupted. This leads to escape of self-reactive T cells into the periphery that can initiate autoimmune responses. Neutrophils can also be activated to undergo degranulation by TGF-β_1 (Balazovich *et al.* 1996) which will contribute to the inflammatory process.

TGF-β_1 recruited Mø have an essential role in phagocytosis of apoptotic cells, a key step towards the resolution of inflammation. By up-regulating CD16 (FcγRIII), the low affinity IgG Fc receptor with its important role in the capture and clearance of particles on monocytes and Mø, TGF-β_1 can regulate early innate responses (Welch *et al.* 1990). TGF-β_1 enhances Mø recognition of phosphatidylserine, expressed on the outer membrane of apoptotic cells (Rose *et al.* 1995). Enhanced phagocytic capacity is associated with increased respiratory burst activity and superoxide generation in response to TGF-β_1 by Mø (Welch *et al.* 1990). This is then followed by inhibition of inflammatory cytokine and mediator production such as IL-1β, IL-8, TNF-α, GM-CSF and LTC_4 (Fadok *et al.* 1998).

Eosinophil clearance is crucial to the resolution of inflammation (Woolley *et al.* 1996) and is predominantly dependent on apoptosis. Apoptotic cell products are phagocytosed by Mø (Woolley *et al.* 1996). TGF-β_1 has been demonstrated *in-vitro* to

enhance the rate of eosinophil apoptosis. This may be through TGF-β_1 mediated inhibition of GM-CSF and IL-5 production (Alam *et al.* 1994). Local tissue IL-3, GM-CSF and IL-5 in particular effectively prolong tissue eosinophil survival and the absence of such factors leads to rapid apoptosis (Yamaguchi *et al.* 1991). In addition TGF-β_1 can inhibit eosinophil release of mediators (Alam *et al.* 1994).

Importantly, Mø activation is followed by down-regulation of the macrophage TGF-β_1 receptor leading to decreased sensitivity to TGF-β_1 mediated signalling (McCartney-Francis & Wahl 1994). This is part of the resolution phase of inflammation. *In-vitro* studies indicate high concentrations of TGF-β_1 lead to deactivation of Mø function such as suppression of the respiratory burst (Oswald *et al.* 1992) and cytokine production (TNF-α as one example) (Tsunawaki *et al.* 1988) (Nelson *et al.* 1991). TGF-β_1 mediated attenuation of MHC class II expression and costimulatory molecules on myeloid DCs (Strobl & Knapp 1999) lead to loss of further T cell activation. TGF-β_1 inhibits proliferation and cytokine production by naïve T cells as well as Th1 and Th2 clones with post-activated T cells undergoing rapid apoptosis in response to TGF-β_1 (Sillett *et al.* 2001).

Thus initially TGF-β_1 initially propagates inflammation but later in the cascade becomes a potent anti-inflammatory agent. Thus consideration of the context of TGF-β_1 signalling is important and is very much dependent on the state of cellular differentiation and the cytokine milieu in which the cell is present. It is therefore likely that the functional outcome of TGF-β signalling in asthma will be influenced by the disease microenvironment and suggests non-human asthma models of TGF-β signalling need to be interpreted with caution.

1.11.6 TGF-β_1 and remodelling

Rapid restoration of tissue structure following injury is essential for the maintenance of organ integrity and function. TGF-β_1 is one of the most fibrogenic factors known and therefore much research has focussed on its role in fibrosis.

Airway epithelium is a very significant source of TGF ligands (Zhang *et al.* 1999). Autocrine effects of such ligands will have important disease implications. TGF-β_1 inhibits epithelial cell proliferation (Fjellbirkeland *et al.* 2003) although the mechanisms of such inhibition have not been fully elucidated. One mechanism by

which TGF-β_1 can inhibit cell proliferation is by induction of the cyclin dependent kinase inhibitor p21^{waf} that leads to arrest of the cell cycle in the G1 phase (Reddy *et al.* 1994). In the TGF KO mice expression of PCNA in inflamed tissues is elevated, indicative of uncontrolled cellular proliferation (McCartney-Francis *et al.* 1996). The finding of markedly increased p21^{waf} expression (Puddicombe *et al.* 2003) in the absence of PCNA expression may then be a result of TGF-β_1 mediated epithelial effects which leads to inhibition of epithelial cell proliferation and therefore repair. TGF-β_1 mediated signalling thus contributes to aberrant epithelial repair.

TGF-β_1 is an important fibroblast chemotactic factor (Postlethwaite & Seyer 1995) and fibroblast numbers have been shown to correlate with TGF-β_1 expression (Vignola *et al.* 1997). The TGF-$\beta_{1\text{-}3}$ isoforms are all potent inducers of fibroblast proliferation with TGF-β_3 demonstrated the most potent (McAnulty *et al.* 1997). The isoforms induce the differentiation of fibroblasts to α-SMA$^+$ myofibroblasts (Thannickal *et al.* 2003) and promote the deposition of ECM components, particularly collagen (Coker *et al.* 1997). All three isoforms can rapidly induce collagen I and III mRNA transcription (Ignotz *et al.* 1987) whilst at the same time limiting intracellular collagen degradation as demonstrated in foetal lung fibroblast *in-vitro* experiments (McAnulty *et al.* 1995). TGF-β_1 leads to rapid synthesis of tenascin (Phipps *et al.* 2002), biglycan (Romaris *et al.* 1991), fibronectin (Ignotz *et al.* 1987) and hyaluronan (Westergren-Thorsson *et al.* 1990). By reducing the synthesis of MMPs, particularly collagenases and stromelysins and by increasing the expression of molecules that inhibit them such as the TIMPs (Zeng *et al.* 1996), as well as plasminogen activator inhibitor (PAI) (Lund *et al.* 1987), ECM degradation is prohibited. TGF-β_1 is one of several mediators with effects on ASM including modulation of proliferation (Cohen *et al.* 1997) and collagen synthesis (Coutts *et al.* 2001). From this background it becomes apparent that it is essential to strictly regulate TGF-β ligand activation and signalling. Any dysregulated function therefore has the potential to lead to impaired inflammatory homeostasis and tissue remodelling.

1.12 Activin

1.12.1 Introduction

Activin, originally identified as a factor that regulates gonadal function by regulation of follicle hormone production (FSH) in the anterior pituitary gland (Ying 1988), has important functions as both a cytokine (Munz *et al.* 1999) and growth and

differentiation factor (Chen *et al.* 2002). Whilst much research thus far has focused on the role of activin in tissue developmental programmes and reproduction, there is increasing interest in activin's role in acute inflammation and the response to tissue repair and injury.

The subunits of activin are called β-subunits which are similar in overall structure to the TGF-β subunits. An activin molecule is comprised of two β-subunits of which four isoforms have been identified in mammals:-βA, βB, βC and βE. The most widely expressed isoforms are βA and βB which dimerise to from homodimeric activin-A (βAβA), homodimeric activin-B (βB:βB) and heteromeric activin-AB (βA:βB). The focus of this study is on activin-A, the most widely expressed of the activin ligands. The critical role of activin in embryogenesis is reflected by its extensive expression in nearly all organ systems. In the developed lung bronchial epithelium, monocytes, macrophages, mast cell, CD4 T cells, airway smooth muscle and vascular endothelium all sources of activin-A (Michel *et al.* 2003). Like the TGF-β isoforms, activin-A is secreted as a prepro-ligand that undergoes enzymatic cleavage to an activated form. Similarly, the ECM provides an important store of activin-A that can be rapidly released in response to injury (Jones *et al.* 2004b).

1.12. 2 Physiological inhibitors of activin signalling

A naturally occurring inhibitor of activin-A, inhibin, shares the same β subunit and antagonises activin-A signalling via effects on ligand synthesis, as well as receptor binding and signal transduction (Ying 1988).

Follistatin, a protein monomer unrelated to activins and inhibins, is the most potent physiological inhibitor of all activins. Follistatin has a high affinity for activin and once bound with it activin undergoes rapid endocytic internalisation and subsequent proteolytic degradation (Hashimoto *et al.* 1997). Recent studies also suggest that follistatin may also bind to the BMPs and inhibit their activity (Iemura *et al.* 1998).

1.12.3 Activin-A and inflammation

It was the observation that activin-A inhibited thymocyte proliferation that first suggested a role for activin-A in immune responses as an inflammatory cytokine (Hedger *et al.* 1989). Activin-A expression is up-regulated in response to IL-1α and

TNF-α (Shao *et al.* 1992) and is released as early as one hour in response to lipopolysaccharide (LPS) in a sheep model of acute inflammation (Jones *et al.* 2004a). Activin-A can inhibit IL-6 (Brosh *et al.* 1995) and therefore inhibit IL-6-dependent B cell proliferation and acute phase protein synthesis. Activin-A also stimulates apoptosis of B and T cells (Hashimoto *et al.* 1997). Activin inhibits IL-1β synthesis (Ohguchi *et al.* 1998). Whether the induction of activin-A in response to inflammation serves to propagate or attenuate inflammatory responses, particularly in a disease setting, remains uncertain.

1.12.4 Activin-A and remodelling

Both inflammatory arthritis and inflammatory bowel disease demonstrate marked activin-A expression. In Crohn's disease, activin-A mRNA expression in both the epithelium and submucosa is correlated with that of IL-1α, a marker of disease severity. It was confirmed by *in-situ* hybridisation (ISH) of activin-A mRNA expression that fibroblasts and inflammatory cells are an important submucosal source (Hubner *et al.* 1997). Hepatic fibroblasts (stellate cells) in animal models of liver fibrosis are immunoreactive for activin-A (De Bleser *et al.* 1997). The functional response in cultured hepatic stellate cells to exogenous activin-A is ECM production, and it maybe that there is a synergistic effect of activin-A with TGF-β_1 to increase ECM synthesis in fibroblasts (Sugiyama *et al.* 1998; Date *et al.* 2000). Human lung fibroblasts can actively synthesise activin-A under basal conditions but up to 3 fold induction by TGF-β_1 can occur (Karagiannidis *et al.* 2006).

There is growing evidence to suggest a role for aberrant activin-A expression in lung disease. Murine models of bleomycin-induced lung fibrosis demonstrate prominent immunoreactive activin-A (Matsuse *et al.* 1995) and this is also the case in human interstitial fibrotic lung disease (Matsuse et al. 1996). The administration of follistatin in the same model was associated with a marked attenuation in the number of inflammatory cells and lung fibrosis, suggesting that activin-A plays a critical role in inflammation and repair. Activin-A is a potent inducer of lung fibroblast proliferation and myofibroblast differentiation (Ohga *et al.* 1996).

Mast cells accumulate at sites of inflammation in response to TGF-β_1 and activin signalling (Olsson *et al.* 2000). Activin A is synthesised de novo in mast cells with allergen challenge and induces ASM proliferation (Cho *et al.* 2003). This is

significant given the finding that mast cell infiltration of ASM is associated with AHR in asthma (Brightling *et al.* 2002). In a mouse model of allergen induced airway inflammation a marked upregulation of activin-A mRNA was seen, with epithelium and inflammatory cells as significant sources. There was a concomitant upregulation of the Type I receptor (ALK-4) for activin. Serum activin-A levels were elevated in severe asthmatics only (Karagiannidis *et al.* 2006). Activin-A is unbound in the circulation and is bioactive (Jones *et al.* 2000).

1.13 Bone morphogenetic proteins

1.13.1 Introduction

The bone morphogenetic proteins (BMPs) are the largest subgroup of structurally and functionally related proteins in the TGF-Superfamily of ligands (Hogan 1996). The BMP ligand system represents a major developmental signalling pathway critical for organ embryogenesis and tissue generation such as in the kidney and the lung (Vukicevic *et al.* 1994a). BMPs are highly conserved in nature as seen in organisms as diverse as the nematode *Caenorhabditis elegans* and humans (Estevez *et al.* 1993), with such conservation consistent with the vital role of BMPs in embryonic development and tissue homeostasis.

BMPs were originally identified on the basis of their ability to induce endochondral bone formation (Wang *et al.* 1988). The BMPs are dimeric molecules linked by an interchain disulphide bond. Each BMP monomer demonstrates the characteristically conserved seven cysteine residues (cysteine knot) common to the TGF-β Superfamily. Each member is synthesised as a 400 amino acid precursor which undergoes post-translational processing to a 110 amino acid mature molecule.

1.13.2 Bone morphogenetic proteins and remodelling

Although first identified in bone, it is now apparent that there is widespread expression in other organ systems such myocardium, kidney, adrenals and smooth muscle, endothelium and lung (Chang *et al.* 2002). The vital function of BMPs in organogenesis is evidenced by the knockout mice. BMP-2 KO mice exhibit severe heart abnormality that is embryonically lethal (Zhang & Bradley 1996) and BMP-4 KO display failure of mesodermal induction (Winnier *et al.* 1995). Such findings suggest that BMPs may play an important role in epithelial-mesenchymal interactions during tissue remodelling.

BMP-1 is unrelated to other BMPs as it does not regulate the growth or differentiation of cells. It serves as a protease for the cleavage of procollagen fibrils as well as the BMP antagonist chordin (Uzel *et al.* 2001). BMP-2, BMP-4 and BMP-6 are the most readily detectable ligands in cultures and therefore have been studied extensively. BMP-2 and BMP-4 share 92% amino acid sequence homology which explains their virtually identical functional repertoire. As essential insight into the role of BMPs as one of the key developmental pathways in tissue repair and organogenesis is beginning to be made, it is becoming apparent that many of the BMP signalling pathways are also fundamental for the maintenance, regeneration and repair of tissue.

In lung branching morphogenesis BMP-4 expression is expressed predominantly in the distal epithelium of the bud outgrowth (branching tips) that grows into the mesenchymal tissue. Mesenchyme derived FGF-10 can induce this expression of BMP-4 (Weaver *et al.* 2000). In the presence of this mesenchymal interaction BMP-4 serves to stimulate branching. The context dependence of signalling is illustrated by the finding that BMP-4 inhibits the growth of isolated epithelium in culture systems (Bragg *et al.* 2001; Shi *et al.* 2001). BMP-4 directly increases the number of α-smooth muscle actin positive parabronchial cells in an *in-vivo* lung explant system (Mailleux *et al.* 2005). In diseases where dysregulated BMP signalling is present as primary pulmonary hypertension (due to vascular smooth muscle proliferation) and non-small cell lung cancer (Kraunz *et al.* 2005), the loss of BMP antiproliferative effects allows progrowth signalling pathways to take-over.

BMP-7 (also known as osteogenic protein 1 or OP-1), identified originally as a potent osteogenic factor from bone, is rapidly gaining prominence as an antifibrotic factor by its ability to antagonise the effects of TGF-β_1. Much of our understanding of BMP-7 has come from work in kidney development and disease. BMP-7 expression is seen at sites of epithelial-mesenchymal tissue interactions with regulatory effects on branching morphogenesis. During lung formation expression is most prominent along the basement membrane (Vukicevic *et al.* 1994b). In kidney development BMP-7 regulates branching morphogenesis and serves as a survival factor for epithelium (Vukicevic *et al.* 1996; Piscione *et al.* 1997). End stage kidney disease is characterised by massive fibrosis driven by TGF-β_1 signalling associated with decreased BMP-7 expression (Ueda *et al.* 2005; Klahr 2003). Exogenous

administration of BMP-7 into experimental systems is able to reverse the fibrotic effects of TGF-β_1, through counteracting TGF-β_1 induced epithelial-mesenchymal transition and is associated with decreased expression of Type I collagen by fibroblasts (Zeisberg *et al.* 2003b; Zeisberg *et al.* 2003a). In lung myofibroblasts BMP-7 inhibits TGF-β_1 mediated collagen expression, α-smooth muscle actin expression and TIMP-2 (Izumi *et al.* 2006). It has been demonstrated in both the kidney (Gould *et al.* 2002) and the gut (Maric *et al.* 2003) that BMP-7 potently reduces inflammation and fibrosis. A selective suppression of the expression of pro-inflammatory cytokines was demonstrated. It is tempting to speculate that BMP-7 may offer similar therapeutic prospects in the lung.

1.14 TGF-β Superfamily signalling

1.14.1 Introduction

Upon activation TGF-β ligands signal via a constitutively active serine-threonine kinase specific Type II receptor that complexes with a Type I receptor which then propagates the signal downstream by phosphorylating receptor-regulated Smads (R-Smads) that translocate to the nucleus in association with Smad4 to initiate gene transcription (Figure 1.5). TGF-$\beta_{1\text{-}3}$ and activin signalling is via phosphorylated (p) pSmad2 and pSmad3 whilst pSmad1, pSmad5 and pSmad8 mediate BMP signals. These R-Smads associate with the common Smad4. Smad6 and7 inhibit further signalling by interacting with the Type I receptor. In mammals only seven Type I receptors and five Type II receptors have been identified. Combinatorial interactions in the receptor complex allow differential ligand binding or differential signalling in response to the same ligand, this being tissue specific. Given that the TGF-β Superfamily are present in an inactive state bound to ECM, signalling analysis is required to detect activity of these factors.

1.14.2 TGF-β Superfamily receptors

The TGF-β ligands are dimeric molecules that signal through the assembly of a receptor tetrameric complex consisting of two Type I receptor molecules and two Type II receptor molecules. The receptor structure is characterised by a ligand binding extracellular domain, a single transmembrane domain and a cytoplasmic serine-threonine kinase domain. The receptor sub-types are distinguished on the basis of the distinct sequence conservation of the kinase domain and in the Type I receptor by the presence of the glycine-serine (GS-box) region immediately up-stream from the

catalytic domain in the juxta-membrane region (Wrana *et al.* 1994). In the receptor complex the Type II receptor has constitutively active kinase activity and phosporylates the GS-box that is critical for the activation of the Type I receptor. Phosphorylation leads to conformational change in the GS box leading to ATP binding and substrate phosphorylation of the downstream R-Smads (Wrana *et al.* 1994).

The Type I Receptor for the TGFβ_{1-3} isoforms is (activin-like kinase) ALK-5 (TβRI) (Franzen *et al.* 1993). The activin group of ligands signal through predominantly ALK-4 (ten Dijke *et al.* 1994a). The BMP ligands signal using ALK-2, ALK-3 and ALK-6) (ten Dijke *et al.* 1994a). ALK-1 is complicated in that it is activated by TGF-β_1 but will signal through the usually BMP-restricted Smad1 and Smad5 (Ota *et al.* 2002). The TGF-β isoforms signal through only the Type II receptor TGFβRII (Lin *et al.* 1992). The activins can signal through either Activin RIIA (ActRIIA) or Activin RIIB (ActRIIB) (Attisano *et al.* 1992). Although BMP signalling is predominantly through BMPRII, signalling can also occur via Activin RIIA and RIIB (Liu *et al.* 1995).

Combinatorial interactions in the receptor complex allow differential ligand binding or differential signalling in response to the same ligand which is tissue specific. There is variation in the affinity of TGF ligands for the different receptor combinations. ALK-5 is activated by TβRII for TGF-β signalling (Franzen *et al.* 1993) whilst the binding of activin to ActRIIA recruits ALK-4. BMP ligand signalling is even more complex. BMP-2 and BMP-4 are related and bind either BMPRII or ActRIIA and ActRIIB with preferential recruitment of ALK-3 or ALK-6 (Aoki *et al.* 2001). BMP-6 and BMP-7 bind either ActIIA or ActRIIB and can preferentially recruit ALK-2 (ten Dijke *et al.* 1994b), but also bind ALK-3 or ALK-6. TGF β_{1-3} isoforms bind TβRII but can also activate ALK-1, especially so in endothelial cells (Ota *et al.* 2002). ALK-1 phosphorylates Smad1 and Smad5, the BMP signalling pathway Smads (Chen & Massague 1999). Thus the differential activation of either ALK-1 or ALK-5 by the same ligand leads to a different functional outcome in the cell. ALK-7 is predominantly expressed in neuroendocrine tissue (Ryden *et al.* 1996) and is used by activins other than activin-A, and is therefore not a focus in this study.

1.14.3 Accessory receptors

Betaglycan is a cell-surface TGF binding protein that promotes binding of for TGFβ_1 and β_2 ligands to the Type II receptor (Andres *et al.* 1991). In addition it acts to promote the interaction of inhibin with Act RIIA and Act RIIB, leading to functional inhibition of activin (Lewis *et al.* 2000). Endoglin is another membrane associated receptor that has a role in regulating the assembly of Type I and Type II receptor complexes for TGFβ_1, TGFβ_3 , activin, BMP-2 and BMP-7 (Barbara *et al.* 1999). In contrast to betaglycan, endoglin expression is cell-specific and serves to inhibit rather than enhance TGF ligand responsiveness. Other decoy receptors such as BAMBI (BMP and Activin Membrane Bound Inhibitor) share sequence homology to Type I receptors and offer an additional level of receptor regulation (Onichtchouk *et al.* 1999).

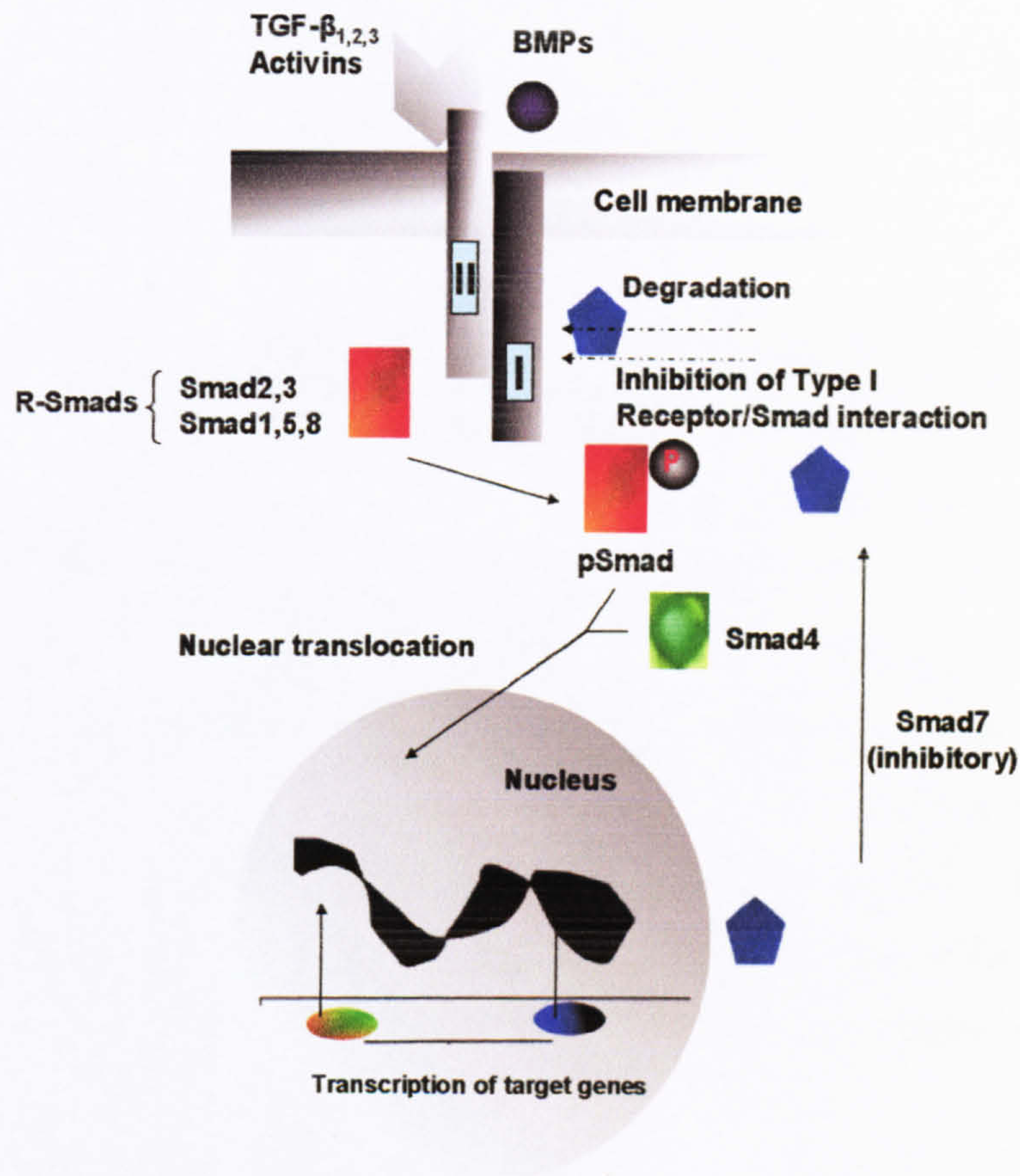


Figure 1.5 Summary of TGF-β Superfamily signalling pathways

TGF-β Superfamily of ligands signal by binding to a constitutively active serine-threonine kinase specific Type II receptor that complexes with a Type I receptor which subsequently propagates the signal downstream by phosphorylating receptor-regulated (R) Smads that translocate to the nucleus to initiate gene transcription.

TGF-β_{1-3} and activin signalling is via phosphorylated (p) pSmad2 and pSmad3 whilst pSmad1, pSmad5 and pSmad8 mediate BMP signals. TGF-β_1 via the Type I receptor ALK-1 can lead to activation of BMP signalling pathways via pSmad1 and pSmad5. R-Smads associate with the common (Co) Smad4. Smad6 and Smad7 expression is rapidly induced in cells in response to TGF-β_{1-3}, activin and BMP ligand signalling. Smad6 and 7 inhibit further signalling by interacting with the Type I receptor. Combinatorial interactions in the receptor complex allow differential ligand binding or differential signalling in response to the same ligand and this is tissue specific. TGF-β ligands are regulated by synthesis but are also bound to ECM proteins as inactive forms which must be activated for example by thrombospondin or $\alpha_v\beta_6$ integrin. Thus signalling analysis is required to detect activity of the factors.

1.14.4 Regulation of receptor activation

Given the critical role of TGF-β signalling pathways it is expected that the point of pathway activation between the Type I receptor and R-Smads will be regulated at several levels.

In the resting state activation of the Type I receptor is prevented by the repressor protein FKBP12 binding to the unphosphorylated GS box. This capping of the Type I receptor phosphorylation site by FKBP12, an FK506 binding immunophilin, prevents activation by the Type II receptor in the basal state (Wang *et al.* 1996). Receptors are further regulated by accessory receptors (such as betaglycan and endoglin) and the substrate anchor proteins such as SARA (Smad anchor for receptor protein). SARA is anchored to the plasma membrane and this is an ideal position for SARA to bind both the receptor complex and either Smad2 or Smad3 (Wu *et al.* 2000). Such interaction serves to stabilise the receptor-Smad complex. Upon phosphorylation of the R-Smad, the SARA–Smad complex dissociates. BMP inactivated Smads are unable to interact with SARA (Tsukazaki *et al.* 1998).

Receptor internalisation and endocytic trafficking of receptors can lead to either activation of signalling by promoting the association of activated receptors with signalling substrates in endosomes or lead to receptor down-regulation by degradation of activated receptor complexes. Both are important mechanisms of regulating signalling pathways in eukaryotic cells (Figure 1.7). Clathrin-dependent pathways are an important mechanism by which receptors are targeted to endosomes and an important pathway for down-regulating many receptors. Another important regulatory mechanism utilises caveolae. Caveolae are formed from caveolin proteins that combine with lipid rafts. Lipid rafts are plasma membrane domains rich in lipids and cholesterol that remain in the liquid phase and recruit hydrophobic proteins linked to plasma proteins. Permanent engagement of a ligand by the receptor induces receptor translocation into the caveolae. TGF-β Superfamily receptors are regulated by both pathways (Di Guglielmo *et al.* 2003). Clathrin-dependent internalisation into early endosomes is important for signal propagation whilst entry into the caveolin positive lipid-rafts leads to receptor degradation and is associated with reduced Smad activation.

1.14.5 Signal transduction by the TGF-β Superfamily

Transcription factors

Transcription factor proteins are nuclear messengers that regulate gene expression. The Smad proteins are a family of transcription factors with a molecular weight of 42 to 65 KDa (Massague *et al.* 2005). Transcription factors initiate gene expression by binding to the regulatory sequences of genes found in the 5' upstream region of the promoter of the target gene. The TATA box (an AT-rich sequence) is found approximately 30 base pairs upstream of the transcription start site of most genes and is essential for positioning the basal transcription machinery at the initiation site of the gene. The promoter is defined by the region of the gene that is bracketed by the TATA box and site of transcriptional initiation termed the Cap site. The promoter region can bind a variety of proteins that direct transcription as well the RNA polymerase II enzyme.

Enhancers are DNA sequence elements that serve to increase the activity of promoters. Remarkably they can be located at great distances from the site of transcription and can contain multiple binding sites for transcription factors. Proteins can bind to the enhancer up or downstream from the promoter but still contact the transcriptional apparatus by looping out of intervening DNA.

Large proteins that bind multiple transcription factors such as the co-activator molecule CREB-binding protein (CBP) and p300 can interact with multiple transcription factors bound to the enhancer and integrate the numerous signals to activate gene transcription. Gene transcription requires DNA unwinding around the histone core. Both CBP and p300 display intrinsic histone acetylation activity that is activated upon binding of transcription factors AP-1 and NF-κB. Histone acetylation leads to DNA, that is coiled round the histone core, unwinding to open up the chromatin structure (Barnes *et al.* 2005). Increased access to DNA sites leads to greater affinity and speed of binding by the transcription machinery and thus more efficient transcription. Smads can directly interact with both CBP and p300, allowing even a greater versatility of signalling targets as discussed below (Janknecht *et al.* 1998).

1.14.6 Smads

It was through studies in Drosophila (Raftery *et al.* 1995; Sekelsky *et al.* 1995) and *Caenorhabditis Elegans* (Savage *et al.* 1996) that insight into the signalling mechanisms downstream from the Type I serine/threonine kinase receptors was first obtained. These studies showed that in *Drosophila Melanogaster Mad* (*mothers against decapentaplegic*) gene and in C.*Elegans* the *Sma* gene coded for a conserved family of TGF-β signalling pathway components with subsequent identification in vertebrates. The genes were thus collectively named as *Smad.*

The Smads are divided into three distinct subfamilies, the receptor-activated Smads (R-Smads) 1,2,3,5 and 8, the common-partner Smads (Co-Smads) of which there is only Smad–4 in mammalian cells and finally the inhibitory (I) Smad6 and Smad7 (Shi & Massague 2003). Smads1,2,3,4,5,8 display two conserved polypeptide domains, termed the Mad homology (MH) 1 domain in the N terminal end and MH2 at the C terminal end which are separated by a proline rich linker region that is less conserved (Figure 1.6). The I-Smads Smad6 and Smad7 are structurally divergent in that they lack the MH1 segment and function with only the MH2 domain.

TGF-$\beta_{1\text{-}3}$ and activin signalling is via Smads2 and Smad3 whilst Smads1, Smad5 and Smad8 mediate BMP signals. Smad2 and Smad3 are activated by ALK-5 (TβRI) and ALK-4 (ActRIB) with Smad1, Smad5 and Smad8 being activated by ALK-1, ALK-2, ALK-3 and ALK-6 (Shi & Massague 2003). In endothelial cells in particular TGF-β_1 can also activate Smad1 and Smad5 through the Type I ALK-1 receptor. Whereas ALK-5 is widely expressed ALK-1 is expressed predominantly in endothelial cells but also at sites of epithelial-mesenchymal interaction (Roelen *et al.* 1997).

The C-terminal of R-Smads display a characteristic -Ser-Ser-Xaa-Ser- motif of which the two latter serine residues are the target for phosphorylation (p) by activated Type I receptors (Abdollah *et al.* 1997). Thus the phosphorylation of the C terminal SXS motif leads to conformational changes in R-Smads, dissociation from the Type I receptor and the formation of a trimeric complex of two R-Smads with one Co-Smad4. Co-Smad4 displays the MH1-linker-MH2 domain structure but lacks the C terminal SXS motif and therefore is not phosphorylated by the Type I receptor (Figure 1.6) (de Caestecker *et al.* 1997). Smad4 interacts with the R-Smads directly via its MH2 region. The heterotrimeric complex of Smad4 with either Smad2 and Smad3 or

Smad1, Smad5 and Smad8 allows a selection of combinatorial interactions and versatility to the signalling process.

The R-Smad-Co-Smad4 complex translocates into the nucleus. At the regulatory sequences of specific genes the complex can participate in the transcription of specific genes by acting directly as a transcription factor as well as activating other transcription factors. Interaction of R-Smads with Smad4 is essential for the formation of nuclear transcription factors rather than nuclear transportation (Liu *et al.* 1997). Inhibitory Smad6 and Smad7 now translocate out of the nucleus and by competing with R-Smads for the Type I receptor provide a negative feedback loop of inhibition on TGF-β Superfamily signalling (Hanyu *et al.* 2001).

1.14.7 Conservation of Smad domains

The biology of Smads as transcription factors is reviewed in detail by Massague *et al.* 2005. The conservation of the MH2 domain in all Smads is a reflection of the importance of this region in interaction with receptors and transcription factors and for the adoption of oligomeric conformations. The MH2 domain also demonstrates transcriptional activator function but this activity is repressed in the resting state by interaction with the MH1 domain. The MH1 domain and linker regions have the capacity to directly bind to DNA as well as other transcription factors. A nuclear localisation signal (NLS) is present on the MH1 domain which can bind a variety of transcription factors (Figure 1.6). The MH2 domain does not bind DNA directly but is important for protein-protein interaction and also contains a nuclear export signal (NES) important for nucleocytoplasmic shuttling. In R-Smads consensus phosphorylation sites in the linker region allows cross talk with MAPK signalling pathways. Such cross-talk with other signalling pathways allows important cell specific TGF-β Superfamily signalling and will be discussed later. The Smad4 C-terminal end of the linker region contains a region called the Smad activation domain (SAD) and allows binding with other transcription factors and is thus essential for signal transduction.

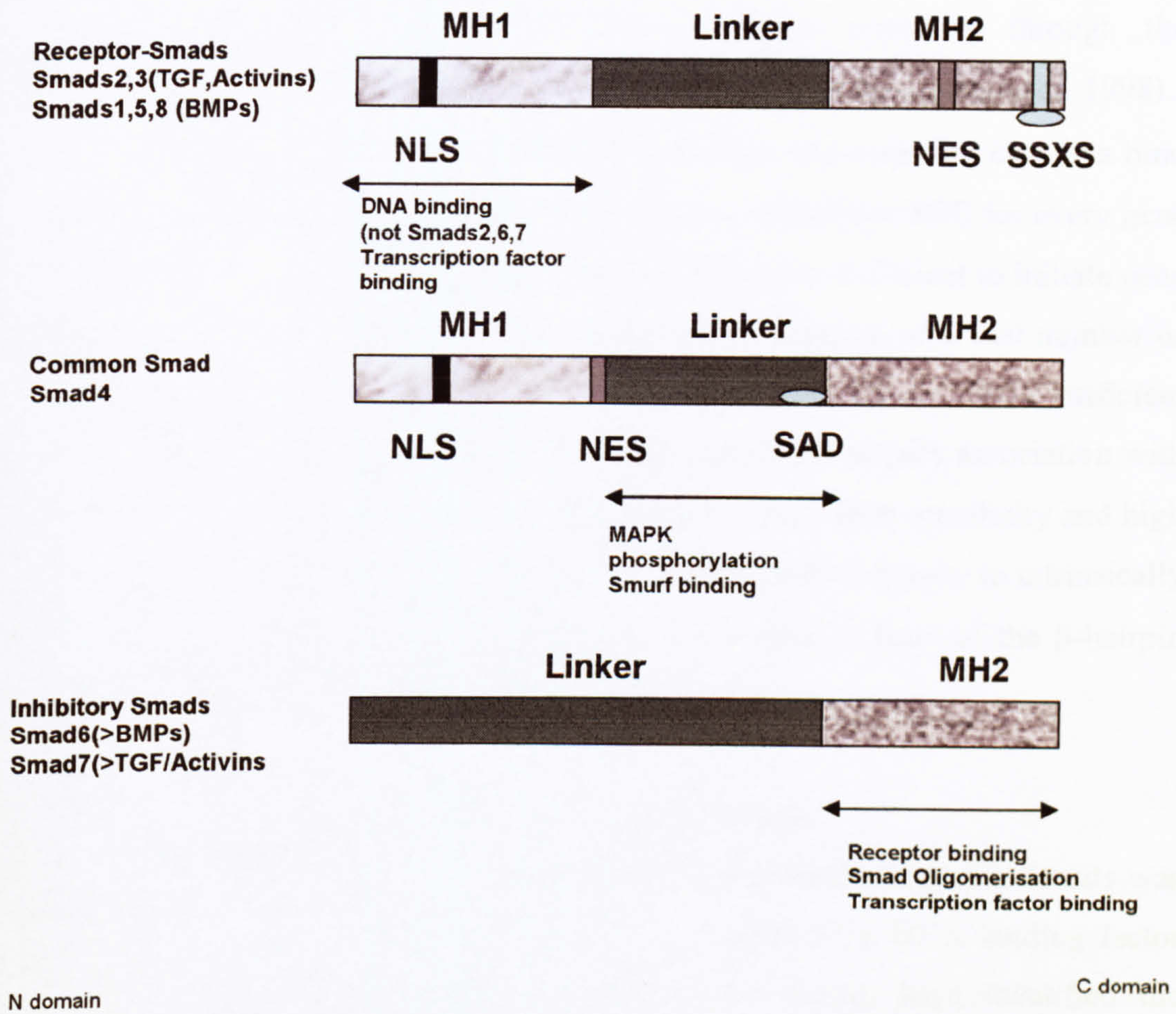


Figure 1.6 Illustration of the structural domains of the 3 classes of Smads in mammalian cells (adapted and further modified from Flanders 2004).

Receptor (R) Smads and Common Smad4 display two conserved domains MH1 and MH2 separated by a Linker region. The inhibitory Smads are structurally divergent by lacking an MH1 domain. R-Smads undergo phosphorylation at the C-terminal SSXS region of the MH2 domain. The conservation of the MH2 domains in all Smads indicates the pivotal role of this region in Smad interaction with receptors, transcription factors and the adoption of oligomeric conformations. The MH1 domain and linker regions can bind DNA directly. In R-Smads consensus phosphorylation sites in the linker region allow cross talk with MAPK signalling pathways. Smad4 C-terminal end of linker region contains the Smad activation domain (SAD) that allows binding of transcription factors. The location of nuclear localisation and export signals (NLS and NES respectively) are shown.

1.14.8 Smads as transcription factors

Once Smad complexes are located to the nucleus they are able interact directly with the DNA. The Smad3 and Smad4 N terminal MH1 domains can directly interact with Smad–binding elements (SBEs) characterised by only four base pairs (5'-GTCT-3' or the reverse complement 5'-AGAC-3'), the interaction occurring through the formation of hydrogen bonds with the two G residues of the SBE (Zawel *et al.* 1998). Smad1, Smad3 and Smad4 share an identical β-hairpin sequence and can thus bind SBEs with similar affinity. It is estimated that there is at least one SBE for every gene in the genome. If Smad binding to SBE sequences alone was sufficient to initiate gene transcription then this could lead to non-discriminate activation of a vast number of genes by Smads. However such interactions are weak and do not allow sufficient promoter selectivity. Thus R-Smad and Co-Smad complexes require association with other transcription factors in order to target DNA sequences with specificity and high affinity. This is particularly so for Smad2 as Smad2 lacks the capacity to intrinsically bind DNA as a result of an extra 30 amino acid sequence in front of the β-hairpin region (Shi *et al.* 1998c).

1.14.9 Smad interactions with DNA transcription factors

It was in *Xenopus* that the role of DNA binding factors interacting with Smads was first described with the FAST/FoxH-1 protein, a winged-helix DNA binding factor (Chen *et al.* 1996). Studies on the mammalian homologue have identified the principles that govern Smad-DNA binding factor interactions. In response to activin signalling the Smad2-Smad4 interacts with the DNA-bound Fox-H1 at an activin response element. The MH2 domain of Smad2 interacts with Fox-H1 whilst Smad4 binds the DNA (Chen *et al.* 1996). The specificity of Fox-H1 for actvin activated Smad2-Smad4 complex and not Smad1/5/8-Smad 4 complexes involved in BMP signalling is a result of few amino acid differences in the Smad2/3 MH2 domain. OAZ, a member of the zinc finger family of proteins, has been identified as a DNA binding factor in the BMP signalling pathway with binding to the Smad1-Smad4 complex (Hata *et al.* 2000). Cell specific expression of such factors as FAST and OAZ thus ensure cell specific responses to activin and BMP ligand signalling. Transcription factors have an essential role in the recruitment of Smads to specific promoters. The remarkable diversity of DNA binding factors with the capacity to undertake DNA transcription in their own right (unlike FAST and OAZ above) that are able to interact with Smad proteins introduces extensive versatility into the

process of gene transcription. Furthermore other important cell specific signalling pathways can directly influence this interaction. Such diverse regulatory interactions explain the complexity of TGF-β Superfamily ligand-induced transcriptional programs and cell specificity of expression.

1.14.10 Smad interaction with co-activators, co-repressors and chromatin modelling factors

Activated (phosphorylated) Smad1, Smad2 and Smad3 can directly interact with the co-activators CREB-binding protein (CBP) and p300 via their Smad C-terminal MH2 domain (Janknecht *et al.* 1998) . Such co-activators bind DNA and have the capacity to interact with and subsequently integrate transcriptional signals from transcriptional factors such as AP-1, NF-κB, c-Jun, CREB, c-Fos, STATS and nuclear hormone receptors such as the glucocorticoid receptor (GR) and retinoic acid. Thus the CBP/p300 integrators serve to bridge Smad–signalling to the basal transcriptional machinery. Furthermore the intrinsic histone acetylase (HAC) activity of CBP/p300 molecules maybe responsible for the Smad–dependent alteration of chromatin structure, leading to altered transcriptional activity.

Smads can also activate transcription by direct interaction and blocking of transcriptional repressors. For example BMP activated Smad1 protein can relieve the repression of osteopontin (a bone ECM component) gene expression by binding to and subsequently dislodging the homeodomain transcription factor Hoxc-8 from its DNA binding elememt (Shi *et al.* 1999). Smads can also recruit co-repressors to DNA and effectively inhibit transcription. An example of this is the homeobox protein TGT interacting factor (TGIF) and its associated histone deacetylase (HDAC) which when recruited by the pSmad2-Smad4 complex to Smad responsive DNA elements results in transcriptional repression of target genes (Wotton *et al.* 1999). Interestingly such repression can affect genes that are normally activated by TGF-β signalling and this may be because TGIF can antagonise activators such as CBP/p300.

In summary, Smads can associate with DNA with low affinity and without strict sequence specificity to regulate gene transcription. However, by interacting with multiple transcription factors or co-activators and co-repressors, which in turn can interact with other multiple cellular signalling pathways, Smad proteins provide a versatile system by which TGF ligands can achieve multiple outcomes in a cell. At

present our insight into signalling by this complex pathway is still at a very basic stage.

1.14.11 Regulation of Smad levels

R-Smads

The E3 ubiquitin ligases, Smurf-1 and Smurf-2 (Smad-ubiquitination-regulatory factor-1), have an important role in the regulation of cellular Smad levels (Figure 1.7). Ubiquitin-proteasome-mediated degradation regulates the level of R-Smads. Inhibition of proteasomal degradation leads to excess nuclear accumulation of R-Smads. However a considerable proportion of the nuclear Smad2 and Smad3 levels do not undergo degradation but are dephosphorylated and shuttled back into the cytoplasm.

1.14.12 Inhibitory Smads

Smad6 and Smad7 inhibit TGF-$\beta_{1\text{-}3}$, activin and BMP signalling. Smad7 is a general and potent inhibitor of TGF-Superfamily signalling (Hayashi *et al.* 1997; Nakao *et al.* 1997) whilst Smad6 preferentially inhibits BMP signalling (Imamura *et al.* 1997) (Hata *et al.* 1998). The levels of Smad6 and Smad7 expression is a determinant of TGF-Superfamily responsiveness and markedly abnormal expression levels of Smad7 in particular have been demonstrated in several inflammatory diseases such as inflammatory bowel disease (IBD) (Monteleone *et al.* 2001) and scleroderma, a disease of widespread skin, organ and vascular fibrosis due to dysregulated TGF-β_1 signalling (Dong *et al.* 2002). Increased expression of Smad7 in IBD tissue was associated with increased inflammation. The use of antisense strategies to abolish Smad7 signalling restores TGF-β_1 responsiveness by the inhibition of inflammatory cytokine production such as TNF-α and IFN-γ (Monteleone *et al.* 2001). In skin biopsies and explanted fibroblasts from scleroderma patients, basal Smad7 expression and the induction of Smad7 was deficient suggesting that aberrant Smad7 expression contributes to the disease process. Using adenoviral constructs to induce Smad7 expression in these fibroblasts, up-regulation of Smad7 levels lead to resultant suppression of TGF-β_1 signalling and a more regulated fibroblast-cell phenotype (Dong *et al.* 2002).

The regulation of Smad6 and Smad7 expression is now beginning to be deciphered. Smad6 and Smad7 expression is rapidly induced in cells in response to TGF-β, activin and BMP ligand signalling (Afrakhte *et al.* 1998) .Unlike R-Smads and Smad4, which are expressed in most, if not all cell types, Smad7 expression is highly regulated by extracellular signals and can be rapidly up-regulated in response to other non-TGF-β Superfamily signalling pathways. IFN-γ activation of the Jak/Stat-1 pathway, TNF-α activated NF-κB and EGF via the MAPK signalling pathways can all influence the expression of Smad7 (Bitzer *et al.* 2000; Ulloa *et al.* 1999). Smad7 is unique in its role of integrating inhibitory signals to TGF-β responses from TGF-β Superfmaily ligands with those from other cellular signalling pathways.

Understanding the regulation of the Smad7 gene promoter is of potential therapeutic importance. The proximal promoter region is the major transcriptional start site of the Smad7 gene and this is characterised by a high G d+ C content termed CpG islands or methylation free islands. These regions of DNA are a nucleosome free which allows easy access to transcription factors. The Smad7 gene promoter displays a R-Smad-Smad4 binding element (SBE) region defined by the palindromic sequence GTCTAGAC that is critical for TGF-β_1 induction of the Smad7 promoter (Brodin *et al.* 2000). Upstream of the transcription sites are Spl binding site clusters. The transcription factors Spl and AP-1 are required by Smad complexes to efficiently interact with the Smad7 promoter and initiate transcription (Brodin *et al.* 2000). Smad6 and Smad7 can regulate TGF-β Superfamily signalling through binding of the Smad-MH2 domain to the Type I Receptor thereby preventing the recruitment and phosphorylation of the R-Smads. Smad7 can also interact with ubiquitin ligases termed Smurfs leading to the degradation of the Type I receptor through proteasomal and lysosomal pathways (discussed later). In addition, Smad6 prevents the formation of BMP-activated Smad1 heteromeric complexes with Smad4 by active competition (Hata *et al.* 1998). Smad7 itself undergoes ubiquitination and degradation but transcriptional induction of Smad7 by TGF-Superfamily members ensures that a steady-state supply of cellular Smad7 is present. Nuclear Smad7 is protected from Smurf-mediated degradation by acetylation of lysine residues but such protection is lost outside the nucleus (Gronroos *et al.* 2002).

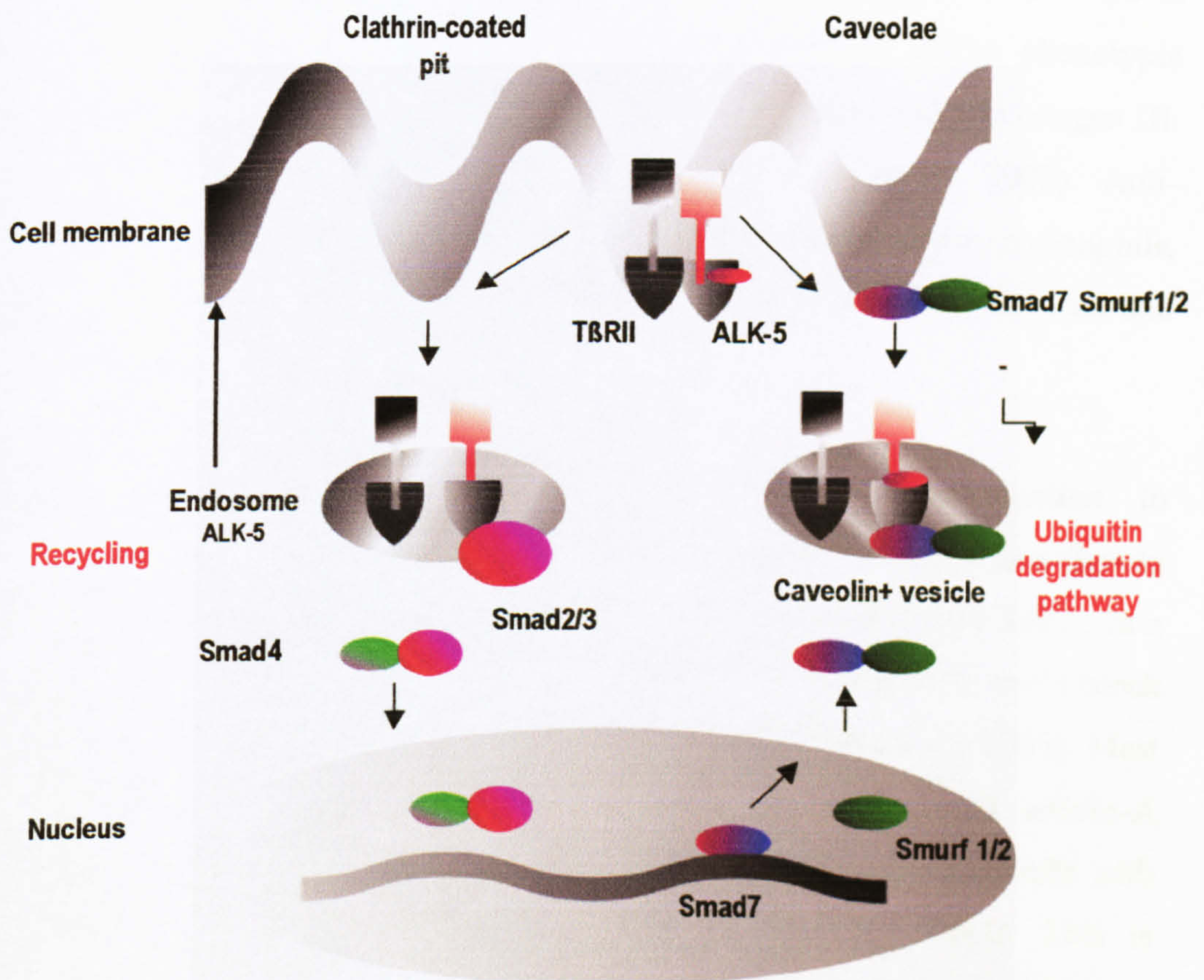


Figure 1.7 Illustration of the two distinct internalisation routes for TGF-β_{1-3} Type I and Type II receptors

Internalisation of the receptor complex into clathrin-dependent early endosomes leads to promotion of ligand signalling. Smad2/3 is presented to the receptors in the endosome complex leading to Smad2/3 phosphorylation. The receptor complex can now be recycled back to the cell membrane surface. The Smad2/3-Smad4 complex upon nuclear translocation leads to Smad7 transcription. Smad7 forms a heterotrimeric complex with Smurf1/2 in the nucleus before translocation back out into the cytoplasm. The Smad7-Smurf complex in the caveolae target the Type I receptor (ALK-5) for degradation through the ubiquitin-mediated proteasomal degradation pathway.

1.15 TGF-β Superfamily signalling in asthma

The role of TGF-β Superfamily signalling in asthma has until recently focussed on the predominant TGF-β_1 isoform and expression patterns in asthma were discussed previously. Both inflammatory cells and structural cells are important sources of the ligand. In particular eosinophils are an important source of TGF-β_1 implicated in remodelling. *In-vitro* co-culture of fibroblasts with eosinophils led to phenotypic change to myofibroblasts and synthesis of ECM proteins tenascin and procollagen III: this was dependent on eosinophil-derived TGF-β_1 (Phipps et al. 2002). Anti-interleukin-5 antibody treatment of asthmatics led to reductions in airway eosinophils, TGF-β_1 expression and RBM staining for the ECM proteins tenascin, lumican and procollagen III (Flood-Page *et al* 2003a).

TGF-β_1 and activin-A stimulate fibroblasts to undergo differentiation to myofibroblasts and to produce collagen, fibronectin and glycosaminoglycans (Ignotz *et al.* 1987; Zhang & Phan 1999; Desmouliere *et al.* 1993; You & Kruse 2002). In a mouse model of allergen-induced airway injury an anti-TGF-β_1 antibody could block development of selected aspects of airway remodelling (McMillan *et al.* 2005). Mast cells accumulate at sites of inflammation in response to TGF-β_1 and activin-A signalling (Olsson *et al.* 2000). Activin-A is synthesised de novo in mast cells with allergen challenge and induces ASM proliferation (Cho *et al.* 2003). This is significant given the finding that mast cell infiltration of ASM is associated with AHR in asthma (Brightling *et al.* 2002).

Bronchial biopsies obtained 24 hours after allergen challenge from mild atopic asthmatics showed significant up-regulation in pSmad2 signalling alongside increases in ECM expression (Phipps *et al.* 2004a). These data support the hypothesis of allergen-induced remodelling and TGF-β signalling, and may suggest allergen induced activation of the epithelial-mesenchymal trophic unit (EMTU): the embryological unit driving airway development which is suggested to be reactivated in airway remodelling.

Other groups have published evidence for activity of TGF-β_1, activin and BMP signalling after airway allergen challenge in a mouse model (Rosendahl *et al.* 2001) (Rosendahl et al. 2002). The TGF-β Superfamily are essential in organogenesis, and BMPs in particular play a role in branching morphogenesis in the developing lung.

Airway remodelling is suggested to involve dysregulated reactivation of the embryological epithelial-mesenchymal trophic unit, making these factors of particular interest. Interestingly, although BMP-2, BMP-4 and BMP-6 were markedly up-regulated in the murine model of allergen exposure, BMP-5 and BMP-7 expression was down-regulated (Rosendahl *et al.* 2002). BMP-2 and BMP-6 has previously been shown to be induced during fibrotic responses (Kaiser *et al.* 1998) whilst BMP-7 is now recognised as regulating the resolution of inflammatory events (Gould *et al.* 2002) and has been shown to counteract the effects of TGF-β_1 in remodelling events (Zeisberg *et al.* 2003).

1.16 Hypothesis

It is hypothesised that:

1. In atopic asthmatics following allergen inhalation challenge there are increases in AHR in individuals with dual asthmatic responses (DARs). Airway infiltration with inflammatory cells as shown by increases in eosinophils, T cells and neutrophils will be more pronounced in these individuals compared to individuals with single early responses (SERs). Since in DARs allergen-induced increased AHR is sustained for days and weeks, there will be persistence of airway inflammation in these individuals.

2. There will be rapid induction of airway remodelling at 24 hours post allergen which in DARs will be in association with increased AHR and cellular inflammation. Some components of airway remodelling will accompany cellular inflammation. Further, at the 7 day time point, when sustained increases in AHR are present, there will be comparable increases in airway remodelling and cellular inflammation

3. Changes in airway remodelling will be associated with activation of TGF-ß Superfamily (i.e TGF-$ß_{1-3}$, activin and BMP) signalling evidenced by rapid increases in pSmad2, pSmad1/5 and Smad7 and altered expression of the Type I and Type II receptors.

1.17 Aims

Based on the hypotheses the aims of the thesis will be:

1. To compare cellular inflammation, airway remodelling and AHR in bronchial biopsies taken at baseline, 24 hours and 7 days after allergen challenge in mild atopic asthmatics.

2. To evaluate the expression pattern of TGF-β_{1-3}, activin-A, follistatin and BMP-2, BMP-4, BMP-7 together with that of Type I (ALK 1-6) and Type II receptors (TβRII, ActRIIA, ActRIIB and BMPRII) and activated phosphorylated R-smads (pSmad2 and pSmad1/5), Co-Smad4, inhibitory Smad6 and Smad7. Expression in bronchial tissue obtained at baseline, 24 hours and 7 days after challenege will be compared to expression in the normal airway.

Chapter 2

Methods

2.1 Ethical statement

The study received prior approval from the Ethics Committee of the Royal Brompton and Harefield Hospital NHS trust. Following patient recruitment written informed consent was obtained in the presence of a witness prior to any procedure being undertaken at the study screening visit. In addition written informed consent was obtained prior to each bronchoscopy in the presence of a witness.

2.2 Volunteer recruitment

Volunteers with asthma and normal healthy controls were recruited by advertisement from both the general public and Imperial College staff.

2.2.1. Asthmatic volunteers

Inclusion criteria

1. Age 18 to 50 years, of either gender or any ethnic group.

2. A clear clinical history of asthma, i.e. intermittent bouts of cough, wheeze and dyspnoea as well as reversible airways obstruction (15% or more increase in peak expiratory flow or FEV_1 to inhaled salbutamol 200 mcg) or airway hyper-responsiveness (methacholine PC_{20} of less than or equal to 8mg/ml).

3. All subjects had positive skin prick tests (wheal size $\geq$3 mm) to at least one common aeroallergen, i.e. grass pollen, house dust mite, cat or have a positive blood RAST to these allergens with a history of bronchospasm on exposure.

4. All subjects were able to provide written informed consent.

6. All subjects were able to follow instructions and completed all requirements.

Exclusion criteria

1. All subjects with an FEV_1 less than 70% of predicted normal.
2. All subjects with symptomatic, uncontrolled/poorly controlled asthma.
3. All subjects taking drugs which interfere with the early or late phase response.
4. Subjects who had taken oral corticosteroids within the previous 2 months, or inhaled corticosteroids within the last month. If patients had mild asymptomatic asthma (FEV_1>80% predicted), and were taking 250 mcg/day or less beclomethasone equivalent, inhaled corticosteroids were stopped for 4 weeks prior to the study (during this period patients were closely monitored with peak flow and symptom diary and weekly contact with the trial physician to check that their asthma did not become unstable [FEV_1<70% or nocturnal symptoms]). Short acting β_2 agonists were withheld for 4 hours before any visit, and long acting β_2-agonists for 12 hours.
5. Vaccination/inoculation within the previous 6 weeks, or vaccination scheduled within the study period.
6. Allergen-immunotherapy in the past.
7. A history of concurrent illness, e.g., autoimmune diseases and other immuno-pathological disease, significant cardiovascular, renal, neurological, gastrointestinal, respiratory (other than asthma and allergic rhinitis), sinusitis, nasal polyps, chronic obstructive pulmonary disease, moderate or severe atopic dermatitis, hepatic or systemic disease (including malignancy).
8. Clinically significant abnormalities in routine haematology, biochemistry or urinalysis.
9. History of drug or alcohol abuse.
10. Subjects who had taken an investigational drug within the last 6 weeks.
11. Women who were pregnant, lactating or not using a reliable form of contraception.
12. Subjects who had smoked within the previous year or more than 5 pack years smoking history.
13. Subjects who had any personal relationship with the investigator

2.2.2. Normal volunteers

Inclusion criteria

1. Age 18 to 50 years, of either gender or any ethnic group.
2. No clinical history of asthma, i.e. intermittent bouts of cough, wheeze and dyspnoea with no reversible airways obstruction (significant changes in peak expiratory flow or FEV_1 to inhaled salbutamol 200 mcg) or airway hyper-responsiveness (methacholine PC_{20} of more than 16mg/ml).
3. No positive skin prick tests (of any size) to any common aeroallergen, i.e. grass pollen, house dust mite or cat . Negative IgE RAST to these allergens.
5. All subjects were able to provide written informed consent.
6. All subjects were able to follow instructions and likely to complete all requirements.

Exclusion criteria

1. Volunteers with an FEV_1 less than 80% of predicted normal.
2. Volunteers taking any form of medication.
3. Vaccination/inoculation within the previous 6 weeks, or vaccination scheduled within the study period.
4. Allergen-immunotherapy.
5. A history of concurrent illness, e.g., autoimmune diseases and other immuno-pathological disease, significant cardiovascular, renal, neurological, gastrointestinal, respiratory (other than asthma and allergic rhinitis), sinusitis, nasal polyps, chronic obstructive pulmonary disease, moderate or severe atopic dermatitis, hepatic or systemic disease (including malignancy).
6. Clinically significant abnormalities in routine haematology, biochemistry or urinalysis.
7. History of drug or alcohol abuse.
8. Subjects who had taken an investigational drug within the last 6 weeks.
9. Women who were pregnant, lactating or not using a reliable form of contraception.
10. Subjects who had smoked within the previous year or more than 5 pack years smoking history.
11. Subjects who had any personal relationship with the investigator

2.3. Study protocol

2.3.1 Asthmatic volunteers

The study protocol is summarised in Figure 2.1. All asthmatic subjects were controlled with as required β_2 agonists only at recruitment and throughout the study period (i.e steroid naïve). Following the screening visit, volunteers recorded symptoms, salbutamol inhaler usage and FEV_1 in a run in period of two weeks before baseline bronchoscopy with bronchoalveolar lavage (BAL) and bronchial biopsy (BB) (FOB1) and throughout the study period. A hand held Piko Device FEV_1 recorder (Ferraris Respiratory Europe Ltd, Hertford, UK) was used with the volunteers fully trained in its use. Volunteers were asked to take measurements in the morning before any reliever medication. Approximately 3 weeks after FOB1 an incremental cumulative inhaled allergen challenge was performed (using allergen pre-determined on basis of skin prick test sensitivity and exposure history) until a 15% fall in FEV_1 was achieved. FEV_1 was recorded for up to 10 hours to document any late asthmatic response (LAR). Allergen challenges were performed with either mixed grass, *Dermatophagoides pteronyssinus* (Allergopharma, Reinbek, Germany) or cat dander (Leti, Madrid, Spain). An incremental dosing scheduled as previously described was used (Taylor *et al.* 2000). A second bronchoscopy (FOB2) with BAL and BB was carried out 24 hours later. A third and final bronchoscopy (FOB3) was carried out 7 days after allergen challenge. Lung function, AHR (methacholine PC_{20} mg/ml) and symptoms were recorded at each visit. Patients with hay fever were studied out of season. AHR and FEV_1 were always measured between 7.00-8.00am on visit days.

2.3.2 Normal volunteers

All normal volunteers underwent only a single baseline bronchoscopy. Spirometry, methacholine challenge using a quadruple dosing schedule and nebulisation of 5mg of salbutumol 30 minutes prior to bronchoscopy with repeat spirometry was performed. The same standard operating protocol for bronchoscopy established for the asthmatic group was followed. All bronchoscopies were performed at 8.30 am. Thus any differences found between the normal and asthmatic airway should be a result of asthma

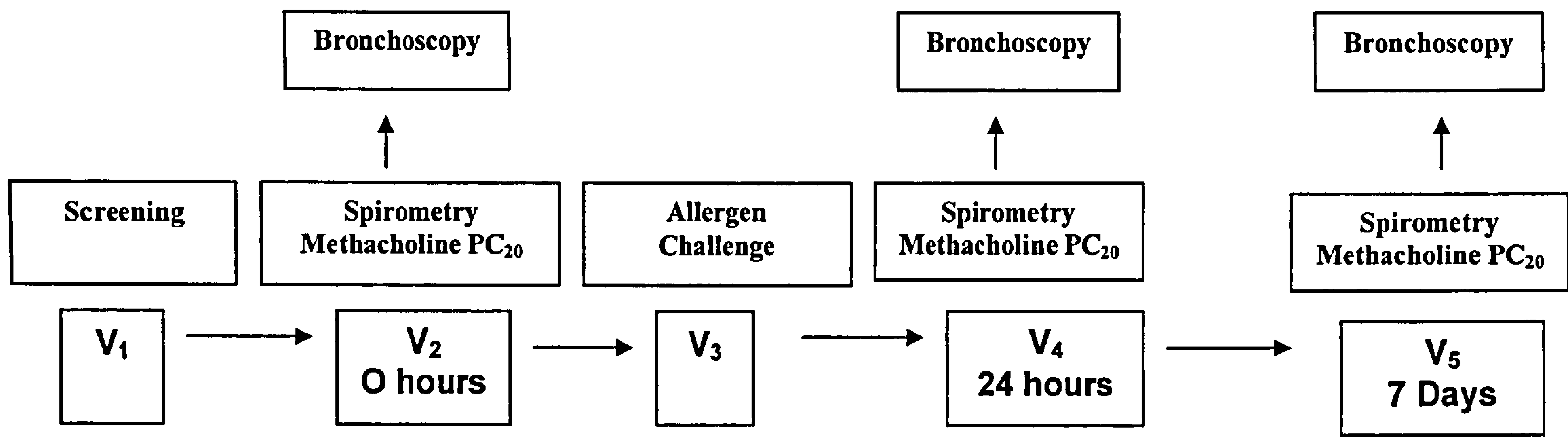


Figure 2.1: Summary of the study design.
Following a screening visit, asthmatics underwent a baseline bronchoscopy at Visit 2 (V_2 Time 0 hours). Visit 4 (V_4) and Visit 7 (V_7) were 24 hours and 7 days after allergen challenge respectively. Spirometry and methacholine-induced AHR was measured prior to each bronchoscopy.

2.4. Clinical methods

2.4.1. Skin prick tests

Skin prick tests (SPTs) were performed on the volar aspect of the forearm using allergen extracts (ALK, Berks, UK) to grasses, trees, cats and house dust mite. Histamine was used as a positive control and normal saline diluent was used as a negative control. Prick test sites were observed for the presence of erythema and weal formation for a total of 15 minutes. Responses were recorded by encircling the weal using a fine fibre tip pen and transferring to a record sheet with adhesive transparent tape. A wheal size $\geq$3 mm was taken as a positive result.

2.4.2. Methacholine challenge

Methacholine challenge was used as a method of measuring AHR in order to define the volunteers into the clinical groups of asthma and normal and as a measure of clinical asthma severity. The protocol was adopted using the consensus guidelines set by the American Thoracic Society (Crapo *et al.* 2000).

Contraindications for Methacholine Challenge Testing

Absolute

1. Severe airflow limitation (FEV_1 <50% predicted or < 1.0 litre)
2. Myocardial Infarction or CerebroVascular Accident (CVA) in last 3 months
3. Uncontrolled hypertension

Relative

1. Moderate airflow limitation (FEV_1 < 60% predicted or < 1.5L)
2. Inability to perform acceptable –quality spirometry
3. Pregnancy and Nursing mothers
4. Current use of cholinesterase inhibitor medication

Volunteers abstained from caffeine products for 12 hours prior to study visits. The asthmatic volunteers were only on salbutamol inhalers at the time of the study and abstained from usage for at least 4 hours before the study visit.

Five Breath Dosimeter Protocol

The five breath dosimeter protocol, first standardised by the National Institutes if Health (NIH) (Chai *et al.* 1975) was used using doubling doses of methacholine according to the standards set in American Thoracic Society guidelines (Crapo *et al.* 2000).

Baseline FEV_1 and diluent Nebulisation

The patient was rested for 15 minutes. The baseline FEV_1 was then measured three consecutive times and the highest value recorded. This value had to be study was more than or equal to 70% of the predicted value in order to proceed to methacholine challenge. All dosimeter inhalations and spirometry were carried out with a nose clip worn. The dosimeter was set to the setting of inhalation time of 1 second, pause time of 6 seconds and a total number of inhalations to 5.

Methacholine was made up using normal saline diluent to the following concentrations: 0.03 0.06 0.125 0.25 0.50 1 2 4 8 16 mg /ml.

At end exhalation using tidal breathing (i.e at functional residual capacity) the patient was instructed to inhale deeply and slowly to maximal capacity from a hand-held nebuliser attached to a breath-activated dosimeter (Mefar, Italy) and then to hold their breath for 6 seconds. This was performed for a total of 5 breaths. FEV_1 measurements at 1 and 2 minutes after inhalation was recorded and the highest value taken. The initial nebulisation was performed with normal saline. If FEV_1 fell less than 10% from baseline value then the volunteer proceeded with the methacholine challenge procedure. If FEV_1 fell by 10% or more following saline inhalation the physician present decided whether to carry on with the procedure or to repeat another diluent challenge or to abort the challenge. Volunteers inhaled doubling doses of methacholine until a $\geq$ 20% fall in FEV_1 from the post saline value was achieved. In order to keep the cumulative effect of methacholine relatively constant each methacholine dose was administered exactly 5 minutes apart. The PC_{20} was calculated using the following formula:

$$PC_{20} = \text{antilog}\left(\log C_1 + (\log C_2 - \log C_1)(20 - R_1)/(R_2 - R_1)\right)$$

C_1= second to last [methacholine] R_1= % fall in FEV_1 after C_1

C_2= final [methacholine] leading to 20% FEV_1 drop R_2=% fall in FEV_1 after C_2

2.4.3 Allergen inhalation challenge

It was first established that the volunteers did not experience an asthma exacerbation or a respiratory tract infection in the preceding 4 weeks. Short-acting β_2 agonists were withheld from the preceding evening. The volunteers were not taking any other medications that could interfere with the early or late phase response.

An incremental allergen challenge protocol as previously published (Taylor *et al.* 2000) was adapted as described below. Fresh dilutions of freeze-dried allergen extract were made up from the stock solution to the predetermined dilutions in the protocol using 0.9 % saline. The allergen solution (starting with the lowest concentration) was administered using a hand-held nebuliser attached to a breath-activated dosimeter (Mefar, Italy). The machine was set to give a delivery time of 1 second per breath. Particles with an aerodynamic mass diameter of 3.5-4.0μm are delivered by the nebuliser at an output of 9μl per breath. The FEV_1 was measured by a dry wedge spirometer (Vitalograph, Buckingham, UK) using an established SOP.

The standard challenge protocol is described. Three spirometric recordings at 1minute intervals were performed and the best FEV_1 was recorded as the baseline reference. In order to precede the FEV_1 must be 70% or more of that predicted. The volunteers now inhaled five breaths of 0.9% saline in a nebulised form from the dosimeter by inspiring slowly from functional residual capacity (FRC) to total lung capacity (TLC) over 3 seconds and then breath holding for 6 seconds. At 2, 3 and 4 minutes a single measurement of FEV_1 is taken and the highest value taken as the post-saline reference. Subjects who did not demonstrate a more than 10% fall in the FEV_1 following saline inhalation now proceeded to allergen challenge using the same method as saline inhalation and stating with the lowest dose. The FEV_1 is taken at 5 and 10 minutes after each allergen dose and the lower of the two values is taken as the response. If the fall in FEV_1 is less than 10% then a 4-fold increment of the previous dose of allergen is administered. If the fall in FEV_1 is more than 10% then only a 2-fold increment of the previous allergen dose is administered. The challenge is terminated once a fall in the FEV_1 of more than 15% of the post saline reference value is achieved (defined as an early asthmatic response (EAR). FEV_1 measurements are taken at 5,10,20,30, 45 and 60 minutes and then every 30 minutes up to 10 hours. A late asthmatic response (LAR) is defined as a fall in the FEV_1 of more than 15% from the post-saline reference value at a single time point between 3 and 7 hours after allergen inhalation. The definition was established prior to the commencement of the study.

2.4.4 Bronchoscopy

A standard operating protocol was established in order to standardise the procedure between the two operators who performed the bronchoscopies.

Subjects received 5 mg of nebulised salbutamol and spirometry performed 30 minutes later before bronchoscopy. Bronchoscopy was performed between 8.30-9.00am on each occasion. Peripheral intravenous access was obtained with a plastic cannula. Lignocaine 2% was sprayed into the oropharynx. All volunteers received atropine 600 μg and midazolam 6-10 mg intravenously. Supplemental oxygen was entrained at 4 litres a minute throughout the procedure and arterial oxygen saturation monitored using pulse oximetry (Nellcor, Pleasanton, CA, USA). The bronchoscope (Olympus IT 40, Olympus Corp., NY, USA) was always introduced through the mouth to minimise any discomfort for the volunteer. Vocal cords and bronchial tree were anaesthesised using aliquots of 2% lignocaine and the minimal amount required was used. The left or right lung was chosen using a randomisation procedure. BAL was performed from either the lingular lobe on the left side or the middle lobe on the right by instilling a maximum of 4 aliquots of 60 mls of warmed sterile saline which was aspirated by gentle suction. Six endobronchial biopsies were then taken from the same side starting from the distal subsegmental carinae and finishing at the carina using Pentax KW-2411S fenestrated cupped forceps (Pentax, Tokyo, Japan). At the end of the procedure a research nurse monitored the patient for another 20 minutes. The volunteers were then allowed to rest for at least 3 hours with measurement of respiratory rate and saturations on air only. At discharge all volunteers received a further 2.5-5 mg of nebulised salbutamol and after 30 minutes spirometry repeated. Paracetamol 1mg to counteract any sore throat or post BAL fever was administered. The study physician reviewed all volunteers and a contact number for the physician was confirmed with the volunteer prior to discharge.

2.5 Laboratory methods

2.5.1 Sample collection

Biopsies for immunohistochemistry (IHC) were collected in phosphate buffered saline (PBS) (Sigma Aldrich, Dorset, UK) and transferred to 4% paraformaldehyde (PFA) (Sigma Aldrich) for 2 hours. The samples were then transferred to 15% sucrose (Sigma Aldrich) in PBS for 1 hour and again into a second vial of 15% sucrose in PBS for at least 1 further hour. The biopsies were then placed in Tissuetek OCT embedding media (ThermoShandon) mounted on card and snap-frozen in isopentane (VWR, Leicester, UK) precooled in liquid nitrogen. Caution was taken to minimise any artefactual damage to the biopsy or generation of air bubbles in the OCT. The samples were clearly labelled and placed in Bijoux containers and stored at –80°C until further use. Biopsies were cut using a Bright Cryostat. 5μm sections were cut and adhered onto superfrost plus microscope slides (VWR). Sections were allowed to air-dry overnight and then wrapped with foil prior to storage at –80°C until further use.

2.5.2 Processing of BAL samples

The BAL was collected in 100ml glass bottles and placed in ice for transfer to the laboratory. The samples were processed within 30 minutes of collection. The BAL fluid was filtered through gauze to remove debris and the filtrate then centrifuged at 800 RPM for 10 minutes at 4°C. The supernatant was stored at -80°C.

The cell pellet was pooled into 20 mls of PBS and a total cell count estimated using a modified neubauer haemocytometer and a Trypan Blue (Sigma Aldrich) stain. The latter also enabled assessment of cell viability. An appropriate dilution to yield a cell count of 0.3 x 10^6 ml was then made. Cytospins were prepared by placing 100μl into each Shandon2 cytospin cassettes (ThermoShandon) and spinning at 450 RPM for 3 minutes. The slides were allowed to air dry for 15 minutes before being fixed by placing in 4% PFA for 15 minutes and then sucrose for a further 15 minutes. The latter step was repeated. The slides were then washed in PBS and allowed to air-dry overnight. The slides were wrapped in aluminium foil and stored in boxes containing silica at -80°C until further use.

2.5.3 Differential cell counts from BAL cytopsins

Using a standard kit from ThermoShandon (Diff-Kwik), cytospin slides were stained by immersing the slide in the order of methyl alcohol for 5 seconds followed by xanthene dye for 5 seconds and finally a thiazine dye for 5 seconds. After a quick rinse in distilled water the slides were left to air-dry overnight before being mounted in DPX medium (VWR). Cells were identified on the basis of morphology and staining colour. In particular lymphocytes are recognised as mononuclear cells with clear blue cytoplasm, eosinophils are bi-lobed cells with reddish orange cells in the cytoplasm. Macrophages are recognised as cells with bright blue-purple cytoplasm and light blue nucleoli.

2.5.4 Principles of immunohistochemistry

All incubations were carried out at room temperature. Washes were performed in PBS unless otherwise stated. Normal human serum 10% (NHS) was used where stated to reduce non-specific binding. All primary antibodies for extracellular markers were incubated for 30 minutes whilst those for intracellular markers were incubated overnight. All incubations were at room temperature unless otherwise stated.

2.5.5Three-step indirect method

An unconjugated antibody binds to the antigen. A secondary antibody directed against the primary antibody is applied. If the primary antibody is made in rabbit or mouse then the secondary antibody must always be directed against rabbit or mouse immunoglobulins, respectively. Finally a third enzyme-conjugated antibody is now added. The purpose of adding in the third antibody is to amplify the signal since several of the enzyme-conjugated antibody can now bind to the secondary and thus localises additional enzyme molecules at the site of tissue antigen of interest leading to greater colour intensity on addition of an appropriate developing substrate-chromogen preparation.

2.5.6 Soluble enzyme immune complex method

This soluble enzyme immune complex method utilises a preformed soluble enzyme anti-enzyme immune complex and is one of the most sensitive immunochemical techniques available. The immune complex is an antibody directed against the enzyme as well as the secondary antibody that is linked to the primary antibody. The primary antibody is added first followed by the secondary antibody (a linker antibody). The soluble enzyme anti-enzyme complex is then added and this too binds to the secondary antibody. A relevant substrate and chromogen preparation is now added. The enzyme can now hydrolyse the substrate to break down products which couple to the chromogen (capture reagent) present to form insoluble coloured dyes enabling the antigen of interest to be localised. Both the primary antibody and enzyme immune complex must be raised in the same species in order for the secondary antibody to link them together. In addition the secondary antibody must be added in excess so that following binding to the primary antibody Fab sites are left free to bind the enzyme immune complex. The soluble enzyme immune complex method is further named on the basis of the particular enzyme immune complex used. The Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) technique was used for the immunohistochemical detection of all antibodies used in this project unless otherwise stated. The APAAP method allows a very high degree of detection given that the APAAP complex is comprised of two enzyme molecules per one antibody against the enzyme. Alkaline phosphatase (AP) catalyses the hydrolysis of a variety of phosphate-containing substances in the alkaline pH range. The enzymatic activity can be localised by coupling a soluble product generated during the hydrolytic reaction with a capture reagent, producing a coloured insoluble precipitate. AP hydrolyses naphthol-phosphate esters (substrate) to phenolic compounds and phosphates. The phenols couple to colourless diazonium salts (chromogen) to produce insoluble coloured azo-dyes. The Fast Red substrate solution, utilised where stated, is based on this reaction and produces a bright red end product. AP turns NBT (nitroblue tertrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) into insoluble NBT formazan (nitroblue tetrazolium formazan). NBT formazan is dark blue and has reflective properties.

2.5.7 Avidin-biotin methods

Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity ($10^{15}M^{-1}$) for the small molecular weight vitamin, biotin. In addition to this high

affinity the avidin has four binding sites for biotin of which only two will bind to biotin. This property was exploited where stated and is termed the avidin-biotin complex (ABC) method and follows the same sequence as described above where the secondary antibody is biotinylated and preformed avidin-biotin complex is added. Commercially available Vectastain ABC-AP kits (Vector Laboratories, Peterborough, UK.) incorporating a secondary biotinylated secondary linker antibody and an avidin-biotinylated complex-alkaline phosphatase (ABC-AP) soluble enzyme immune complex was used where stated.

2.5.8 Enumeration of inflammatory cells

Detection of monoclonal antibodies

Cellular infiltration of bronchial tissue by inflammatory cells was evaluated using the following mouse monoclonal antibodies: eosinophils-anti-MBP (BMK-13, in house) at 1/30 dilution and from Dako (High Wycombe, UK), elastase (Clone NP57, neutrophils) at 1/250, CD3 (Clone T3-4B5, $CD3^+$ T cells) at 1/25, CD4 (Clone MT310,$CD4^+$ T cells) at 1/20, CD8 (Clone DK 25, $CD8^+$ T cells) at 1/20, CD68 (Clone EBM11, macrophages) at 1/40 and tryptase (Clone AA1, mast cells) at 1/80 dilution. The slides were allowed to thaw at room temperature before unwrapping and being placed in a humidity chamber. The primary mouse was antibody appropriately diluted in PBS and applied to the section .The sections were left in the humidity chamber for 30 minutes after which they were washed twice in PBS for 5 minutes each. The sections were then incubated with a rabbit-anti-mouse secondary antibody (Dako) diluted to 1:30 in 10% normal human serum (NHS)/PBS for 30 minutes in a humidity chamber. The slides were then washed in PBS. A tertiary antibody layer of mouse antibody conjugated to the APAAP complex (Dako) diluted to 1:30 is now added and the slides again incubated in a humidity chamber for 30 minutes before washing. The slide were developed using Fast Red (Sigma) chromogen for signal visualisation over 20 minutes. Washing the slides in tap water terminated the reaction. Appropriate positive and negative controls were used. The sections were then counter-stained with haematoxylin and mounted with glycergel (Dako). Given that Fast Red end products are alcohol soluble, it is essential to use an aqueous mounting medium (Dako).

Congo Red stain

In addition, infiltration of eosinophils was determined using the Congo Red stain (Grouls & Helpap 1981). This was to ensure that ECM deposition with MBP from degranulated eosinophils was not mistaken for individual eosinophils. Congo red is an azo-dye, derived from benzidine that turns red in the presence of alkalies and blue in the presence of acids. In addition, the alkaline Congo Red technique stains amyloid and elastin orange-red. Briefly the sections were incubated in 0.5% Congo Red (VWR) made up in ethanol/0.1M glycine for 5 minutes at room temperature. The sections are then successively rinsed in 70% v/v ethanol until the tissue background cleared and finally mounted in glycergel (Dako).

2.5.9 Enumeration of remodelling markers

Collagen markers

HSP-47

Mouse monoclonal anti-HSP-47 (Clone M16.10A1, Stressgen) diluted to1/100 in NHS 10% saponin 0.1% buffer was incubated overnight with tissue sections. Following several washes in 0.1 % saponin/PBS the sections were then incubated with a rabbit-anti-mouse secondary antibody (Dako) diluted to 1:30 in 10% NHS-0.1% saponin-PBS for 30 minutes in a humidity chamber. The slides were then washed in 0.1% saponin-PBS. A tertiary antibody layer of mouse antibody conjugated to the APAAP complex (Dako) diluted to 1:30 was added and the slides again incubated in for 30 minutes before washing. Fast Red (Sigma) as chromogen for signal visualisation was used.

Procollagen

Procollagen I (Chemicon, Harrow, UK) rat monoclonal antibody diluted to 1/2000 in NHS 10% saponin 0.1% buffer was incubated overnight with tissue sections. A rat Vectastain ABC kit was used according to the manufacturer's instructions with a biotinylated secondary antibody diluted in 0.1% saponin-PBS buffer incubated with tissue sections for 30 minutes. Following repeated washes in 0.1% saponin-PBS buffer, the sections were then incubated with the ABC-AP complex as described and developed with Fast-Red.

α-Smooth Muscle Actin

Mouse monoclonal antibody to α-smooth muscle actin (Clone 1A4, Dako) diluted at 1/100 and mouse monoclonal antibody to vimentin (Clone V9, Dako) diluted at 1/100 in 10% NHS -0.1% saponin-PBS was incubated with tissue sections overnight. A mouse Vectastain -ABC kit was used according to the manufacturer's instructions as detailed earlier and the reaction developed with Fast Red.

2.5.10 Enumeration of TGF-β Superfamily and growth factor ligands

TGFβ $_{1\text{-}3}$ isoform expression was analysed by detecting mRNA and is explained in the *in-situ hybridisation* section.Tissue expression of activin-A was detected using a goat polyclonal antibody (R & D Systems, Abingdon, UK) diluted at 1/75, BMP-2 using a mouse monoclonal (Clone 100221, R & D Systems) at 1/50 dilution, BMP-4 and BMP-7 using goat polyclonal antibodies (R & D Systems) at 1/50 and 1/75 dilution in 10% NHS 0.1% -saponin -PBS buffer respectively. Appropriate Vectastain-ABC kits were used according to the manufacturer's instructions and the reaction visualised using the Fast Red chromogen. Follistatin (polyclonal goat, R & D Systems) and VEGF (mouse monoclonal, Clone 23410, R & D Systems) were similarly detected.

2.5.11 Double staining immunohistochemistry

Inflammatory cell expression of growth factors activin-A and BMP-7 was determined using a double staining technique. Essentially, sections were stained for growth factors as described previously except the sections were pretreated with hydrogen peroxide 0.33% for 20 minutes to block endogenous peroxidase activity and then washed in 0.1%-saponin-PBS solution. After overnight incubation detection was completed using a goat Vectastain-ABC kit but the reaction developed using 3, 3'-diaminobenzidine (DAB) chromogen that produces a brown end product. The reaction was terminated by a quick plunge in tap water followed by PBS. An avidin-biotin blocking step was performed using a VectaKit and the cell phenotypes identified as described earlier using an appropriate anti-mouse Vectastain-ABC kit with the reaction visualised using Fast Red chromogen that produces a red end product. Double stained cells were seen as brown-red in colour.

2.5.12 TGF-Superfamily signalling detection

Antibody details

The antibodies directed against the Type I and Type II receptors and Smads were a kind gift from Prof. P.Sideras, Athens Biomedical Institute, Greece. Briefly, polyclonal antibodies were raised in rabbits against synthetic polpypeptides as previously described (Rosendahl *et al.* 2001; Rosendahl *et al.* 2002; Franzen *et al.* 1993). These antibodies have been previously been validated in human tissue (Nakao *et al.* 2002). Antibodies against Type I receptors ALK-4 (ActR-IB), ALK-5(TβR-I), ALK-2, ALK-3, ALK-6 and Type II receptors ActRIIB and BMPRII were raised using peptides that corresponded to the divergent intracellular juxtamembrane domains. TβRII and ActRIIA Type II receptor antibodies were raised using synthetic peptides that corresponded to the divergent carboxy termini. Antibodies directed against Smad2, Smad4, Smad6 and Smad7 were raised using peptides that corresponded to the variable proline-rich linker region. Antibodies to pSmad2 and pSmadl/5 were raised by coupling peptide KKK-SSpMSp (where Sp stands for phosphorylated serine residue) and peptide KKK-NPISpSVpS (pS standing for terminally phosphorylated serine residue) respectively. The specificity of antisera was confirmed using immunoprecipitation and Western blot analysis on receptor and Smad transfected COS cells in the collaborator's laboratory.

Staining protocol

Optimal dilutions were determined for each antibody used. Sections were incubated with the primary antibody made up to the appropriate concentration in NHS 10% - 0.1% saponin- PBS buffer overnight. All subsequent washes were with 0.1% saponin-PBS buffer. A rabbit VectaStain ABC-AP kit was then used as described previously and developed using Fast Red substrate. Omission of the primary antibody was used as a negative control. No immunoreactivity was seen in sections stained with omission of the primary antibody.

2.5.13 Immunofluorescence and confocal microscopy

Reticular basement membrane tenascin and procollagen III

Sections were incubated overnight with either a mouse monoclonal antibody directed against tenascin (Clone T2H5, Monosan, Uden, Netherlands) at 1/20 dilution or a rabbit polyclonal antibody directed against procollagan III (Chemicon International, Harrow, UK) diluted to 1/1000 in NHS 10% saponin 0.5% PBS. Following repeated

washes in PBS/0.5% saponin, the tenascin stained sections were incubated with a rabbit anti-mouse FITC (fluorescein isothiocyanate) conjugated antibody (Dako) at 1/30 dilution for 30 minutes and the sections washed thoroughly with 0.5% saponin PBS. The procollagen III sections, after washing, were incubated with a biotinylated goat anti-rabbit secondary antibody at 1/30 dilution (Dako) for 30 minutes. Following thorough washing the sections were incubated with a streptavidin-Alexa Fluor 594 tertiary antibody (Invitrogen) at 1/1250 dilution for 30 minutes and followed by stringent washing. All sections were mounted in fluorescent medium (DAKO) containing 4', 6-diamidino-2-phenylindole (DAPI) as a counterstain. DAPI shows blue fluorescence on binding to DNA (deoxyribonucleic acid) upon excitement with ultra-violet (UV) radiation generated by an argon-ion laser. Cell nuclei are thus visualised allowing orientation of the tissue section. Negative controls were established by omitting the primary antibody during the experiments.

A Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) was used to acquire images. Standardised microscope settings were used to allow comparison of immunoreactivity between paired tissue sections and measurements were analysed using Scion Image analysis software (Scion Corporation, Frederick, Maryland) as previously published (Flood-Page *et al.* 2003a; Phipps *et al.* 2004a) . The thickness of immunoreactivity at the RBM was calculated by drawing a line perpendicular to, and across, the band of immunoreactivity in the RBM at 20μm intervals over the length of the biopsy. Image analysis software was used to measure the length of the line (thickness) and the mean density along the line (pixels per square microns). The values were averaged over the whole length of the RBM to give the mean thickness x density of immunoreactivity. The product of thickness and density (*txd*) was taken as a measure of expression of the ECM protein in the RBM. The intraobserver analysis error was less than 10% as previously reported (Flood-Page *et al.* 2003a).

2.5.14 Myofibroblast expression of ALK-4

The phenotype of fibroblast-like cells that express the activin Type I receptor ALK-4 was confirmed using double immunofluorescence. Sections were incubated simultaneously with a primary Cy3-conjugated goat anti-mouse antibody against α-smooth muscle actin (Sigma) at 1/500 dilution and a primary rabbit anti-mouse ALK-4 polyclonal antibody (gift Prof. Sideras, Athens) at 1/100 dilution in 10% NHS 0.5% saponin PBS buffer overnight. After extensive washing the sections are incubated

with a secondary swine anti-rabbit FITC-conjugated antibody (Dako) at 1/30 dilution for 30 minutes, washed extensively before mounting fluorescent medium with DAPI (Dako). To ensure non-specific binding was not present the primary antibody was excluded in control sections during the IHC procedure.

2.5.15 *In-situ* hybridisation (ISH) immunogenic detection

Introduction

In-situ hybridisation (ISH) is an elegant technique by which gene expression can be localised to microanatomy. An immunogenic detection procedure with non-radioactive FITC-labelled probes ISH kit (Biognostik, Göttingen, Germany) was used to evaluate TGF-$\beta_{1\text{-}3}$ isoform expression. FITC-probes are single-stranded phosphodiester DNA oligonucleotides. The double-FITC-labelled probes contain a FITC group at the 5' and 3' ends of the nucleotides. FITC confers excellent immunogenic propereties equivalent to biotin and digoxigenin. Three FITC-labelled probes, each designed with a sequence directed against a different region of the same target mRNA was used for TGF-β_1, TGF-β_2 and TGF-β_3 isoform detection. Simultaneous use of three different probes for each isoform target mRNA allowed enhanced signal intensity.

***ISH* protocol**

Proteinase K Digestion

Briefly, the tissue sections were allowed to thaw to room temperature and then placed on the surface of a humidity chamber warmed to 37^{0}C. Proteinase K (Promega,UK) 20 µg/ml diluted in TES (50mM Tris-HCL (ph=7.4), 10 mM EDTA, 10 mM Nacl) was used to increase the accessibility of the mRNA. Each section was covered with 30µl and the sections incubated at 37^{0}C for 15 minutes.The sections were now washed briefly in PBS and fixed in 1% PFA for 5 minutes. The sections are immersed in sterile water for 5 minutes, air-dried for a further 5 minutes before proceeding to the pre-hybridisation step.

Pre-Hybridisation

This step is essential to prevent background staining. The buffer provided in the kit (hybrid-buffer) is heated to 95^{0}C in a water bath to clear precipitates and then rapidly cooled in ice to around 40^{0}C. The hybrid-buffer is added in excess to fully cover the sections and then incubated at 30 ^{0}C in an air-tight humidity chamber for 3 hours.

Hybridisation

The probes are added to the hybrid-buffer (prepared as before) at 60μl per 1000μl of hybriprobe. It is important to briefly vortex this mixture before use.

The hybrid-buffer is now gently removed from the sections and the sections are now covered with 25 μl of the probe solution. This step must be performed quickly to ensure that no drying of the sections takes place at this stage. The sections are incubated for 10-16 hours at 30 ^{0}C in an air-tight humidity chamber.

Post-Hybridisation Washes

This step was performed to remove any partially hybridised probe. Mismatched hybrids form less stable complexes compared to perfectly matched complexes.

By manipulating the salt concentration, wash times and temperatures it is possible to remove mismatched probes and therefore decrease staining background. Sections are rinsed twice for 30 seconds each in 1x SSC which removes most of the excess probe and then a further 5 minutes in 1x SSC at room temperature. A more stringent wash in 0.1x SSC at 37 ^{0}C for 15 minutes is performed twice.

Blocking Step

This is essential to block non-specific binding sites for the antibody.

Following a quick plunge in PBS and removal of excess solution from around the section, the section is now incubated with Block Buffer (in excess) for 10-20 minutes. The reaction is terminated with a quick plunge in PBS.

Anti-FITC-Alkaline Phosphatase Antibody (Anti-FITC-AP) Incubation

Excess PBS is removed from around the section. A separate commercial FITC-detection kit (DAKO) is now used. The anti-FITC-AP antibody is added in excess to cover the section and now incubated for 2 hours in a water containing chamber to ensure the slides do not dry out. The reaction is terminated by three 5 minute successive washes in PBS. Excess solution is again removed from around the sections before adding nitro blue tetrazolium (NBT)/ 5-bromo-4-chloro-3-indoxyl phosphate (BCIP) substrate solution (Sigma) in excess. The sections were left for at least 20 minutes before inspection for colour change. Once developed the reaction was quenched by plunging into water. Nuclear Red (Dako) was used as a counterstain. The slides were mounted in glycergel.

Positive and Negative Control

Control sections were run simultaneously with each experiment to validate that the system itself is functional and to also validate the specificity of the detection signal.

A poly d (T) probe served as a positive control whilst a random probe without target (supplied with kit) was used as negative kit.

2.6 Cell counting

The sections were counted blinded to the clinical details. The numbers of positively and negatively stained epithelial cells were counted along the entire basement membrane (BM) of each section using a squared eyepiece graticule (Olympic Corp., Lake Success, NY, USA). When comparing epithelial expression between normals versus asthma, the cell counts were expressed as % positive cells. Cell counts were also expressed as the number per unit length of BM (positive or negative cells/ mm BM). Positive cells below the BM were determined by counting the whole section and expressed as cells per square millimetre of biopsy. All counts were performed using an Olympus BH-2 Microscope (Olympus Corp., Lake Success, NY, USA). On selected samples the slides were counted in duplicate and the coefficient of variation was $< 5\%$.

2.7 Statistical analysis

The methacholine PC_{20} was log transformed before analysis and expressed as the geometric mean ± range. Cell counts are expressed as the median (inter-quartile range) unless otherwise stated. Changes in FEV_1 following allergen challenge are graphically illustrated using the mean (SEM).

The Mann-Whitney U test was used to compare non-paired data. All paired within-subject data was analysed using the Wilcoxon signed rank test. 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 remodelling markers. Data was analysed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA). Significance was accepted as $p<0.05$.

The data in Chapter 3 and 4 was also analysed using a mixed modelling approach to assess the change over time of the selected parameters. This was performed by the Institutional Statistician, NHLI Division, Imperial College London. In this model

patients were entered as a random effect, with time as a fixed effect. The change in each variable measured is presented together with the 95% confidence intervals (CI). Significance was accepted as $p<0.05$.

Chapter 3

Dissociation of Cellular Inflammation from AHR

3.1 Introduction

Allergen exposure in atopic asthma is associated with an inflammatory influx alongside an increase in AHR and bronchial obstruction. The clinical events in an allergen provocation model setting is similar to symptomatic attacks of asthma associated with an airway acute inflammatory response precipated by outdoor allergens and pollutants.

Bronchial provocation tests involve the controlled administration of allergen by inhalation in order to determine the pattern and magnitude of the evoked airway response. Careful standardisation with attention to both technical and non-technical factors is essential for the interest of safety (to avoid excessive bronchoconstriction) and to enable accurate result interpretation. By employing a more longitudinal approach for airway sampling, the model of allergen-provoked asthma offers a unique opportunity to understand the relationship of cellular inflammation to AHR. Following allergen inhalation challenge of atopic asthmatics there are increases in AHR which are far more pronounced in individuals who experience dual (early and late-phase) asthmatic responses (DAR) rather than single early responses (SER) (Cockcroft *et al.* 1977). Furthermore allergen inhalation challenge produces infiltration of airway mucosal inflammatory cells as shown by increases in eosinophils, T cells and neutrophils in BAL fluid (Robinson *et al.* 1993; Metzger *et al.* 1986) and the lamina propria of the airway wall (Beasley *et al.* 1989). Some studies have indicated that airway inflammation is more pronounced in the DAR group indicating that infiltrating leukocytes, especially eosinophils, maybe related to the pathogenesis of both the late asthmatic reaction (LAR) and AHR (Dorman et al. 2004a). Following allergen inhalation a decrease in the FEV_1 of more than 15 % of the post saline reference is defined as an early asthmatic reaction (EAR). A late asthmatic reaction (LAR) is defined as a fall in the FEV_1 of more than or equal to 15% from the post-saline reference value at a single time point between 3 and 7 hours after allergen inhalation (Dorman *et al.* 2004a). Since in the DAR group allergen-induced increased AHR is sustained for days and weeks (Taylor *et al.* 1979; Cockcroft *et al.* 1977) it is reasonable to speculate that in these individuals, there will be a persistence of airway inflammation if inflammation drives AHR. Thus by sampling the airway a week after allergen challenge it is possible to analyse the relationship of sustained AHR to inflammation.

Thus asthmatic individuals that demonstrate dual asthmatic responses (i.e the DAR group) will demonstrate pronounced airway cellular inflammation. The sustained allergen-induced increases in AHR will be associated with persistence of airway inflammation.

In this chapter bronchial biopsies obtained at baseline and 24 hours and 7 days after allergen challenge, according SER and DAR status, were examined in terms of cellular infiltration for the numbers of bronchial mucosal MBP^+ eosinophils, $CD68^+$ macrophages, $CD3^+$, $CD4^+$, $CD8^+$ T cells, $elastase^+$ neutrophils and $tryptase^+$ mast cells. The time-course relationship of inflammatory changes to that of AHR was then analysed. In addition BAL cell numbers for eosinophils, neutrophils, macrophages and monuclear cells were also obtained.

3.2 Results

3.2.1 Cellular inflammation and AHR

Of the 15 volunteers that entered the study, 1 volunteer presented with increase in AHR compared to the screening visit associated with unexpected allergen exposure and another volunteer developed coughing post-allergen challenge such that it was not possible to be certain about her exact single or dual asthmatic response status. These two individuals were therefore excluded from this section of the study analysis.

3.2.2 Volunteer characteristics

Volunteer details are summarised in Table 3.1 for thirteen volunteers. Four volunteers (3 male and 1 female), median age (range) of 25.5 (19-26) years, median FEV_1% predicted 97.65% (range 76.7-114.7) demonstrated single early responses (SER) and 9 volunteers (5 males and 4 females), median age 24 (19-46) years, median FEV_1% predicted 97% (88.70-118.2) had dual asthmatic responses (DAR). The DAR has been defined as a fall in the forced expiratory volume in one second (FEV_1) of 15% from the baseline value between 3 and 7 hours.

One volunteer in the DAR group did not complete the final bronchoscopy and the paired-data analysis has taken this into account. The combined changes of the SER and DAR groups in FEV_1 post allergen challenge are presented in Figure 3.1. The maximal median (range) decrease in FEV_1 3-10 hours after allergen challenge in SER was -10.50 (-6 to -12) % and in DAR was -35.5 (-16 to -42) %. The individual volunteer responses to allergen challenge are summarised in Figure 3.2 for SER and Figure 3.3 for DAR.

3.2.3 Single early responders (SER)

Given that there were only 4 volunteers with SER it was not possible to perform statistical analysis on this group of volunteers. There was no observed trend in either the FEV_1 or PC_{20} following allergen challenge (Figure 3.4). The median change in FEV_1 % from baseline was 2.06% at 24 hours and 4.17% at 7 days post allergen. The methacholine PC_{20} (geometric mean) at baseline bronchoscopy was 3.076 mg/ml and 4.2mg/ml and 2.31 mg/ml 24 hours and 7 days post allergen respectively.

3.2.4 Dual asthmatic responders (DAR)

The airway responses to allergen at 24 hours and 7 days after allergen are summarised in Figure 3.5. The median fall in % FEV_1 from baseline in DR was -17.31 (-21.65- -2) % at 24 hours ($p=0.02$) and -5.1 (-8.3- +7.8) % at 7 days (p=ns) (Figure 3.5A).

Figure 3.5B illustrates that AHR was markedly increased in the DAR group at both 24 hours and 7 days compared to baseline after allergen challenge. The methacholine PC_{20} decreased from 1.631 (0.94-2.82) mg at baseline to 0.3889 (0.1477-1.029) mg/ml at 24 hours ($p=0.004$) and 0.4050 (0.26-0.66) at 7 days ($p=0.02$).

	Volunteer	Age	Sex	FEV_1% Predicted	PC_{20} Methacholine mg/ml	Allergen Specific IgE (IU/Iml)	Allergen Dose (BU)	Maximum FEV_1 % decrease EAR	Maximum FEV_1 % decrease LAR
SER	1	24	M	95.1	7.6	54.9	390	-23	-9
	2	25	M	76.7	0.41	100.5	31.25	-17	-6
	3	26	F	100.2	4.6	25.5	13.32	-39	-12
	4	19	M	114.7	3.12	6.34	375	-26	-8
DAR	5	23	F	95.7	6.16	36.4	400	-30	-42
	6	31	M	101.9	0.88	100.2	175	-19	-36
	7	46	M	118.2	8	11.6	162	-27	-25
	8	21	F	88.7	1.74	20.5	1325	-41	-39
	9	44	M	101.2	2.89	3.63	412	-36	-36
	10	27	F	97	1.08	62.10	1325	-39	-32
	11	19	F	90.3	1.4	0.93	5325	-56	-16
	12	29	M	92.9	0.61	8.53	2125	-46	-35
	13	22	M	95.13	3.2	54.80	390	-23	-23

Table 3.1: Summary of volunteer demography and responses to allergen challenge

A decrease in the FEV_1 of more than 15 % of the post saline reference was defined as an early asthmatic reaction (EAR). A late asthmatic reaction (LAR) was defined as a fall in the FEV_1 of more than 15% from the post-saline reference value at a time point between 3 and 7 hours after allergen inhalation.

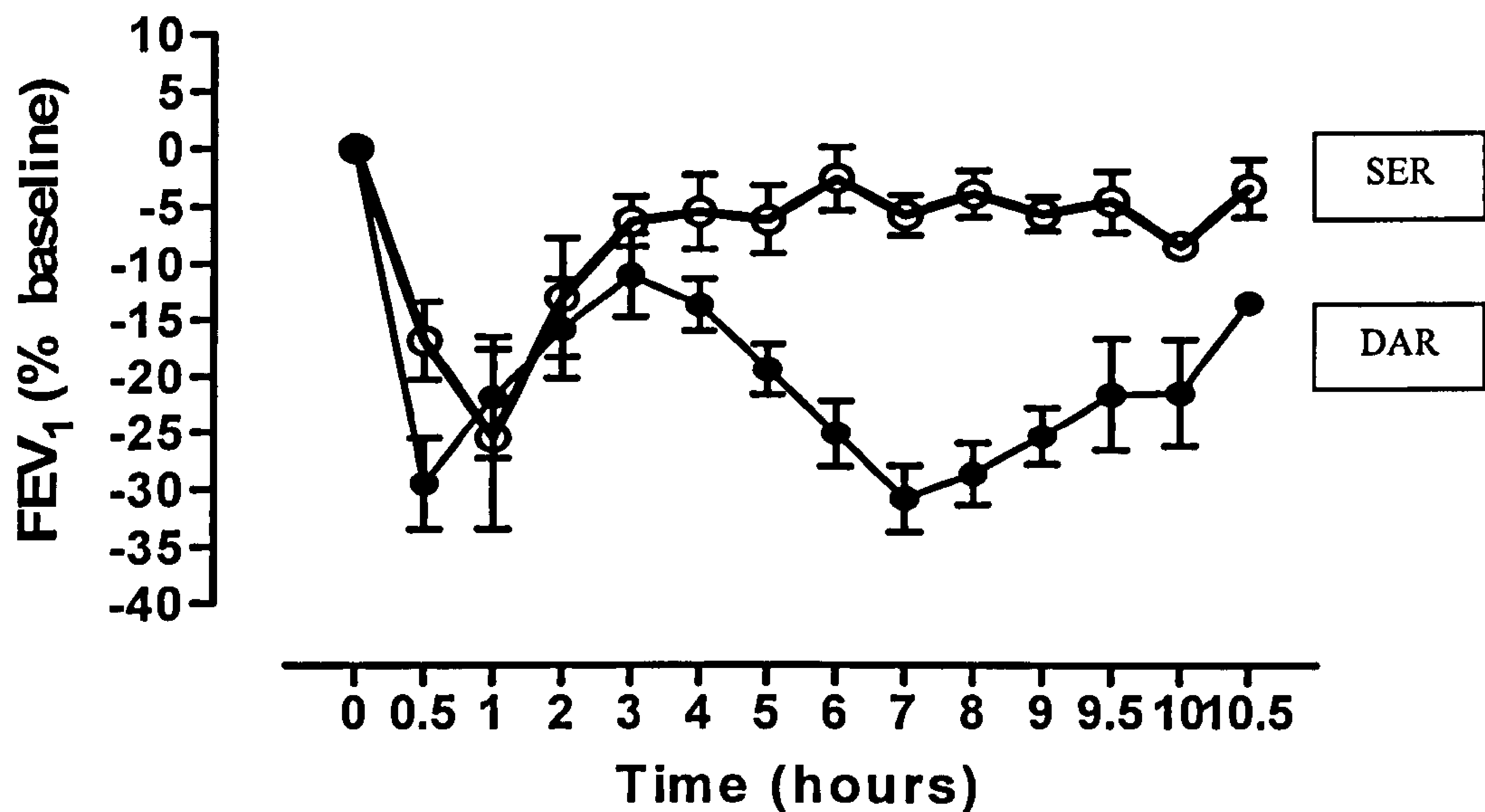


Figure 3.1: Summary of the effect of allergen challenge on the airway response

The changes in FEV_1 are expressed as the mean (SEM) in single early and dual asthmatic responses respectively. Following allergen challenge there was a fall in the FEV_1 of at least 15% from the baseline defined as the early asthmatic reaction followed by a second fall in FEV_1 by at least 15% from the baseline 3-7 hours later defined as the late asthmatic reaction. After a maximal fall in the FEV_1 for the dual asthmatic responses (DAR) at the 7 hour time point a gradual improvement in the degree of airway obstruction is seen, although FEV_1 fails to return to baseline levels.

SER=Single Early Response
DAR=Dual Asthmatic Response

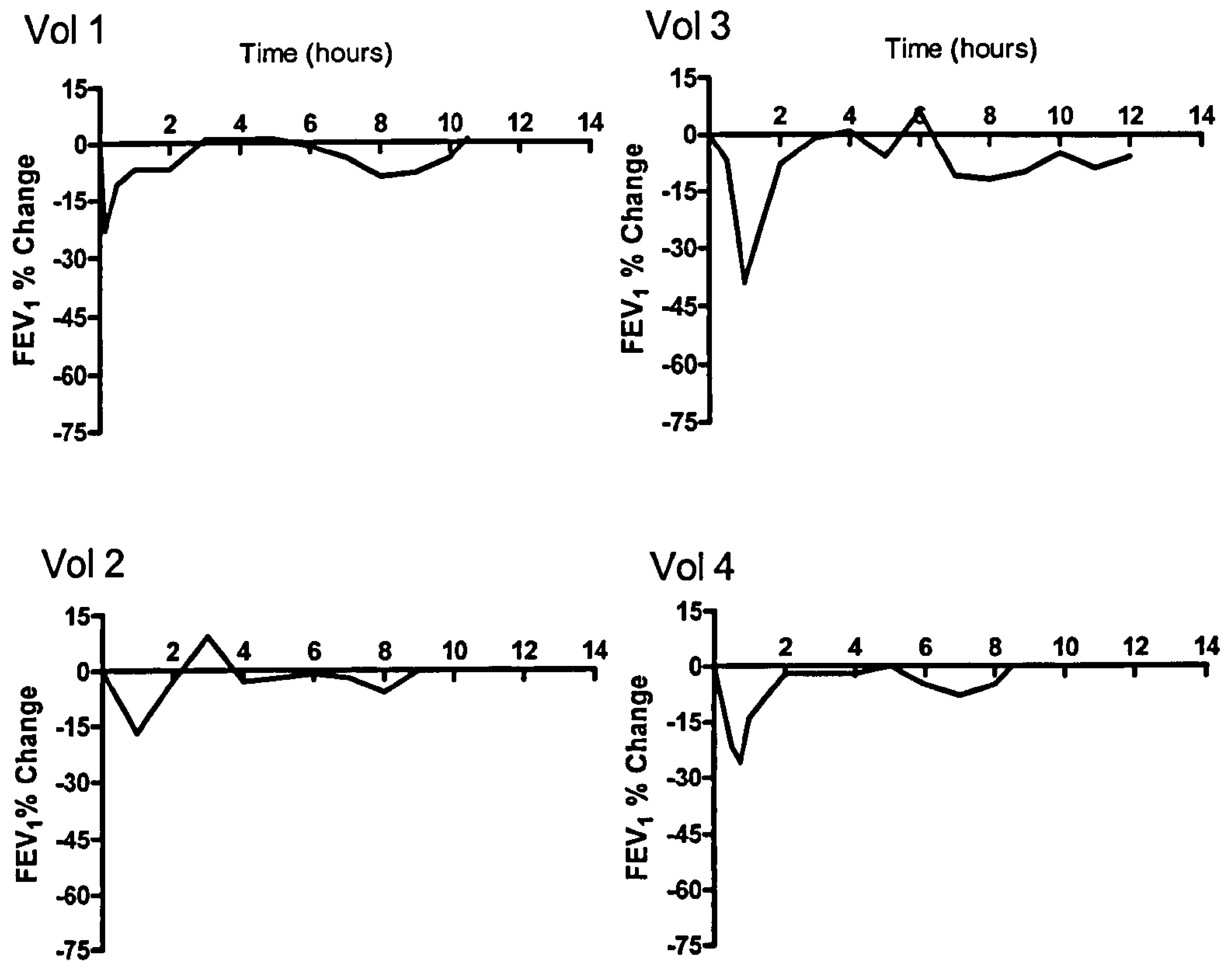


Figure 3.2: Summary of airway responses to allergen challenge in single early responders

The allergen solution (starting with the lowest concentration) was administered using a hand-held nebuliser attached to a breath-activated dosimeter. The challenge was terminated once a fall in the FEV_1 of more than 15% of the post saline reference value was achieved (defined as an early asthmatic reaction) (EAR). FEV_1 measurements were taken at 5,10,20,30, 45 and 60 minutes and then every 30 minutes up to 10 hours. The FEV_1 returns towards baseline values over the course of the day in the single early responders (SER).

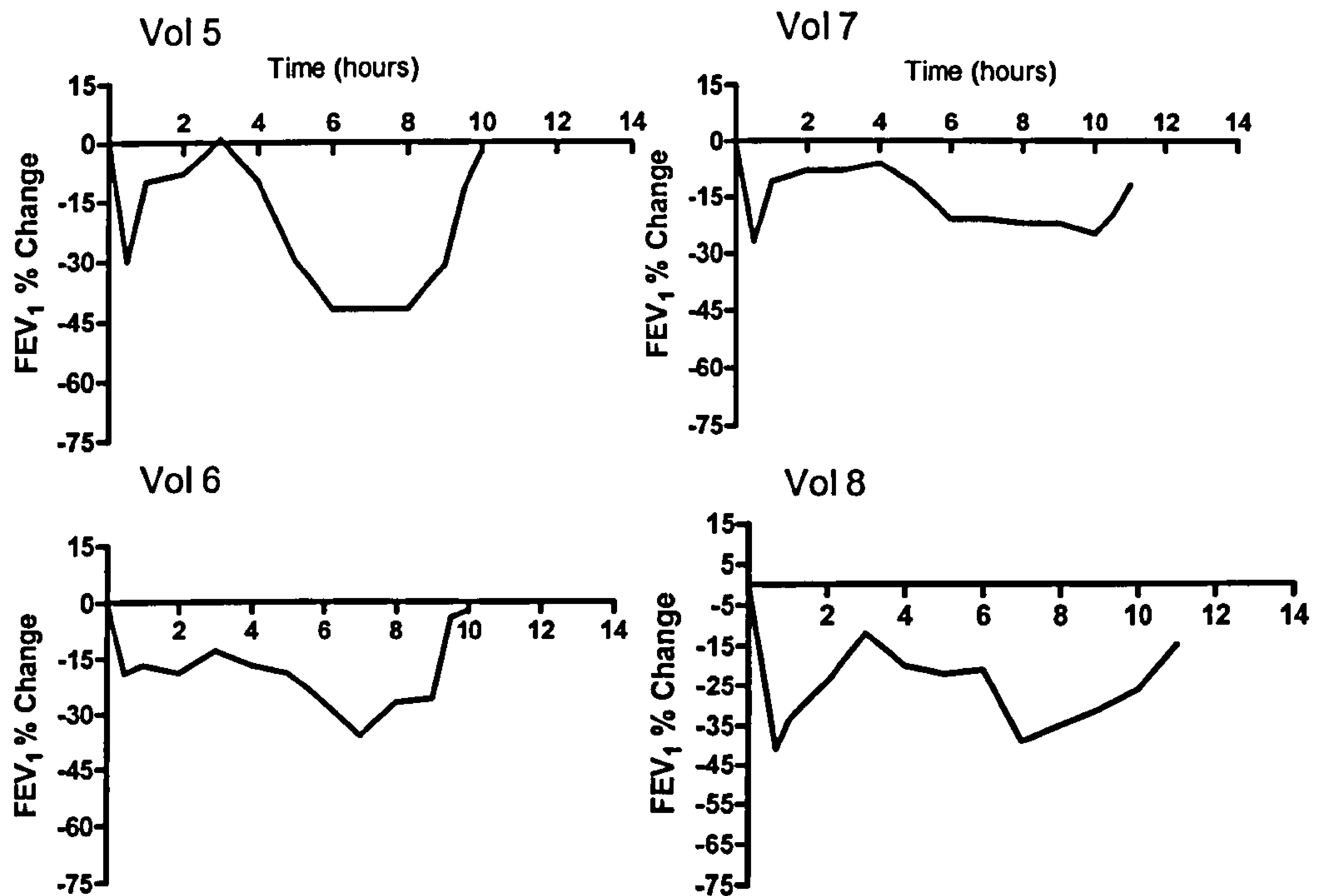


Figure 3.3: Summary of airway responses to allergen challenge in the dual asthmatic responders

The allergen solution (starting with the lowest concentration) was administered using a hand-held nebuliser attached to a breath-activated dosimeter. The challenge was terminated once a fall in the FEV_1 of more than 15% of the post saline reference value is achieved. FEV_1 measurements were taken at 5,10,20,30, 45 and 60 minutes and then every 30 minutes up to 10 hours.

The immediate fall in the FEV_1 of at least 15% from the baseline value was defined as the early asthmatic reaction (EAR), is maximal within 30 minutes and resolves between 1 and 3 hours. The FEV_1 then returns towards baseline values before there is a sustained fall through the course of the day. The late asthmatic reaction (LAR) was defined as a fall in the FEV_1 of more than or equal to 15 % from the baseline value seen between 3-7 hours post-allergen.The volunteers demonstrate a maximal fall in the FEV_1 around 6-8 hours post allergen before a sustained improvement was seen. At this time nebilised β_2 agonist was given which rapidly reversed the airway obstruction before the volunteer was discharged.

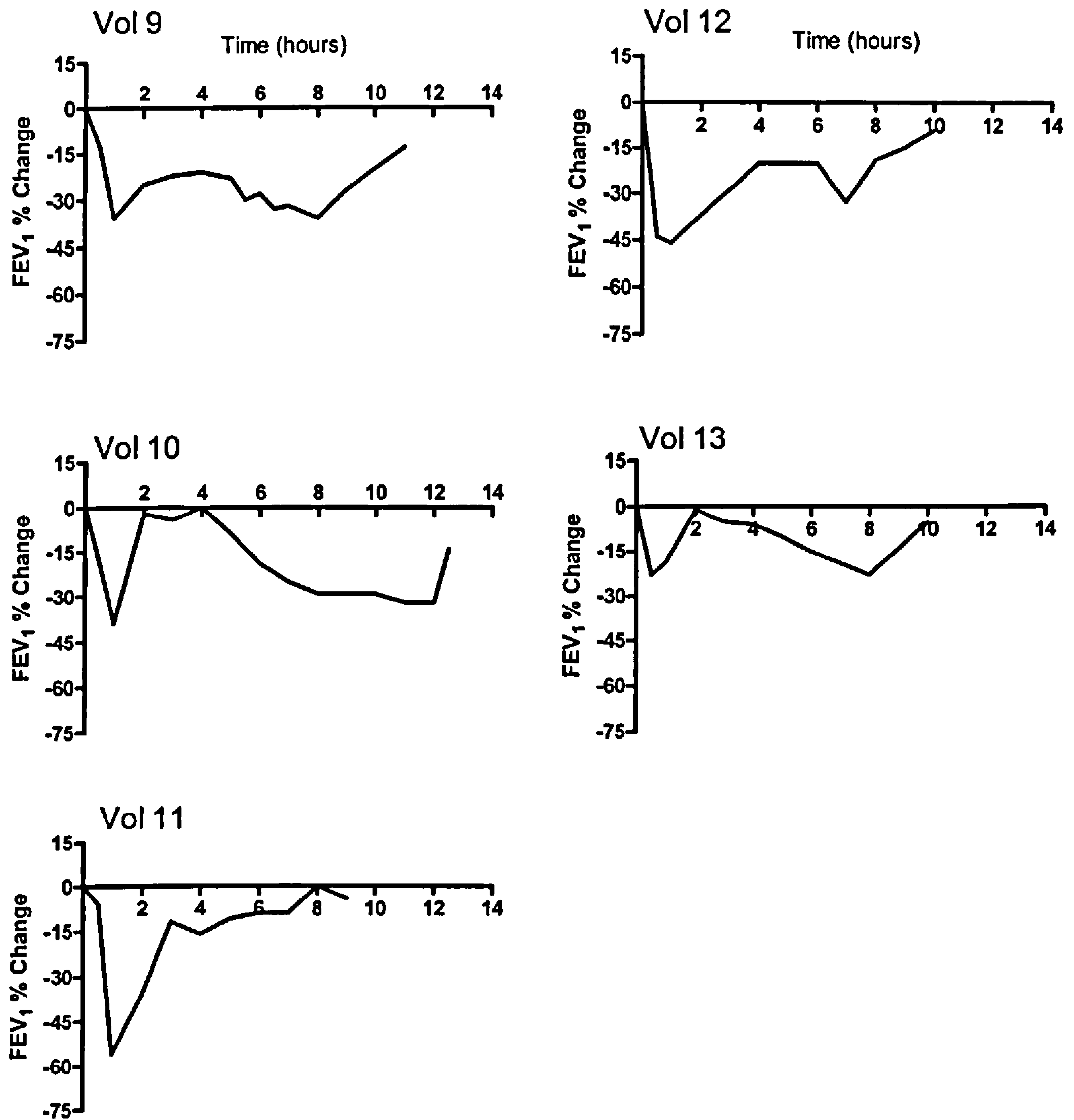


Figure 3.3 (cont.): Summary of airway responses to allergen challenge in the remaining dual asthmatic responders

The late asthmatic reaction (LAR) was defined as a single fall in the FEV_1 of more than or equal to 15 % from the baseline value 3-7 hours post-allergen challenge. The volunteers demonstrate a maximal fall in the FEV_1 around 6-8 hours post allergen before a sustained improvement is seen. At this time nebilised β_2 agonist was given which rapidly reversed the airway obstruction before the volunteer was discharged.

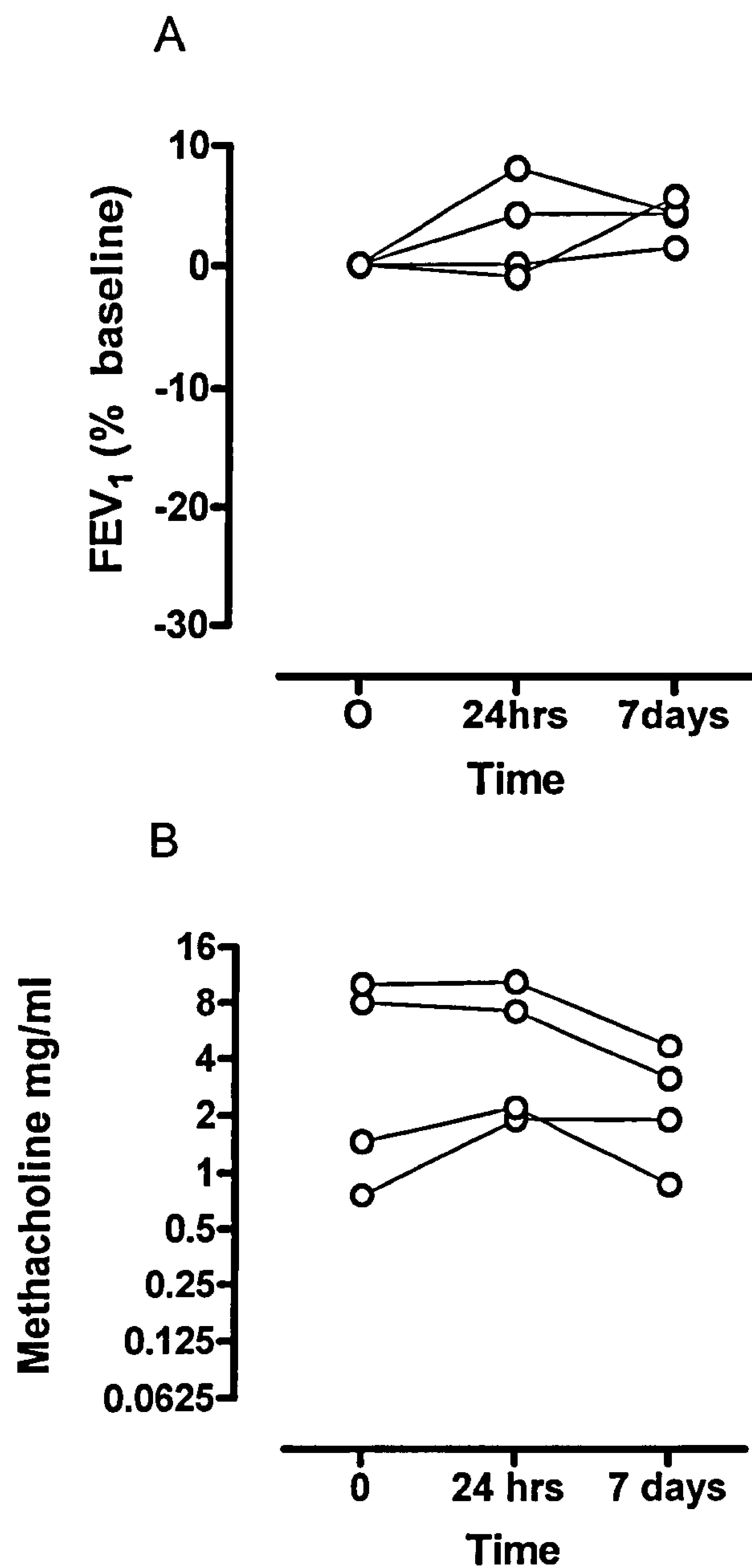


Figure 3.4: Summary of the effect of allergen challenge on the airway response in single early responders (SER)

The effect of allergen inhalation on the FEV_1 and PC_{20} expressed as a percentage change from the baseline value at Visit 2 (Time 0) and Visit 4 (24 hours allergen challenge) and Visit 5 (7 days after allergen challenge) in dual asthmatic response (DAR) group). There is no change in FEV_1 (A) or AHR (B) at either time point after allergen challenge.

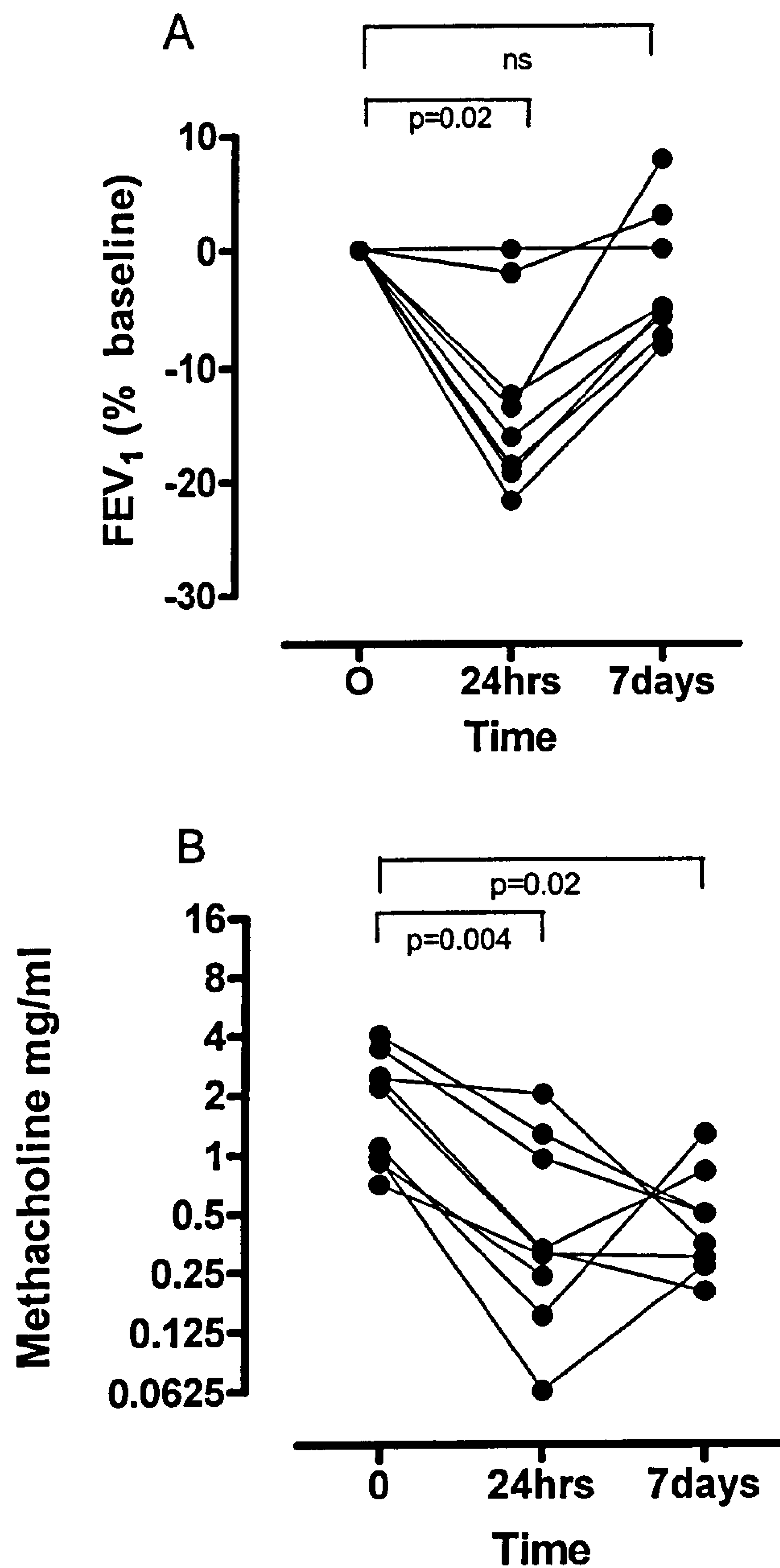


Figure 3.5: Summary of the effect of allergen challenge on the airway response in dual asthmatic responders (DAR)

The effect of allergen inhalation on the FEV_1 expressed as a percentage change from the baseline value at Visit 2 (Time 0) and Visit 4 (24 hours allergen challenge) and Visit 5 (7 days after allergen challenge) in dual asthmatic response (DAR) group. There is a significant fall in FEV_1 24 hours after allergen (A). However, the increased AHR exhibited at 24 hours is sustained 7 days after allergen challenge (B).

3.2.5 Airway inflammation in dual asthmatic responders (DAR).

The cellular median counts (inter-quartile range) are summarised in Table 3.2 and the total cell counts for each volunteer are illustrated in Figure 3.6 for DAR group. In the DAR group a significant increase in MBP^+ eosinophils was observed at 24 hours after allergen challenge (p=0.02) but returned to baseline at 7 days. $CD68^+$ macrophages and elastase+ neutrophils were significantly increased in DAR at 24 hours only (p=0.01 and p=0.03 respectively). Similarly the DAR group demonstrated a significant increase in $CD3^+$ T cells from baseline at 24 hours following allergen challenge (p=0.004). At the 7 day post-allergen time point cellular counts returned to baseline levels. DAR as a group failed to demonstrate any significant increase in either $CD4^+$ or $CD8^+$ T cells at 24 hours and 7 days after allergen challenge.

On combining the cell count data for both the SER and DAR groups (Figure 3.7) the increase in MBP^+ eosinophils from baseline at 7.8 (3.8-31.85) (median ± interquartile range) to 35.50 (22.95-52.50) was highly significant at 24 hours (p=0.002) but not at 7 days. The combined data for macrophages was also highly significant at 24 hours (p= 0.0004). The increase in $CD4^+$ and $CD8^+$ T cells now reached statistical significance with p=0.05 and p=0.03 respectively at 24 hours following allergen, whilst the increase $CD3^+$ T cells was highly significant (p=0.0002).

Given that extracellular MBP staining may be misinterpreted as intact eosinophils (Figure 3.8B) the counts were repeated using Congo Red. The mucosal eosinophila was highly significant at 24 hours post-allergen (p=0.0078) for the DAR group (Figure 3.9).

Mast cells were counted in both the whole section and in association with smooth muscle. Individual total cell counts are illustrated in Figure 3.10A (whole tissue) and 3.10B (smooth muscle). No significant increases in mast cell numbers was observed in the DAR group. Mast cells infiltrating airway smooth muscle is illustrated in Figure 3.11A with confirmation of the presence of smooth muscle in the biopsy by staining for smooth muscle actin (Figure 3.11B).

3.2.6 Bronchoalveolar lavage (BAL)

Table 3.3 summarises the cellular kinetics of inflammatory cells as identified in bronchoalveolar lavage (BAL) fluid. Individual cell counts are presented as percentage cell counts. In the DAR group, the eosinophils increased from 0.53 (0.04-0.89) % at baseline to 3.15(0.8-7.7) % at 24 hours (p=0.02) and remained elevated at 2.0 (1.5-5.1) % at 7 days (p=0.02). There was a reciprocal decrease in the number of macrophages in the DAR group post-allergen (p=0.04). There was no significant change in mononuclear cell percentage (assumed to be T and B cells) or neutrophils. BAL mast cells were generally low in number and could not be identified on the basis of simple morphology. Given the low volunteer number of volunteers in the SER group no statistical analysis could be performed but an upward trend in eosinophils, mononuclear cells and neutrophils at both 24 hours and 7 days after allergen challenge was noted.

Mucosa

		Dual Asthmatic Responders			Single Early Responders	
Cell Type	**0 Hours**	**24 Hours**	**7 days**	**0 Hours**	**24 Hours**	**7 days**
MBP+ Eosinophils	4.7 (1.7-26.65)	36 * (23.25-58.5)	8 (4.5-22)	12.10 (7.8-20.10)	26.6 (21.4-39.2)	28.8 (13.4-42.85)
CD68+ Macrophages	30.5 (2.65-42.7)	40.0* (16.55-94.65)	34.75 (21-57)	21 (14-28)	56.15 (36.5-76.1)	40.55 (19.65-47.9)
CD3+ T cells	27.30 (22.4-64)	86.60* (55.7-107.3)	43 (15.45-57.9)	41.75 (30.55-49.10)	99.95 (68.2-159.1)	66.75 (50.15-82.80)
CD4+ T cells	18.2 (2.85-31.30)	40.00 (21.5-60.45)	32.8 (3-39.70)	37.35 (17.85-42.6)	55.35 (13.35-104.4)	52.7 (23.9-65.75)
CD8+ T cells	2.4 (0.6-6.25)	14.20 (1.45-33.10)	4 (0-13.95)	12 (1.5-18.25)	8.6 (1.8-53.35)	12.6 (2.65-16.55)
Elastase+ Neutrophils	22.0 (13.45-27.30)	29.4* (22-38)	21.15 (16.15-31)	27.65 (18.25-38)	25.5 (22-37.800	27 (21.35-30.10)
Tryptase+ Mast Cell	20.7 (11.6-34.85)	20.25 (4.75-35.65)	17.35 (9.3-25.9)	3 (1-12)	12 (6-14)	3 (1-4)

Table 3.2: Summary of mucosal cellular inflammation

The cell counts (cells/mm^2) are expressed as the median (inter-quartile range) for single early responders and dual asthmatic responders. * indicates $p<0.05$ compared with the cell count at baseline for DAR group.

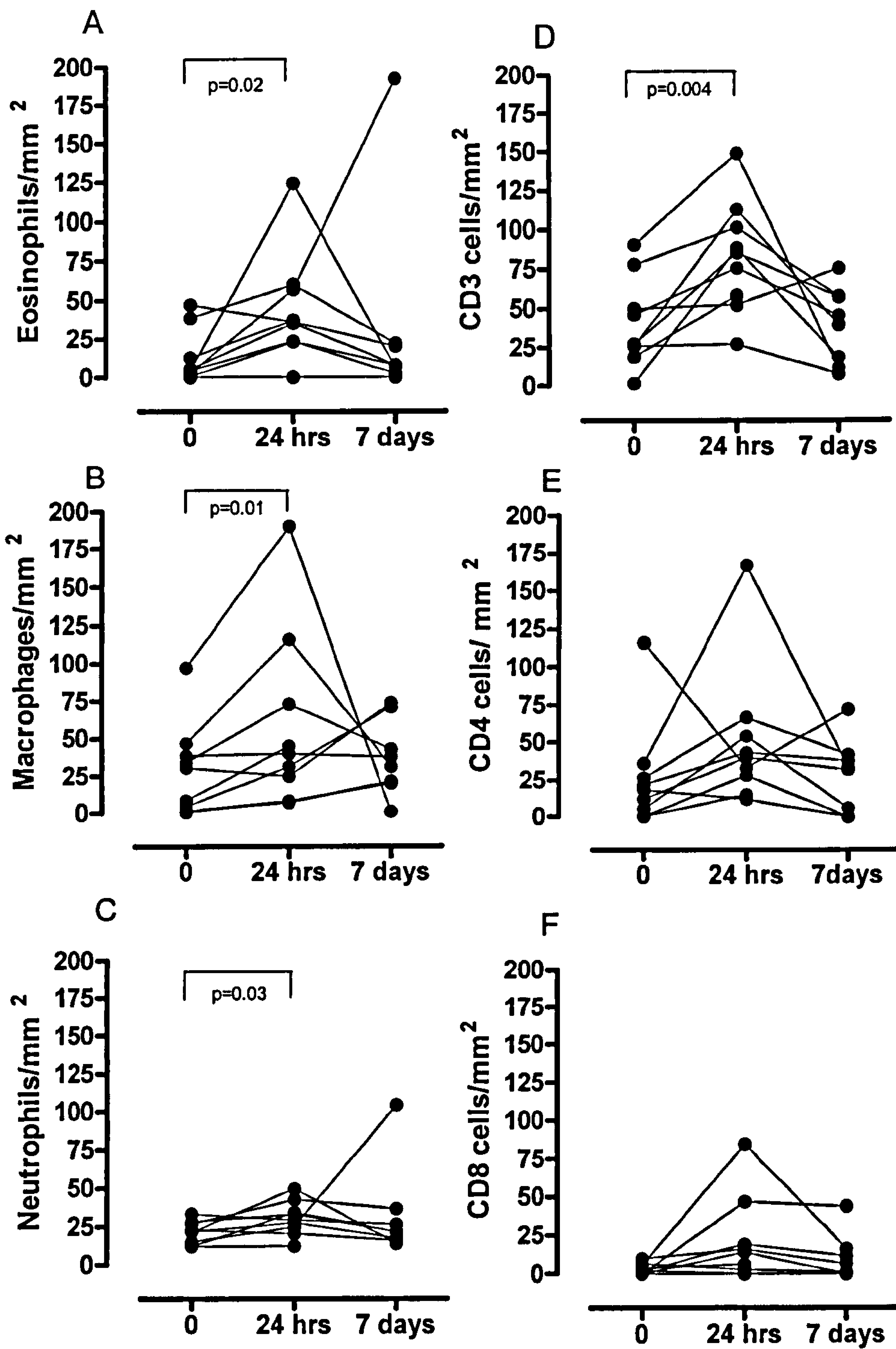


Figure 3.6: Summary of the changes in cellular inflammation in response to allergen challenge in the dual asthmatic response (DAR) group

Positive inflammatory cells are expressed as cells/mm^2. Significant differences between time points were analysed using the Wilcoxon signed-rank test.

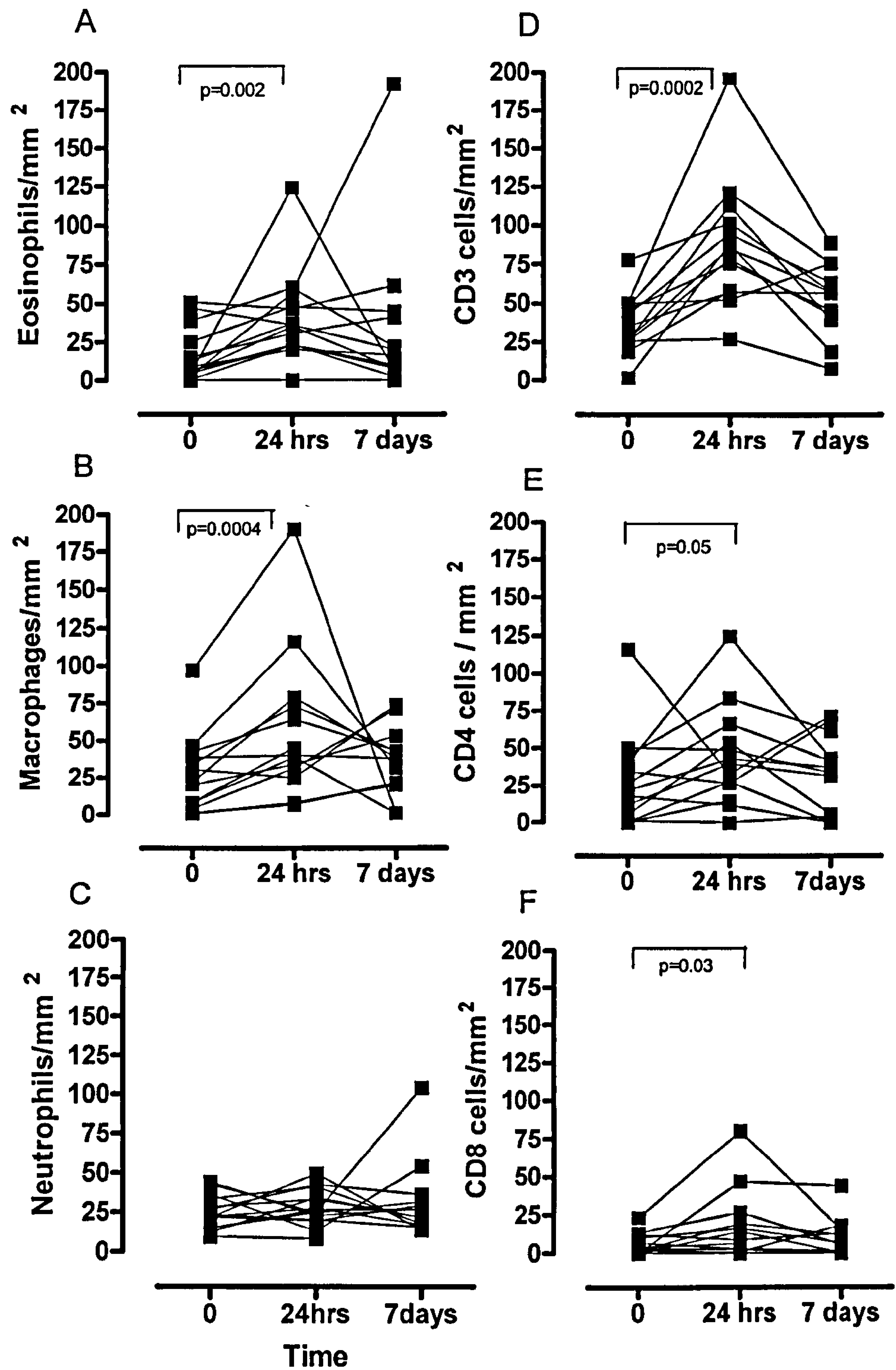


Figure 3.7: Summary of the changes in cellular inflammation in response to allergen challenge in all thirteen volunteers combined

It can be seen that SER individuals also demonstrate increased cellular recruitment to the airway albeit less marked than the DAR group, leading to further significant p values for MBP^+eosinophils (A) (p=0.002), $CD68^+$ macrophages (B) (p=0.004) and $CD3^+$ T cells (D) (p=0.0002). This data is consistent with the view that the SER and DAR are not a dichotomous group but a continuum.

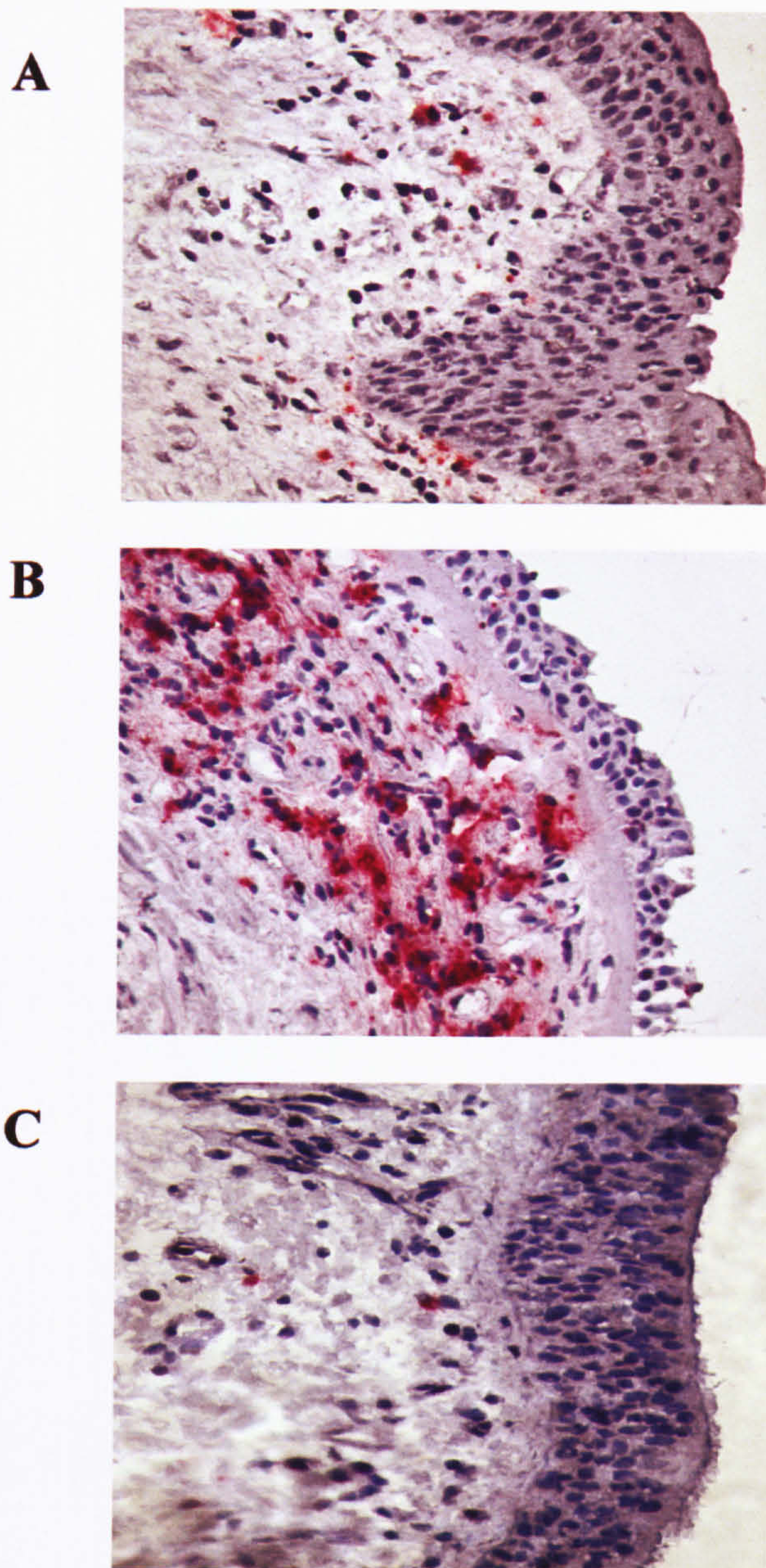


Figure 3.8: Representative photomicrographs of MBP⁺ eosinophils in a selected volunteer

At baseline (A) eosinophils are present but low in number. At 24 hours post-allergen (B) there is a marked increase in MBP^+ eosinophils with evidence of explosive degranulation. At the 7 day-time point (C) mucosal eosinophil numbers approach baseline levels. Sections were stained with a monoclonal anti-MBP antibody and developed using the APAAP system.

MBP=Major basic protein

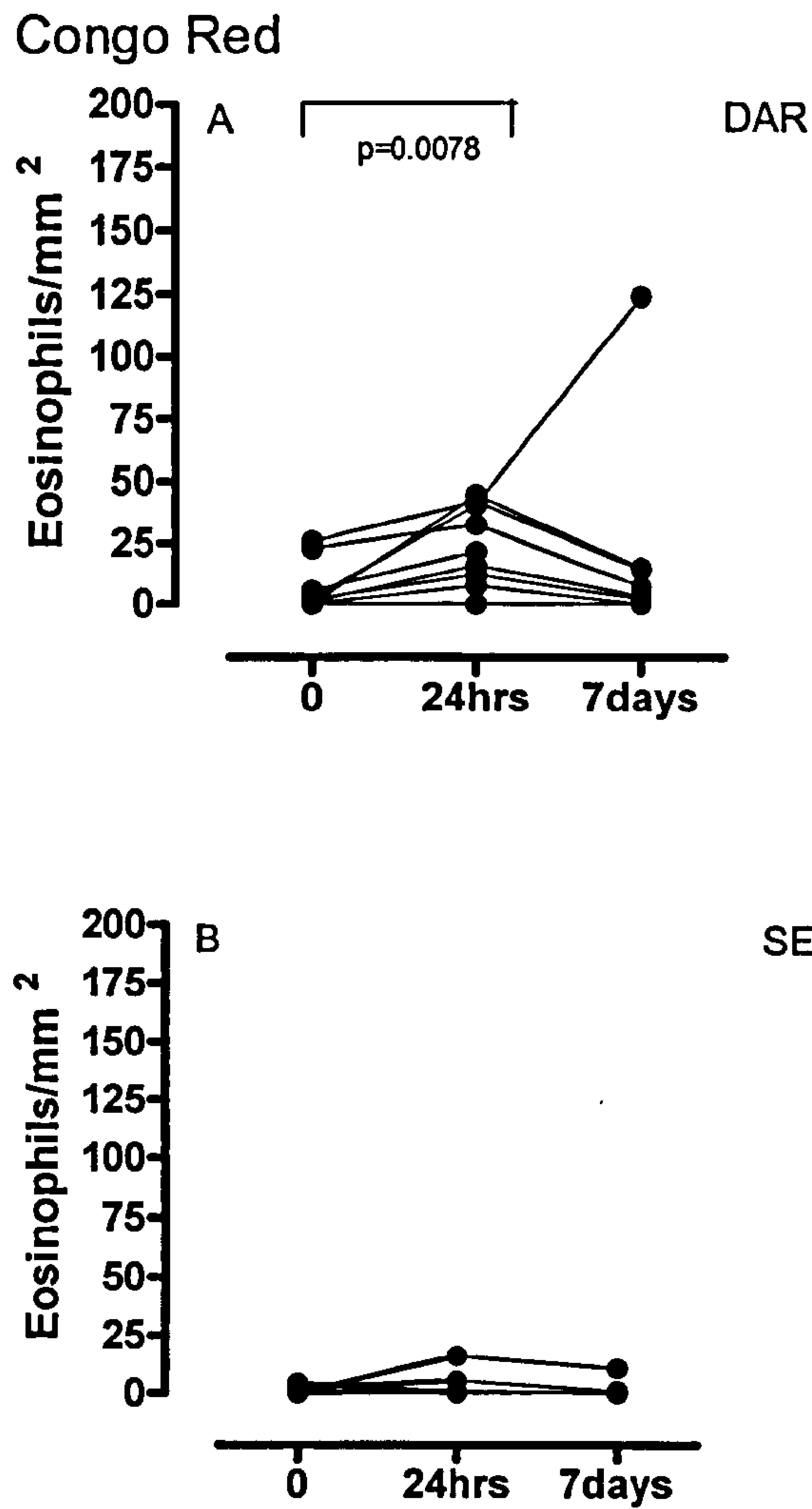


Figure 3.9: Congo Red staining determination of airway eosinophilia

Given that extracellular MBP staining may be misinterpreted as intact eosinophils the counts were repeated using Congo Red. The mucosal eosinophila is highly significant at 24 hours post-allergen in the DAR group (p=0.0078) (A). Positive inflammatory cells present are expressed as cells/mm^2.

MBP=Major basic protein

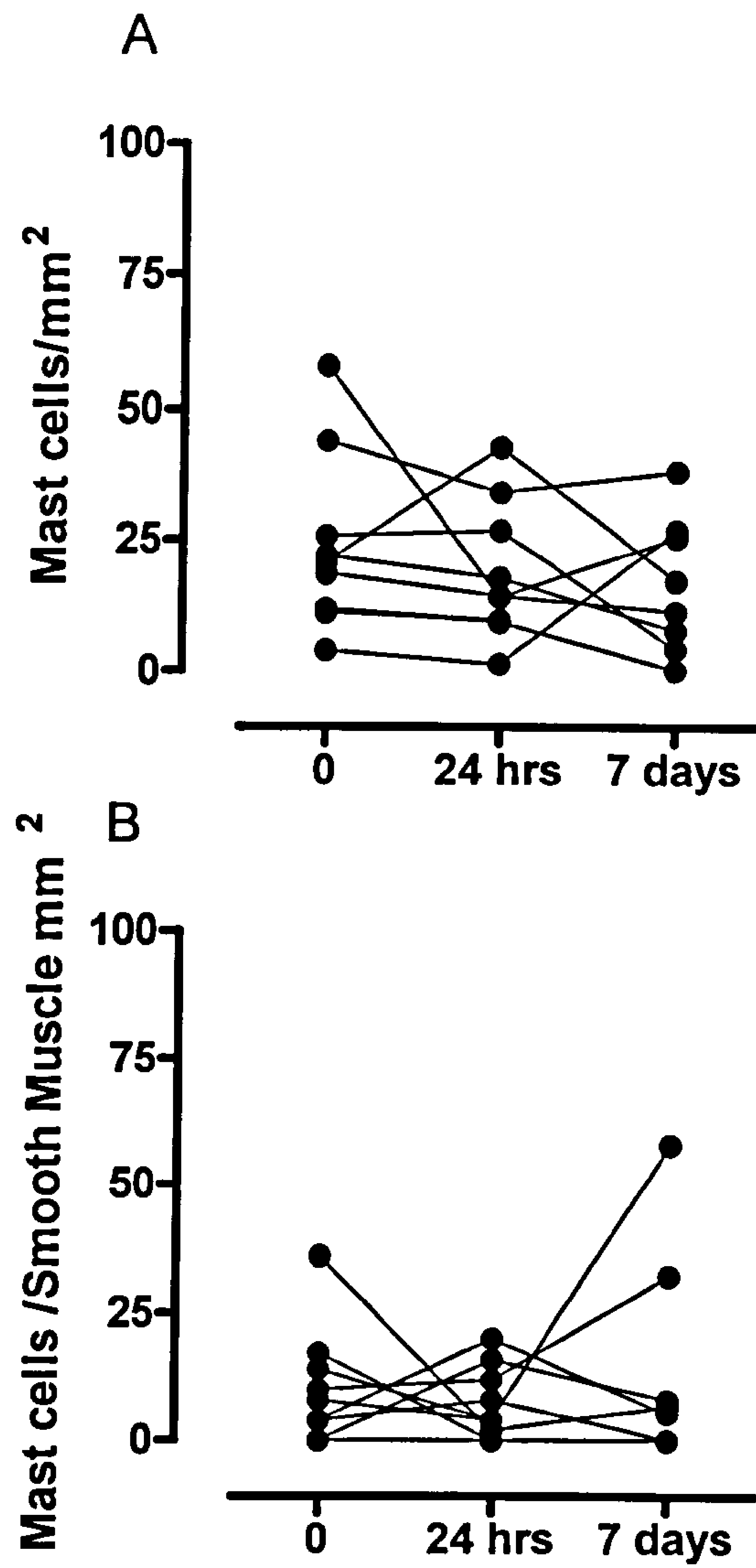


Figure 3.10: Changes in mucosal mast cell numbers

Figure A shows total mast cell numbers (cells/mm^2) infiltrating the mucosa and Figure B shows mast cell numbers infiltrating the airway smooth muscle areas in the DAR group.

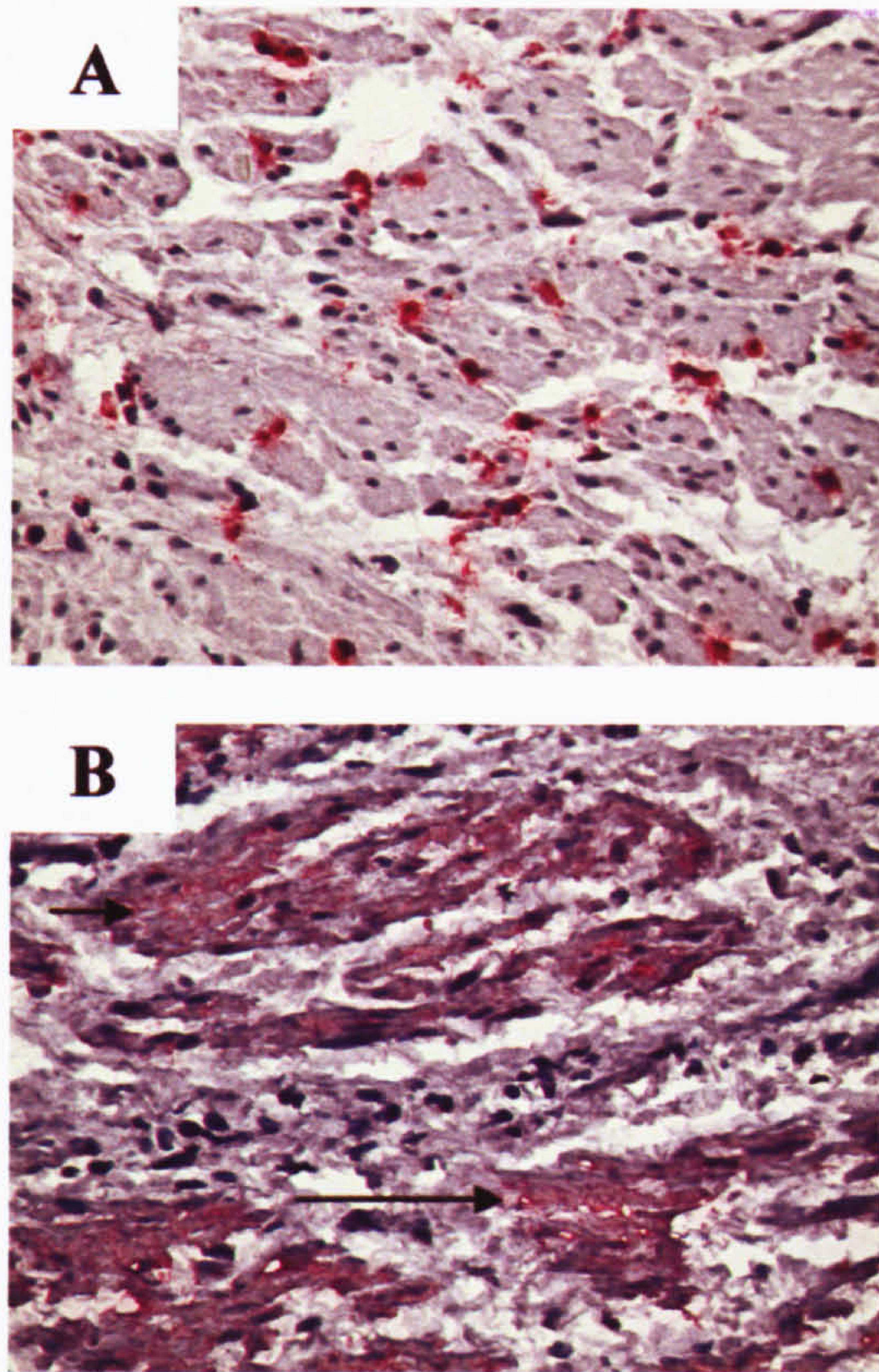


Figure 3.11: Representative photomicrograph of tryptase^{+} mast cells infiltrating airway smooth muscle in a selected asthmatic volunteer

Mast cells were counted specifically in the smooth muscle (A). Confirmation of the presence of smooth muscle in the biopsy was confirmed by staining for smooth muscle actin seen as filamentous red bands (arrowed) in smooth muscle areas delininated as purple areas by H and E staining (B).

	Dual Asthmatic Responders			Single Early Responders		
	0 Hours	24 Hours	7 days	0 Hours	24 Hours	7 days
Total Cells 10^6/ml	0.75 (0.45-1)	0.85* (0.65-1.48)	0.95 (0.2-1.3)	0.7 (0.45-1.3)	0.65 (0.35-0.9)	0.85 (0.6-1)
Eosinophils %	0.53 (0.04-0.89)	3.15* (0.8-7.7)	2* (1.15-5.1)	1.95 (1.3-3)	2.75 (1.3-3.05)	2.15 (0.65-3.7)
Macrophages %	88.93 (86.52-92.4)	73.97* (66.9-87.7)	85.27 (78.2-89.4)	89.47 (86.32-90.43)	85.40 (84.57-90.07)	88.75 (83.25-95.40)
Mononuclear cells %	7.86 (6.13-12.49)	7.4 (1.7-19.9)	11.07 (5.83-17.93)	6.93 (5.53-11.52)	10.67 (2.7-15.72)	12.20 (2-16.82)
Neutrophils %	0.3 (0.1-1.2)	0.6 (0.2-4.8)	0.6 (0-0.9)	0.2 (0.1-1)	1.1 (0-3.1)	0.8 (0.4-1.3)

Table 3.3: Summary of BAL cellular kinetics

Cells were identified on the basis of morphology and staining colour. In particular eosinophils were bi-lobed cells with reddish orange granular staining in the cytoplasm. Macrophages were recognised as cells with bright blue-purple cytoplasm and light blue nucleoli. Lymphocytes were recognised as mononuclear cells with clear blue cytoplasm. BAL mast cells are generally low in number and could not be identified on the basis of simple morphology.

3.3 Discussion

In this chapter it is shown that increases in AHR 24 hours post-allergen challenge is associated with cellular inflammation. By sampling the airway at the 7 day time point it is shown that allergen-induced increases in airway inflammation in the dual asthmatic response (DAR) group is virtually resolved by seven days, whereas at that time the increases in AHR still persist. It is therefore possible to dissociate increases in AHR from cellular inflammation.

Using the LAR to separate out SER and DAR as two separate dichotomous groups allows researchers to focus on asthmatics with greater or lesser allergen-induced increases in inflammation and AHR and evaluate the mechanisms that lead to increased inflammation and AHR. Such a separation is based completely on an arbitrary definition which can vary within the same research group as an FEV_1 fall of 15% or 10% from baseline (Dorman et al. 2004a) (Wood *et al.* 2002). It is more likely that the SER and DAR are the ends of a continuum and the development of the LAR in an individual maybe dependent on the dose of allergen delivered to provoke the EAR (Ihre *et al.* 1988). In turn the development of the EAR is dependent on the level of specific allergen sensitivity and the underlying degree of AHR (Cockcroft *et al.* 2005). Safety concerns limit all studies as to what level of allergen is given to provoke the EAR which may then determine whether or not a LAR develops. The 'continuum' concept may explain why the SER individuals both in this study and others (Wood *et al.* 1998; Gauvreau *et al.* 1999; Dorman *et al.* 2004b) demonstrate eosinophil and other inflammatory cell recruitment to the airway, although to a less significant degree compared to the DAR volunteers.

Allergen-injected atopic skin has also served as an important immunological model of allergic inflammation. It has been shown that there is a dose-dependent relationship between allergen-dose and continuation of the skin reaction to the formation of a skin late-phase response (LPR) (Frew & Kay 1988a) seen as an erythematous, indurated swelling that peaks between 6-24 hours and is resolved by 72 hours (Frew & Kay 1988b; Frew & Kay 1988a). Time course studies in skin confirm granulocyte infiltration as early as 2 hours post-allergen (Ying *et al.* 1999) with eosinophils and neutrophils peaking as early as 6 hours which remain elevated as far as 72 hours later. The peak of $CD3^+$ cells (predominantly $CD4^+$ $CD45RO^+$ memory immunologically primed T cells) was seen at 24 hours (Ying *et al.* 1999) and elevated cells numbers

were still present at 48 hours. Eosinophils, T cells, neutrophils and macrophages remain elevated as far as 96 hours post-intradermal allergen (Tsicopoulos *et al.* 1994).

Confidence in the safety and tolerability of fibre-optic bronchoscopy (FOB) has allowed sampling from the airways after allergen challenge at different time points. Studies evaluating airway responses after challenge in biopsies confirm significant eosinophilia and T cell recruitment at 6 hours (the highest numbers of cells are found at this time point) which were sustained at 24 hours and 48 hours (Bentley *et al.* 2003). Th2 cytokine expression can be correlated with the preceding airway narrowing during the LAR (Robinson *et al.* 1993) with increased IL-13 expression (Huang *et al.* 1995). Our volunteers demonstrated marked increases in $CD3^+$ T cell numbers 24 hours following allergen challenge. This increase is consistent with allergen activation of T cell-mediated inflammation. Analysis in terms of $CD4^+$ and $CD8^+$ confirmed only a trend towards increases in airway $CD4^+$ cells post-allergen in that it did not reach statistical significance. This was somewhat surprising, particularly given the essential role of T cells in the LAR as confirmed by the ability of peptides to directly induce the LAR (Ali *et al.* 2004). Whilst it is not possible to directly compare studies of allergen with peptide, such work does nevertheless suggest a significant causal role for the T cell in the LAR. Other studies have also failed to achieve statistical significance for the paired increases in $CD4^+$ cells after allergen challenge (Bentley *et al.* 1993). It is possible that the failure to achieve statistical significance is attributable to not evaluating allergen-specific $CD4^+$ T cells that are recruited.

The significant increase in macrophages (Mø) in response to allergen may be an indication of their role as antigen-presenting cells (Larche *et al.* 1998) . The status of Mø as professional phagocytes enable them to play a critical role in inflammatory resolution by clearance of apoptotic inflammatory cells (Gregory & Devitt 2004). Phagocytosis by Mø recognition of membrane phosphatidylserine on apoptotic cells is associated with the production and secretion of TGF-β_1 that subsequently serves to reduce airway inflammation (Huynh *et al.* 2002; Otsuka *et al.* 2004). Mø secrete large amounts of TGF-β_1. Given that the induction of airway remodelling in response to allergen induced injury is seen as early as 24 hours (Phipps *et al.* 2004a) after allergen challenge, Mø infiltration seen in our model at 24 hours might also contribute airway repair with consequent fibrosis.

We did not demonstrate marked increases in airway neutrophils after allergen challenge. Neutrophil recruitment is related to the concentration of endotoxin in the allergen used for challenge (Alexis *et al.* 2004) (Hunt *et al.* 1994). We confirmed very low levels of endotoxin (data not given) to be present in the *Fel D1* (*Leti,* Madrid) preparation. The endotoxin level data in HDM and grass allergen extract (Allergopharma) was not available from the company. Although no measure of basophils was made in this study previous work has shown that, compared to eosinophils, there were minimal increases in bronchial mucosal basophil numbers after allergen challenge (Macfarlane *et al.* 2000). In contrast to the upper airways and the skin, basophils were not a prominent cell in the airways of asthmatics.

BAL enables the sampling of cells from the luminal side of the airway barrier. In normal volunteers 10-20 million cells can be recovered in a lavage volume of around 100mls. Macrophages (mø) are the predominant cell population recovered and comprise 80-90% of the total cell population with lymphocytes contributing 10-20%. The total contribution of eosinophils and neutrophils were less than 1% whilst mast cell populations were less than 0.5%. In airway biopsies, lymphocytes were the predominant cell type in the normal airway mucosa. Mø contribute to 20% of the total cell population whilst mast cells contribute less than 10%. Neutrophils can vary in number but eosinophils are rarely present in normal individuals and if present are in an inactivated state. Thus the information obtained from BAL provides different but complementary information.

Allergen studies in the lung have confirmed significantly increased cellular inflammation in individuals with DARs. In an early study (De Monchy *et al.* 1985) analysis of BAL just after the EAR and during the LAR confirmed marked eosinophilia at both time points. BAL analysis 4 and 24 hours after whole lung allergen challenge (Metzger *et al.* 1986) confirmed increases in eosinophil and neutrophil percentages as early as 4 hours with a reciprocal decrease in macrophages. Further studies confirmed early eosinophilia (Rossi *et al.* 1991) and that it is sustained at 24 hours (Metzger *et al.* 1986; Robinson *et al.* 1993). Eosinophils were of an activated phenotype. Diaz et al confirmed increased BAL neutrophils, eosinophils and also lymphocytes 6 hours post-allergen in DAR group (Diaz *et al.* 1989). In a separate time-course study by Metzger et al confirmed increased BAL neutrophils, eosinophils and T cells at 48 hours post allergen with sustained eosinophils and T cells at the 96

hour time point (Metzger *et al.* 1987). The kinetics of cellular inflammation is provided in a detailed induced sputum study (Gauvreau *et al.* 1999) showing significant increases in luminal eosinophils, macrophages, neutrophils and metachromatic cells (presumed mast cells and basophils) significantly elevated at 7 hours. Only luminal eosinophils and macrophages remained significantly elevated at 24 hours, 2 days, 4 days and 7 day time points.

BAL eosinophila is the most characteristic cellular abnormality finding in asthma ranging from 2-11%. The cells demonstrate an activated phenotype. Whole lung allergen challenge is associated with moderate increases in BAL eosinophila within 4-6 hours after allergen that is sustained at 24 hours, particularly in DAR group individuals. Direct comparisons of past studies are difficult to compare to the findings from this study due to different types of bronchial challenge applied and variation in allergen stock and dosing schedules.

In this study the increase in BAL eosinophilia was sustained at both 24 hours and 7 days post-allergen despite the mucosal eosinophil counts returning to baseline levels. Luminal exit may be more effective than apoptosis as an eosinophil clearance mechanism (Erjefalt *et al.* 2004; Uller *et al.* 2001). The continued exit at 7 days suggests that clearance, whilst a rapid process, is also a sustained process. The changes in BAL macrophage numbers can be variable. Whilst some studies have shown no changes (Diaz *et al.* 1989) others have shown increases (Dupuis *et al.* 1992), but sampling was at 6 hours post inhalational allergen challenge in the former while segmental allergen challenge was used in the latter study. We observed a small but significant reciprocal decrease in the individual macrophage counts (expressed as a percentage), consistent with the observation by Hunt *et al* (Hunt *et al.* 2002). Given that macrophages undergo activation with increased adhesiveness post-allergen, it is possible increased adherence to the airway lumen or even the collecting vial leads to an underestimation of the cell numbers.

Despite the fact that the size of the LAR and associated increase in AHR has been shown to correlate with the degree of airway eosinophilia (Wardlaw *et al.* 1988), whether the eosinophil is involved in the development of the LAR is controversial. A single infusion of anti-IL-5 (eight volunteers in each group) did not abolish the LAR despite marked reduction in sputum and blood eosinophilia (Leckie *et al.* 2000). The

kinetics of eosinophil recruitment in the skin model has been shown to be dissociated from that of the skin LPR (Phipps *et al.* 2002). In addition eosinophil depletion using an anti-IL-5 antibody did not affect either the magnitude of the cutaneous swelling that defines the LPR (Phipps *et al.* 2004b) suggesting that the eosinophil may not be causal for the development of the LPR. However cyclosporin-A can block the LAR but not the EAR (Sihra *et al.* 1997) suggesting an important role for activated T cells. Inhalation of allergen-derived T cell peptides can lead to the LAR (Haselden *et al.* 1999) which again supports a role for T cells in the LAR. Activated eosinophils ($EG2^+$) and macrophages ($CD68^+$) recruited in response to allergen express the high affinity IgE receptor FcεRI which may explain the ability of IgE to independently induce the LPR.

The original observation of Cockcroft and his group (Cockcroft *et al.* 1977) that allergen inhalation increases AHR in DAR individuals is sustained for at least 7 days is confirmed in this study. This observation is reminiscent of the sustained increase in airway symptoms related to AHR in asthmatic patients following allergen exposure. The temporal association between increased inflammatory cell infiltration and increased AHR at 24 hours after allergen challenge (Dorman *et al.* 2004b; Brusasco *et al.* 1990; Dorman *et al.* 2004a) may suggest a causal relationship. However, the current findings suggest that increases in airway inflammation do not need to be sustained for persistence of increased AHR. It is therefore possible that the mechanism underlying the acute increases in AHR at a time of intense airway inflammation may be different to that which sustains AHR long-term. Inflammatory cells are important sources of vasoactive mediators, cytokines and cysteinyl leukotrienes, particularly LTC_4, which can induce bronchoconstriction, mucus hypersecretion and increased microvascular permeability that will all contribute to inflammation associated increases in AHR. Inflammatory cell derived products may also lead to structural cell activation that is sustained beyond resolution of cellular inflammation to baseline levels. For example a so called IL-13-cysteinyl leukotriene-ASM axis has been identified where IL-13 may not only influence ASM contraction directly via IL-13R expressed on ASM (Laporte *et al.* 2001), but also via release of leukotrienes such as LTD_4 that act directly on ASM (Panettieri *et al.* 1998).

The exact role of the eosinophil in relation to AHR has recently been questioned by the finding that anti-IL-5 (mepolizumab) given both on single occasion and in a

separate study on three consecutive occasions failed to have any effect on AHR (Leckie *et al.* 2000; Flood-Page *et al.* 2003b). However mepolizumab only reduced bronchial mucosa eosinophils by only 55 % in the latter study. Until studies are performed where airway eosinophilia is completely abolished it is difficult to comment on the exact contribution eosinophils may make to AHR. Another feature of these two studies is that AHR was measured in response to histamine. It has been shown that AHR measures using indirect challenge such as with adenosine better reflects AHR related to cellular inflammation (Van den *et al.* 2001). A significant role for eosinophils in airway repair is now established and it is possible that eosinophil infiltration at 24 hours in our model is again related to airway repair responses (Kay *et al.* 2004). Recruited macrophages and T cells may also be related to their role in tissue repair. In idiopathic pulmonary fibrosis at least it has been suggested that $CD8^+$ T cells and $CD68^+$ macrophages in particular contribute to the severity of disease (Daniil *et al.* 2005).

Significant insight into the role of inflammation in the mechanism of AHR, particularly the role of the eosinophil, can be obtained by our experience of allergic rhinitis (AR) and eosinophilic bronchitis (EB). ARs can demonstrate marked airway eosinophilia similar to asthma in response to allergen challenge but with no obvious airway functional change (Lopuhaa *et al.* 2002). Inflammatory changes in EB can be indistinguishable from asthma despite the complete absence of AHR (Brightling *et al.* 2002). These findings support the assertion that inflammation in itself is not responsible for AHR. It may be that cellular inflammation but must occur in the context of a specific (as yet undefined) mucosal environment for AHR to develop. The micro-location of the inflammatory components may also be important. For example airway smooth muscle (ASM) infiltration by mast cells may be an important determinant of AHR in asthma as it was a defining difference between baseline asthma and eosinophilic bronchitis (Brightling *et al.* 2002). The suggestion that a mast-cell induced smooth muscle cell myositis is a determinant of AHR in asthma is intriguing. In our study we were unable to demonstrate any increased trafficking of mast cells into the smooth muscle areas. These findings do not necessarily contradict a role for mast cells in baseline AHR and these cells may contribute to allergen increases in asthma by degranulation products acting directly on ASM leading ASM activation and contraction.

Several studies have failed to demonstrate convincing correlation between inflammation and AHR with several studies having found no convincing association (Djukanovic *et al.* 1990; Ollerenshaw & Woolcock 1992; Iredale *et al.* 1994; Crimi *et al.* 1998; Brusasco *et al.* 1998). Whilst it is likely that the factors such as asthma heterogeneity and AHR measures with direct acting agents may have influenced such results, overall the evidence from human studies does not support the view that cellular inflammation, particularly eosinophil infiltration, directly relates to intrinsic or sustained AHR. This does not exclude inflammatory events contributing to the initiation and propagation of AHR.

Animal models of disease provide insight into what aspects of cellular inflammation and cytokines may contribute to AHR. Studies where Th2 effector cytokine activity has been blocked, particularly IL-4, IL-5 and IL-13 have questioned further the exact role airway inflammation has in the induction and maintenance of AHR. IL-4 deficient mice still develop AHR with allergen challenge despite markedly reduced airway inflammation (Rankin *et al.* 1996). The results with IL-5 are conflicting. The reduction in peribronchial eosinophila in models using IL-5 deficient mice (Foster *et al.* 1996) or IL-5 blocking antibodies (Hamelmann *et al.* 1997) is associated with reduction in AHR. Similarly, epithelial transgenic over-expression of IL-5 recapitulates many of the pathological features of asthma and demonstrate AHR to methacholine in the absence of allergen exposure (Lee *et al.* 1997). These findings implicate eosinophilic inflammation as contributing to AHR. Yet Humbles *et al* elegantly demonstrated using a GATA-1 knock out mouse model that, despite complete ablation of the eosinophil lineage, development of allergen–induced AHR is unaffected (Humbles *et al.* 2004). This conflicting finding suggests that eosinophils are not effector cells in AHR and may offer an explanation for the somewhat disappointing results found in trials of anti-IL-5 in human asthma.

There is increasing evidence that IL-13 is able to induce AHR in the absence of inflammatory cell infiltration. IL-13 blockade inhibits AHR despite the presence of inflammatory cells and continued IgE synthesis. It appears that T and B cells are not a requirement for IL-13 induced AHR (Grunig *et al.* 1998). Pre-treatment of mice with the potent granulocyte inhibitor vincristine (Singer *et al.* 2002) did not prevent the induction of AHR by IL-13 whilst studies in eotaxin/IL-5 double knock-out mice again confirmed IL-13 induction of AHR (Yang *et al.* 2001) in the absence of

eosinophilia. Over expression of only epithelial STAT6 in IL-13 transgenic mice induced AHR in the absence of eosinophils again suggesting eosinophils do not participate in the development of AHR (Kuperman *et al.* 2002). The interpretation of these studies must be that IL-13 can induce the entire pathophysiological changes of asthma through its combined effects on the structural cells themselves. This is independent of traditional inflammatory cells. Such cells may still contribute to the disease process by the synthesis and release of IL-13 but inflammatory cells are not essential for the induction or maintenance of AHR. In asthma it is possible that allergen induced injury activates the synthesis and release of IL-13 from resident airway structural cells such as epithelium, ASM and fibroblasts which can then act on ASM in the absence of inflammatory cell infiltration. Activation of IL-13 signalling is evident as early as 24 hours after allergen challenge in human asthma (Phipps *et al.* 2004a). There is convincing data that AHR may in fact be a direct effect of IL-13 on ASM or by regulating the production of several mediators that can regulate the contraction or relaxation of ASM. IL-13 has been shown to reduce β2 receptor induced relaxation of ASM via MAPK pathway (Laporte *et al.* 2001). IL-13 also contributes to the severity of AHR indirectly via its positive feedback interaction between cysteinyl leukotriene and TGF-β_1 production that are known to induce ASM hyperplasia (Espinosa *et al.* 2003).

It is believed that the development of the LAR occurs as a continuum within and between individuals, possibly related to the dosage of the allergen used (Ihre *et al.* 1988).Although SER numbers are small it still possible to see an upward trend for the recruitment of eosinophils, macrophages and $CD3^+$ T cells in our population. There is however evidence to suggest that acute increases in AHR in response to allergen may to some extent be dependent on T cell recruitment and activation as has been demonstrated on several occasions by $CD4^+$ and $CD8^+$ depletion studies where the development of acute allergen induced transient increases in AHR was absent (Gavett *et al.* 1994). Using blocking anti-$CD4^+$ and anti-$CD8^+$ antibodies and an acute and chronic model of allergen exposure, Leigh *et al* were able to show that the acute increases in AHR, whilst T cell dependent, sustained AHR associated with chronic allergen exposure did not require the presence of either $CD4^+$ or $CD8^+$ (Leigh *et al.* 2004b).

An important observation is that the fall in FEV_1 was significant at 24 hours in the DAR group but approached baseline values at 7 days. Thus it appears that although AHR does not follow changes in absolute cell numbers, changes in FEV_1 may do, as has been outlined before in previous studies (Bousquet *et al.* 1990; Walker *et al.* 1991). Increased airway wall oedema and vascularity, mucus hypersecretion and cellular debris associated with the peak of inflammation at 24 hours post-allergen leads to increased airway obstruction in the DAR group. Such acute airways changes will diminish with inflammatory cell resolution. The clinical implication from this work is that measuring cellular inflammation does not allow any insight into asthma severity in terms of AHR.

Thus inconclusion persistent inflammatory cell infiltration of the bronchial mucosa does not appear to be essential to sustain allergen-provoked increases in AHR. This may reflect persistence of the end effects of inflammation such as neural, vascular and mucosal remodelling processes which in turn have downstream effects contributing to AHR. It maybe that there are mechanisms for AHR in response to allergen challenge that are not due to airway inflammation perhaps indicating that there are different components to AHR. Acute increases in response to allergen maybe initiated by inflammation whereas sustained AHR may result from a different mechanism

3.4 Summary of Chapter

In this chapter it was investigated whether sustained AHR in DARs was associated with sustained increases in cellular inflammation. The numbers of eosinophils (MBP^+ and Congo Red^+), macrophages (CD 68^+), neutrophils ($elastase^+$), mast cells ($tryptase^+$) and T cells ($CD3^+$, $CD4^+$, $CD8^+$) were counted in bronchial biopsies from mild atopic asthmatics (n=14) at baseline, at 24 hours and at 7 days after allergen inhalational challenge. AHR (methacholine PC_{20}) and FEV_1 were also measured at these time points. In DARs (n=9) AHR was markedly increased 7 days after allergen challenge. However eosinophils, macrophages, neutrophils and $CD3^+$ and $CD8^+$ T cells were significantly elevated only at 24 hours and returned to baseline values by 7 days. Thus the data suggests that persistent infiltration of further inflammatory cells to the airway does not appear to be essential for the maintenance of increased AHR following allergen inhalation in dual asthmatic responders.

Chapter 4

Airway Remodelling

4.1 Introduction

In this chapter the expression of remodelling markers in asthmatic and normal volunteers are described as is their relationship to inflammation and AHR. Whilst it is now becoming accepted that airway remodelling is an acute event and is up-regulated at the same time as inflammation in response to allergen-induced airway injury in asthma, it remains uncertain as to whether remodelling is sustained, particularly the relationship of remodelling in the presence or absence of cellular inflammation to AHR.

A previous study has shown that bronchial biopsies from mild atopic asthmatics who develop dual asthmatic responses (DAR) obtained 24 hours after allergen challenge show significant increases in RBM tenascin deposition and HSP-47 expression (a marker of induction of collagen synthesis) with localisation to airway fibroblasts (Phipps *et al.* 2004a). There was evidence of IL-13 and TGF-β Superfamily signalling in the epithelium and fibroblasts at this time point. Airway infiltration by myofibroblasts in the DAR group was seen as early as 24 hours after allergen challenge (Gizycki *et al.* 1997). This data supports the hypothesis of allergen-induced remodelling suggesting that allergen-induced activation of the epithelial mesenchymal trophic unit (EMTU), the embryological unit driving airway development, is rapidly reactivated during the process of airway remodelling. Separate studies on the effect of eosinophil depletion on decreasing ECM components (Flood-Page *et al.* 2003a), together with the effects of steroids on attenuating selected aspects of remodelling (Laitinen *et al.* 1997) suggests that some aspects of airway remodelling may be a consequence of inflammatory events. Therefore it is possible that airway remodelling is an acute response to allergen-induced airway injury. Some aspects of remodelling will be related to inflammatory cell infiltration whilst other aspects will be related to structural cell activation.

For these reasons the aim of this chapter to confirm that allergen inhalation leads to rapid induction of remodelling markers at 24 hours but in addition determine whether this up-regulation is maintained or resolved at the 7 day post-allergen time point. The results will be compared with the degree of AHR at these time points.

4.2 Results

4.2.1 Allergen–induced activation of airway remodelling

The modulation of expression of airway remodelling markers in response to inhaled allergen is summarised in terms of cellular median counts (inter-quartile range) in Table 4.1 for all thirteen volunteers and Table 4.2 for the DAR group and SER group separately. Data for individual volunteers are illustrated in subsequent figures (stated in text below).

The induction of tenascin in the RBM was markedly increased from baseline (Figure 4.1A) 24 hours post allergen (p=0.0007) but returned to baseline values at the 7 day post allergen time point as can be seen from the confocal images (Figure 4.2A, B and C respectively). The increase at 24 hours significantly correlated with eosinophil infiltration only (r=0.61, p=0.03) (Figure 4.1C). We and others have previously shown that tenascin expression in normal airways is either minimal or absent, in contrast to asthma. This finding is illustrated from a normal volunteer who participated along side the negative control in images in Figure 4.2D and 4.2E respectively.

A discrete layer of cells with fibroblast-like appearance were identified, on the basis of morphology, below and parallel to the RBM and in the deeper layers of the submucosa that stained for the collagen chaperone HSP-47. Expression of HSP-47 was also evident in the epithelium. We did not detect any HSP-47 expression in fibroblast-like cells in the 6 normal volunteers suggesting basal transcription of HSP-47 (and hence collagen) is minimal under basal conditions in healthy adults. With allergen challenge there was significant up-regulation of both procollagen I (p=0.001) and HSP-47 (p=0.02) in airway cells counted in the whole biopsy at the 7 day time point (Figure 4.3A and 4.3B respectively) in the 13 asthmatic volunteers.

There was significant up-regulation of HSP-47 in fibroblast-like cells at the 24 hour time point (p=0.02) (Figure 4.3C) but not airway epithelium, confirming our previous published findings (Phipps *et al.* 2004a). At 7 days following allergen exposure, a marked and sustained increase was evident with an almost 12-fold increase in the median number of HSP-47^{+}-fibroblast numbers (p=0.0001) (Figure 4.3C and Figure 4.7C).

The morphology of fibroblast-like cells staining for HSP-47 could be readily identified (Figure 4.7D). It was not possible discriminate fibroblast-like cells on the basis of morphology when using procollagen I staining given that procollagen is both intra and extracellular, making cell morphology indistinct (Figure 4.7E) and therefore the counts for procollagen I may include cells other than fibroblasts. The expression of α-SMA in fibroblast-like cells was taken as evidence of myofibroblast transformation. There was no difference in the number of myofibroblast-like cells between normal and asthmatic volunteers (Figure 4.4A). There was a significant increase in myofibroblasts numbers at 24 hours ($p=0.02$) which was sustained at 7 days ($p=0.03$) (Figure 4.4B). These spindle-like cells were independent of any vascular or glandular structure and therefore unlikely to represent vascular smooth muscle or glandular tissue that may express α-SMA.

4.2.2 The relationship of airway remodelling to AHR

The dual asthmatic response (DAR) group demonstrated markedly increased AHR at both 24 hours and 7 days as stated in Chapter 3. We further determined whether the DAR group alone would have significant expression of remodelling markers at these time points.

The expression of RBM tenascin increased at 24 hours after allergen challenge ($p=0.004$) but approached baseline levels again by Day 7 (Figure 4.1B). The expression of procollagen III in the RBM did not change at the 24 hour post allergn point consistent with a previous study from the group (Phipps *et al.* 2004a). However, at the 7 day time point there was a significant increase in RBM procollagen III ($p=0.01$) (Figure 4.9A and Figure 4.10 Images A-C). The increase in procollagen I remained highly significant at the 7 day time point ($p=0.0078$) and HSP-47^{+} expression remained significant at both 24 hours ($p=0.04$) with further highly significant increases at 7 days ($p=0.0078$) (Figure 4.5 A and 4.5 B). The increase in AHR (PC_{20}) was significantly correlated with HSP-47 expression in the group as a whole at 24 hours ($r=-0.6$ $p=0.02$) but this association was not significant at 7 days ($r=-0.5$, $p=0.08$). The DAR group only demonstrated a marked correlation between PC_{20} and HSP-47 expression at 24 hours ($r=-0.8$, $p=0.02$) but this association did not persist to 7 days ($r=-0.23$, $p=0.4$) (Figure 4.6).

In the previous chapter it was demonstrated that inflammation and AHR dissociate at the 7 day time point. Thus inflammation and remodelling can also be dissociated at a time point when AHR and remodelling remain associated.

The data for the DAR group was also analysed using a mixed modelling approach to see if there were significant changes over time in the ten variables that changed at either or both the 24 hour or 7 day post allergen time point in the study (i.e. PC_{20}, FEV_1, MBP^+ eosinophils, $CD68^+$ macrophages, $CD3^+$ T cells, $elastase^+$neutrophils, RBM tenascin, RBM procollagen III, procollagen I^+ cells and HSP-47^+ cells). Such an approach had added the advantage of using all the data (including the patient for whom there was no 7 day follow-up). It also allowed us to quantify the effect of time. The statistics from the mixed model approach is summarised in Table 4.3. It can be seen that such analysis leads to similar conclusions as that obtained from the paired analysis data disussed earlier. The change in PC_{20} remained significant but the overall change in FEV_1 was not significant. The change per day for the other variables was only significant for procollagen I^+ cells, RBM procollagen III and HSP-47^+ fibroblasts.

4.2.3 Vascular endothelial growth factor (VEGF)

Infiltrating inflammatory cells were identified as an important source of VEGF although epithelium was also contributory. There were also cells with fibroblast-like morphology present staining positive for VEGF. No expression was seen in smooth muscle cells. Expression of VEGF was significantly more in the asthmatic group compared to normal volunteers (Figure 4.11A). No significant change in the expression level of VEGF following allergen challenge was detected in terms of IHC, with median baseline expression of 252 (102-400) cells/mm^2 at baseline, 296 (184.5-480) cells/mm^2 at 24 hours and 324 (192-424) cells/mm^2 at 7 days post-allergen (Figure 4.11B).

4.2.4 Epithelial-mesenchymal transition

There was no significant difference in the level of epithelial expression for HSP-47, vimentin or α-SMA between normals and asthmatics at baseline. On allergen challenge expression of vimentin in epithelial cells did not change significantly from baseline at 30.40 cells/mm^2 (15-4-44.90) to 32 cell/mm^2 (6.3-49.9) at 24 hours and 19.55 cell/mm^2 (9.85-43.35) at 7 days (all p=ns). Whilst an up-regulation of HSP-47 from baseline levels at 52.54 cells/mm2 (32-150) was evident at both 24 hours at 90.50 cells mm^2 (43.34-139.7) and 98 cells/mm^2 (44-149.7) at 7 days, the increases did not reach statistical significance. Similarly no change in the number of epithelial cells expressing α-SMA was seen (data not shown).

Marker	O hours	24 hours	Significance	7 days	Significance
Tenascin RBM units	**841 (652-930)**	**1330 (793.4-1989)**	**p=0.0007**	**942 (407-1160)**	**ns**
PC I$^+$ all cells/mm^2	**88.0 (26-115)**	**125.7 (57.2-228.5)**	**p=0.08**	**264.0 (116.8-348.8)**	**p=0.001**
PC III RBM units	**2190 (1631-2871)**	**2543 (1875-3502)**	**ns**	**2208 (1515-4158)**	**ns**
HSP-47$^+$ all cells/mm^2	**53.24 (33-112)**	**104.7 (61-173.8)**	**ns**	**166.7 (87.56-368)**	**p=0.02**
HSP-47$^+$ fibroblasts cells/mm^2	**2 (0-8)**	**10.67 (1-34.7)**	**p=0.02**	**51.56 (16-173.8)**	**p=0.001**
α-SMA$^+$ fibroblasts	**16.0 (8.85-48)**	**43.0 (31.10-69)**	**p=0.02**	**47.95 (23.35-97.35)**	**p=0.03**

Table 4.1: Expression of tenascin and procollagen III in the RBM and cellular expression of procollagen I, HSP-47 and α-SMA in asthmatic volunteers

Positive cells were determined by counting the whole section and expressed as cells per square millimetre of biopsy. All counts were performed in a blinded fashion using an Olympus BH-2 Microscope (Olympus Corp., Lake Success, NY, USA). RBM ECM expression was via confocal microscopy and Scion Image Analysis software as decribed separately. On selected samples the slides were counted in duplicate and the coefficient of variation was $< 5\%$. Cell counts are expressed as the median (inter-quartile range). Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as $p<0.05$.

		Dual Asthmatic Responders			Single Early Responders	
Remodelling Marker	0 Hours	24 Hours	7 days	0 Hours	24 Hours	7 days
Tenascin RBM units	854.2 (386.5-1055)	1368 * (1023-1989)	942 (571.6-1343)	813 (782.1-887.3)	1060 (883.3-1867)	938 (648-1097)
PC III RBM units	2051 (1374-2657)	2483 (1550-2965)	2465 * (1845-3570)	2810 (2233-2129)	3221 (2129-4300)	2048 (1301-3543)
PC I + all cells/mm^2	88.0 (13.3-124.4)	125.7 (57.2-266.5)	232 * (64-332)	78.4 (43.5-115.5)	138.5 (62.95-184)	296.8 (199.2-368.8)
HSP-47+ all cells/mm^2	48 (33-118)	121.6 (79.43-173.8)	202.7* (123.6-520)	62.67 (28-112.7)	41.6 (10.10-213)	110.8 (54.67-168.7)
HSP-47+ fibroblasts/mm^2	2.67 (1-12)	16* (4.4-34.7)	83.56* (36-452)	0 (30.55-49.10)	5.33 (0-61.34)	16 (4.7-40.55)
α-SMA+ fibroblasts/mm^2	13.3 (4-49.15)	37.7 (28.70-59.20)	47.95 (13.5-97.35)	20.55 (16-36.55)	62 (41-115.4)	56.85 (32.85-181.4)

Table 4.2: Summary of changes in cellular expression of markers related to airway remodelling markers expressed as dual (n=9) and single early responders (n=4).

Positive cells were determined by counting the whole section and expressed as cells per square millimetre of biopsy. Cell counts are expressed as the median (inter-quartile range). Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as $p<0.05$.

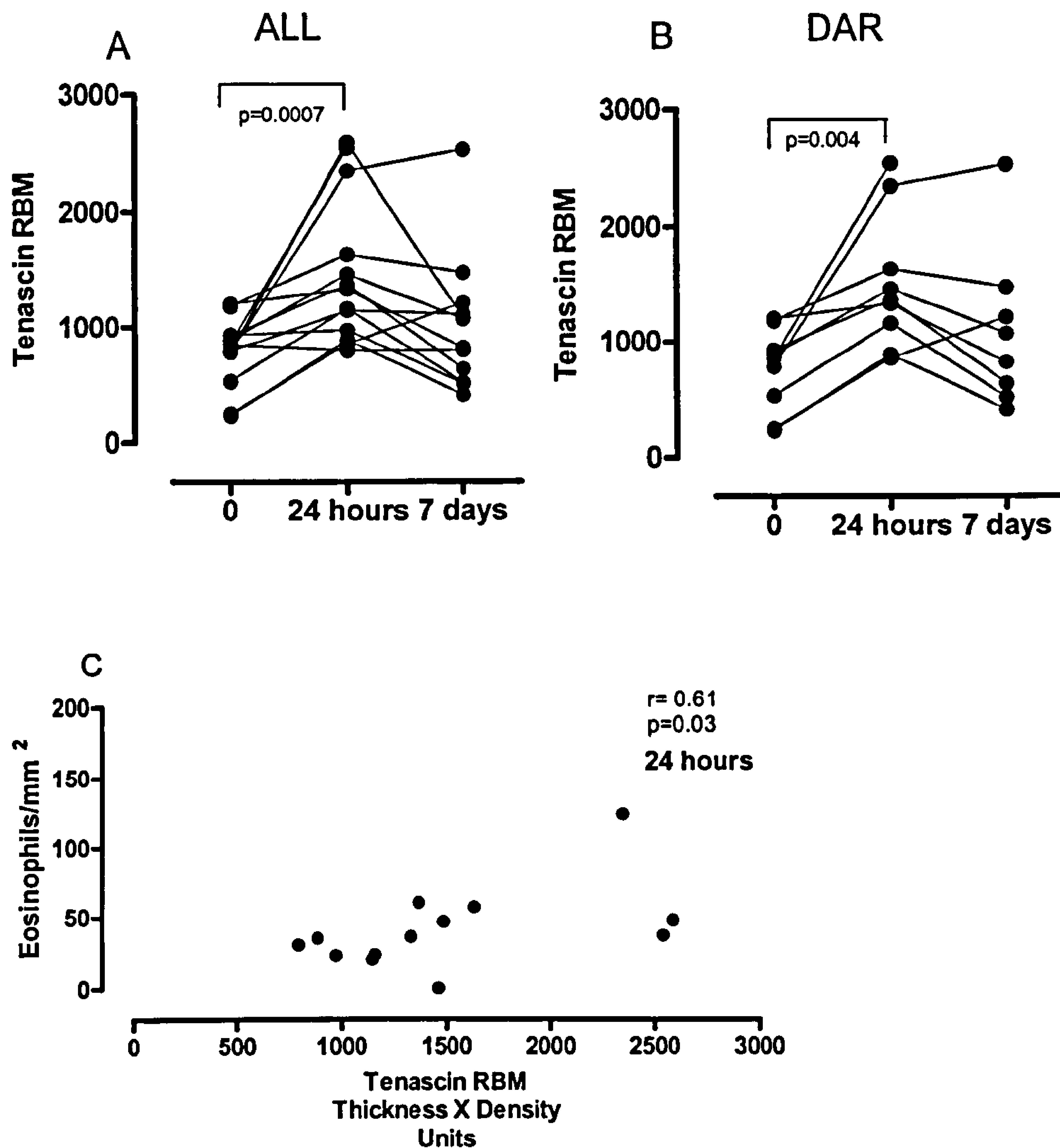


Figure 4.1: Time course of expression of tenascin at the reticular basement membrane (RBM)

The immunoreactivity of tenascin at the RBM in asthmatic volunteer group at baseline (Time 0 hours) and in response to allergen challenge (Time 24 hours and 7 days) is presented as paired data. Using Scion image software, the immunoreactivity of RBM is expressed as the product of thickness of the RBM (measured as the distance of a line drawn perpendicular to the band of immunoreactivity in the RBM) and average density of this line, averaged over the whole thickness of the RBM. The median increase was statistically significant at 24 hours (p=0.0007) (A). The dual asthmatic responder group (DAR) demonstrated significant increases (p=0.004) (B). Significant differences between time points were analysed using the Wilcoxon signed-rank test. The increased expression of tenascin following allergen challenge at 24 hours only correlated to the increase in eosinophils (Spearman r=0.61, p=0.03) (C).

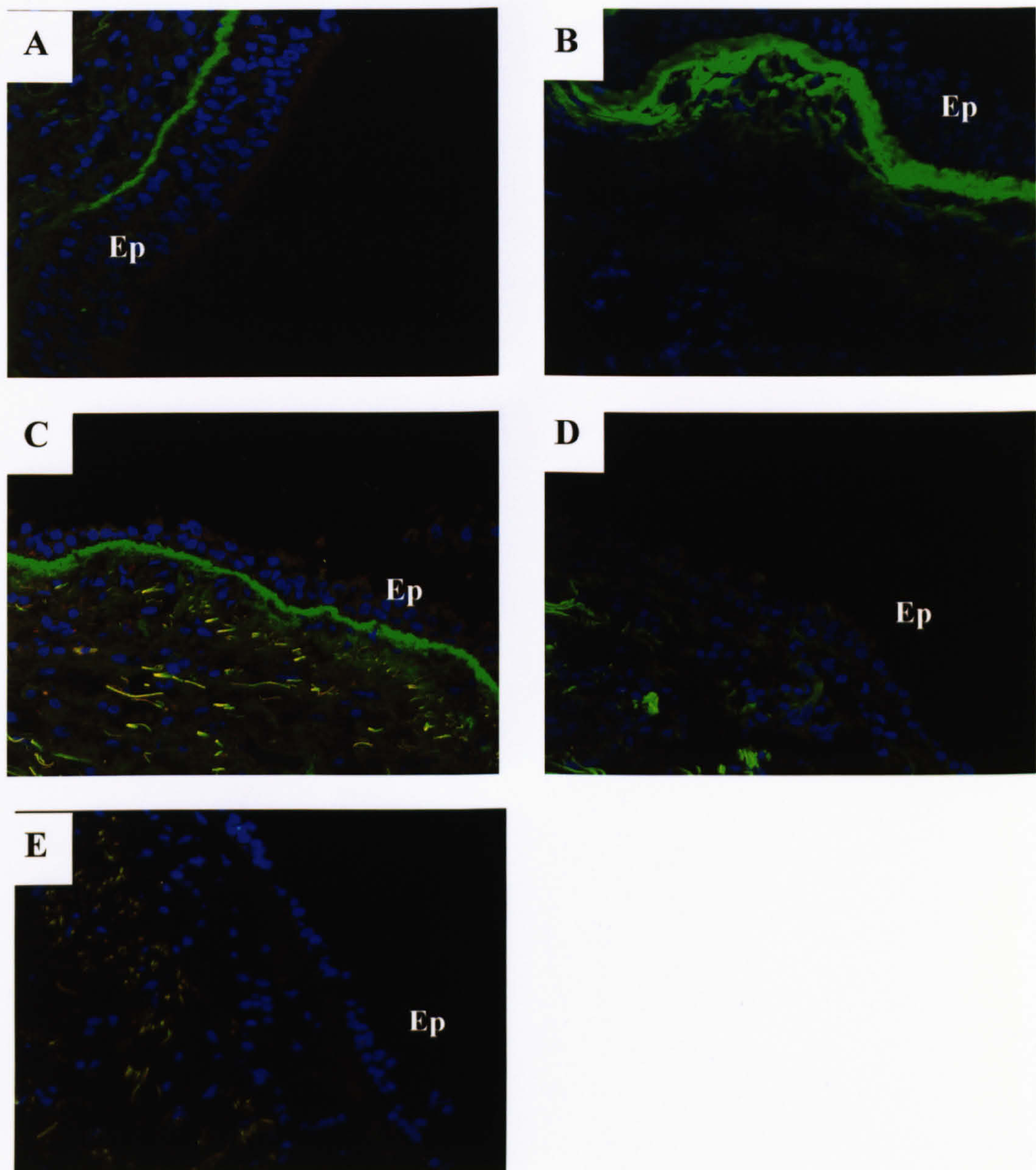


Figure 4.2: Tenascin immunoreactivity of the reticular basement membrane (RBM)

The expression of tenascin increased from baseline (A) at 24 hours (B) following allergen challenge, returning to baseline levels at 7 days (C). Tenascin expression in a normal volunteer (D) and the negative control (E) where the primary antibody was omitted is also shown. Sections were counterstained with DAPI (blue nuclei). The epithelium is labelled Ep in order to provide orientation for the section. For measurement of immunofluorescent tenascin staining, the confocal microscope settings were standardized to allow comparison of immunoreactivity intensity between different sections (density). Measurements were analysed using a Scion Image Analysis software package. The thickness of immunoreactivity in the RBM area was calculated by taking multiple measurements over the length of the biopsy (more than 100 measurements) at 10 μm intervals. Briefly, at each measurement, a line was drawn perpendicular to, and across, the band of immunoreactivity in the RBM and image analysis software used to quantitate the length of the line (thickness) and its intensity (density). The values were averaged over the whole length of the RBM to give the mean product of thickness and density of immunoreactivity.

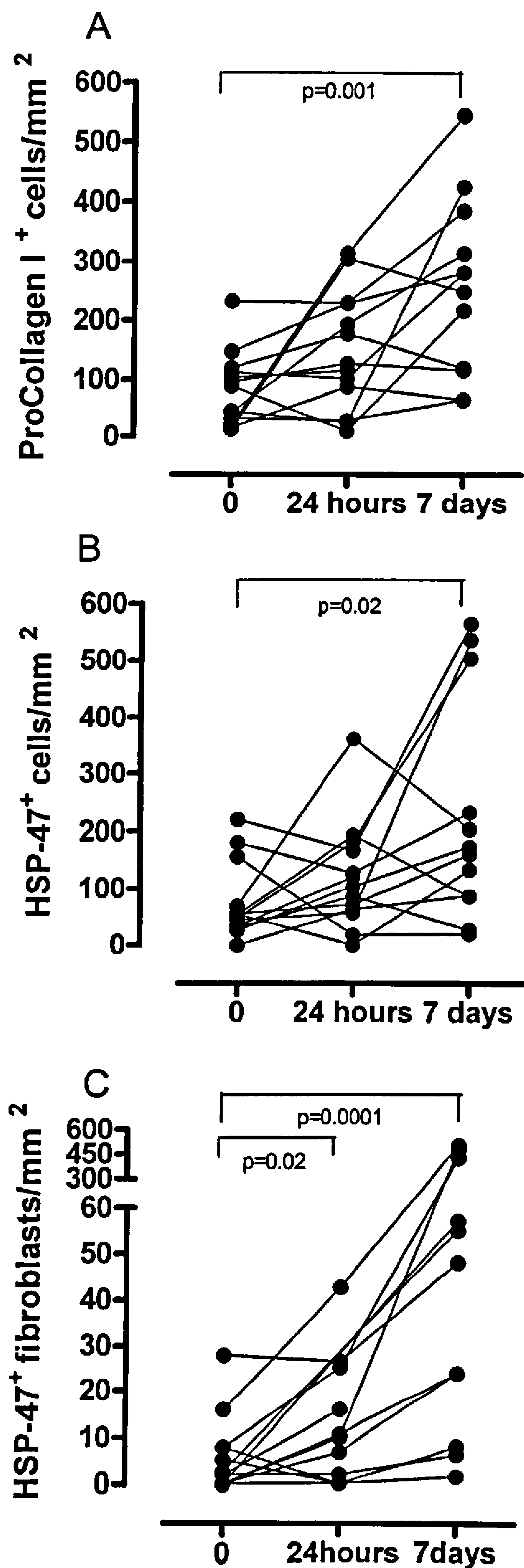


Figure 4.3: Time-course of collagen expression in all asthmatic subjects

The results are expressed as the number of cells expressing procollagen I (A) and HSP-47 (B) as cells per mm^2. The marked expression of HSP-47 in fibroblasts (C) was significant as early as 24 hours post allergen.

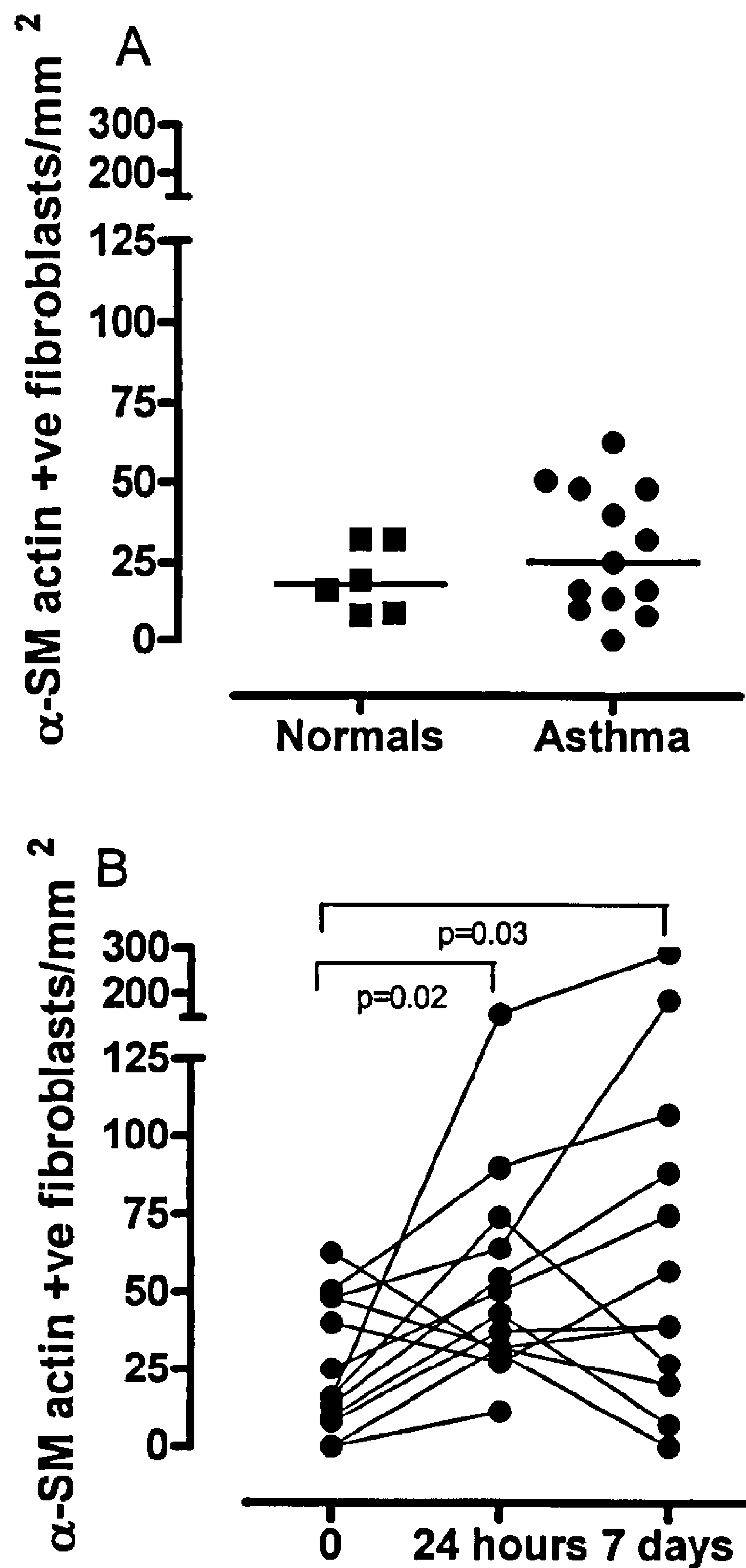


Figure 4.4: Analysis of expression of α-smooth muscle actin in fibroblast-like cells

No difference in the expression number of fibroblast-like cells expressing α-smooth muscle actin (α –SMA) was observed between normal and asthmatic volunteers (A). Significant increases in α-SMA positive fibroblast–like cells were observed 24 hours and 7 days after allergen (B). The Mann-Whitney test was used to compare non-paired data. All paired within-subject data was analysed using the Wilcoxon signed rank test. The results are expressed as the number of positive cells per mm^2. Fibroblasts were identified morphologically as being fusiformic in shape with elongated nuclei. Immunoreactive positive cells identified as leukocytes within this zone were not counted

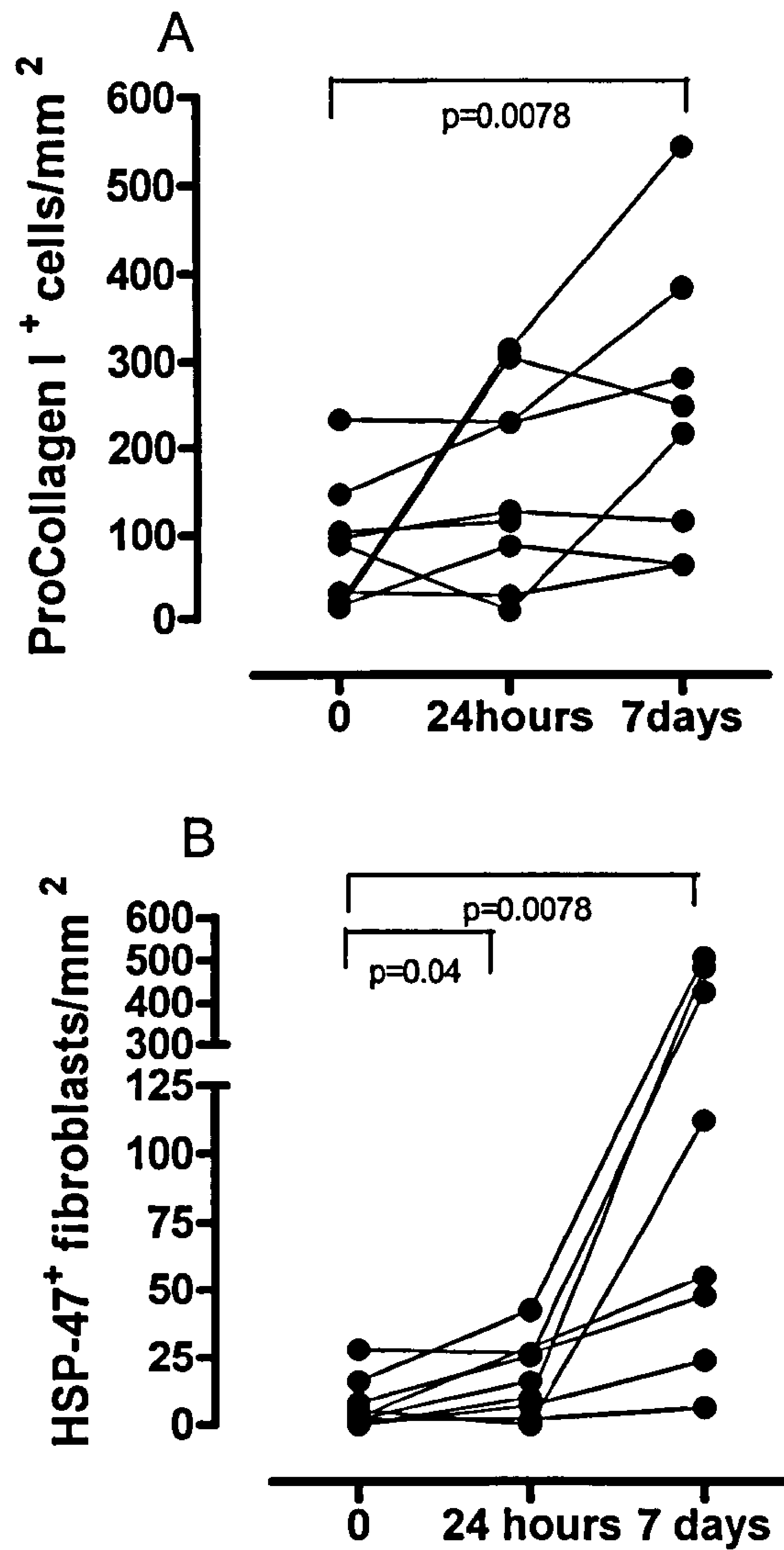


Figure 4.5: Analysis of procollagen I and HSP-47 expression according to dual asthmatic response (DAR) status

The results are expressed as the number of positive cells per mm^2. Fibroblasts were identified morphologically as being fusiformic in shape with elongated nuclei below the basement membrane. Positive cells were determined by counting the whole section and expressed as cells per square millimetre of biopsy. Immunoreactive positive cells identified as leukocytes within this zone were not counted.

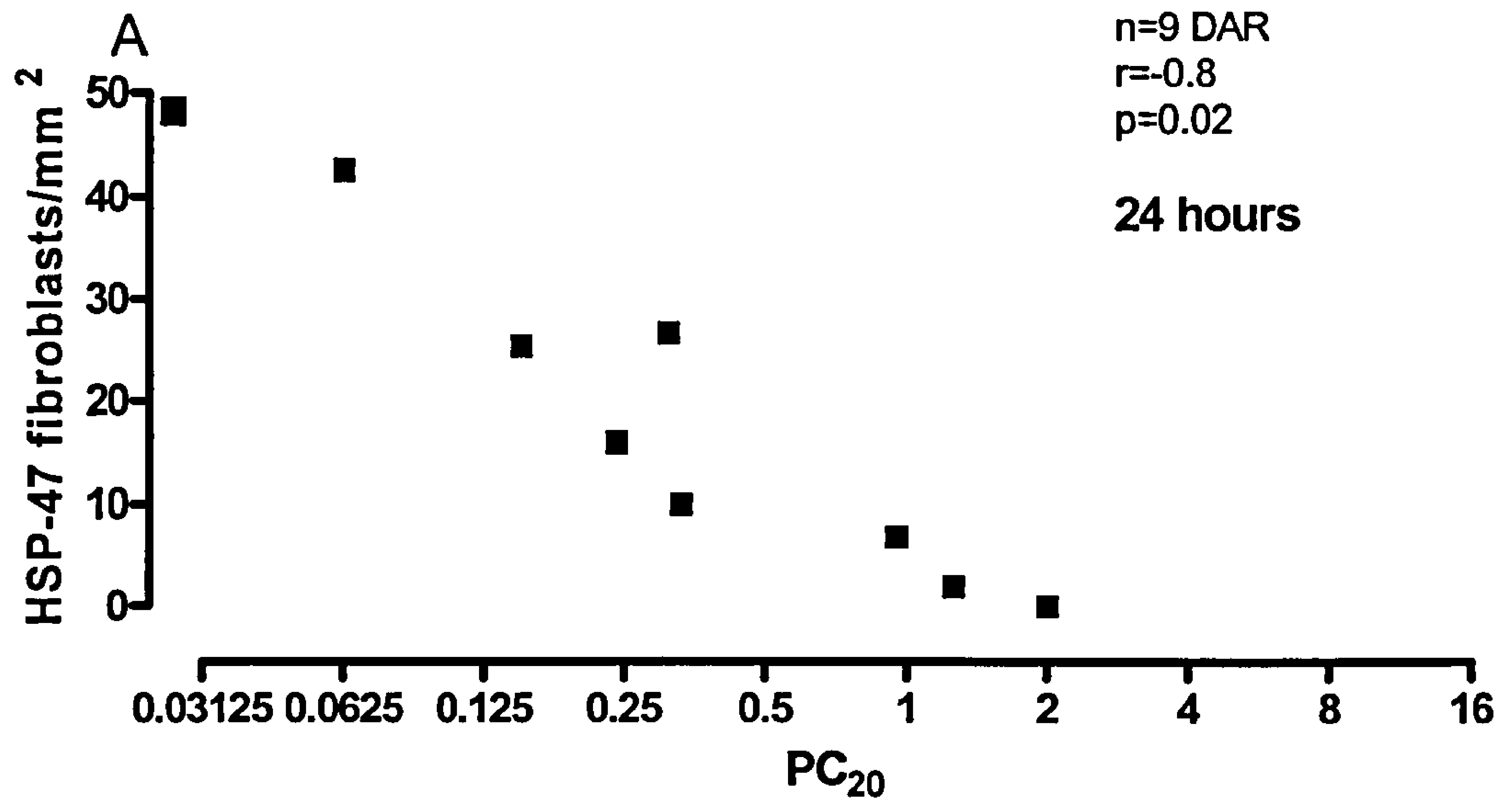


Figure 4.6: HSP-47 fibroblasts vs PC_{20} (AHR)

Correlations were performed between PC_{20} (AHR) against cellular counts and remodelling markers on specific time point measures, using Spearman's correlation. 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. AHR did not correlate with either cellular inflammation or RBM tenascin and procollagen III expression.

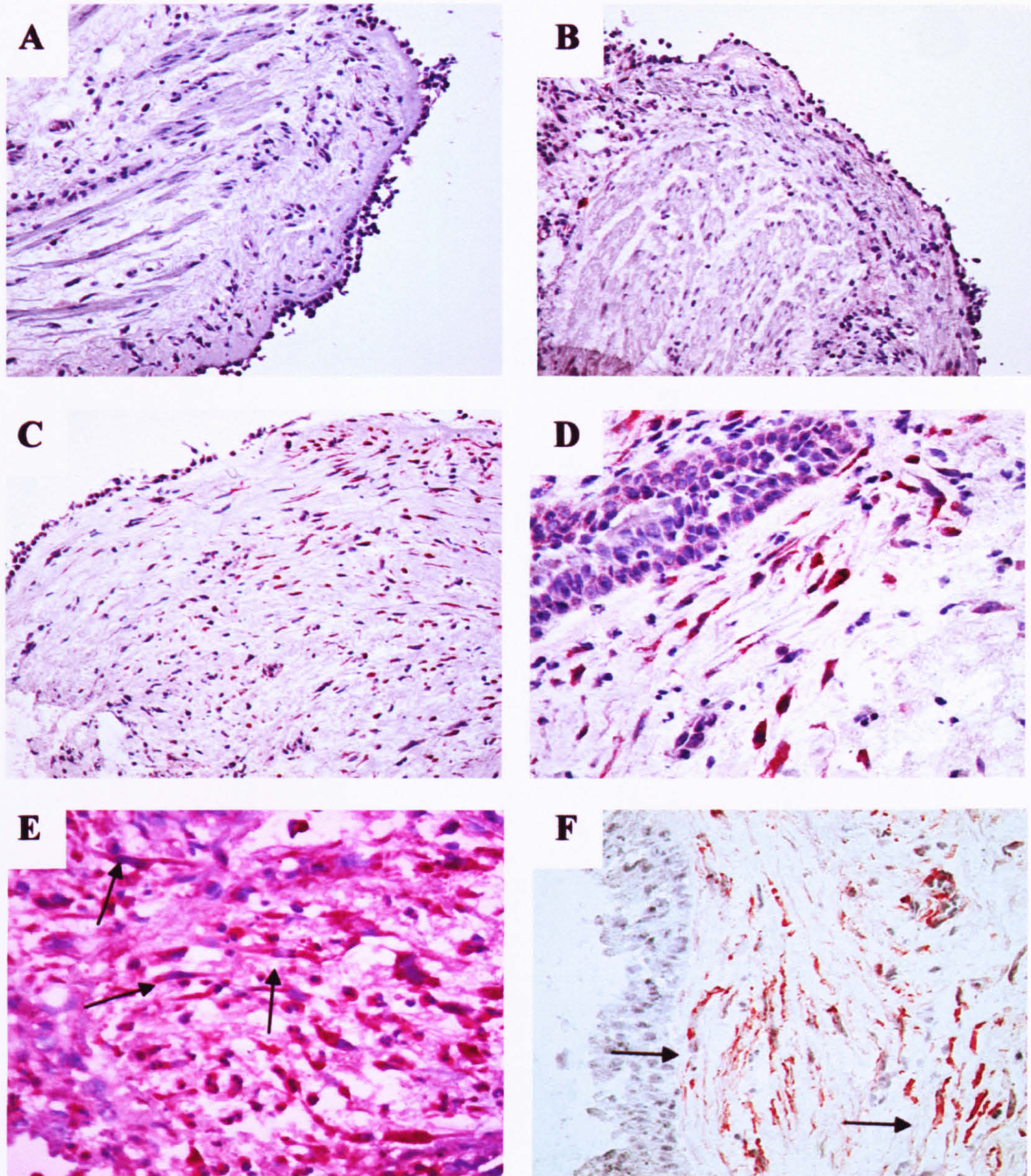


Figure 4.7: HSP-47, procollagen I and α-SMA immunoreactivity in fibroblast-like cells

HSP-47 immunostaining of paired bronchial biopsies obtained at baseline (A), 24 hours (B) and 7 days (C) after allergen challenge in a representative volunteer is shown (x 40 magnification). Fibroblasts were identified morphologically as being fusiformic in shape with elongated nuclei as shown by the cells staining for HSP-47 (x100) (D). Cellular procollagen I and α-SMA immunoreactivity in submucosal fibroblast-like cells (arrowed) (E and F respectively) is also shown (x100 and x40 respectively). Immmunoreactivity was detected by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method. The phosphatase substrate fast red was used to develop the reaction enabling signal visualisation. Immunoreactive positive cells identified as leukocytes within this zone were not counted.

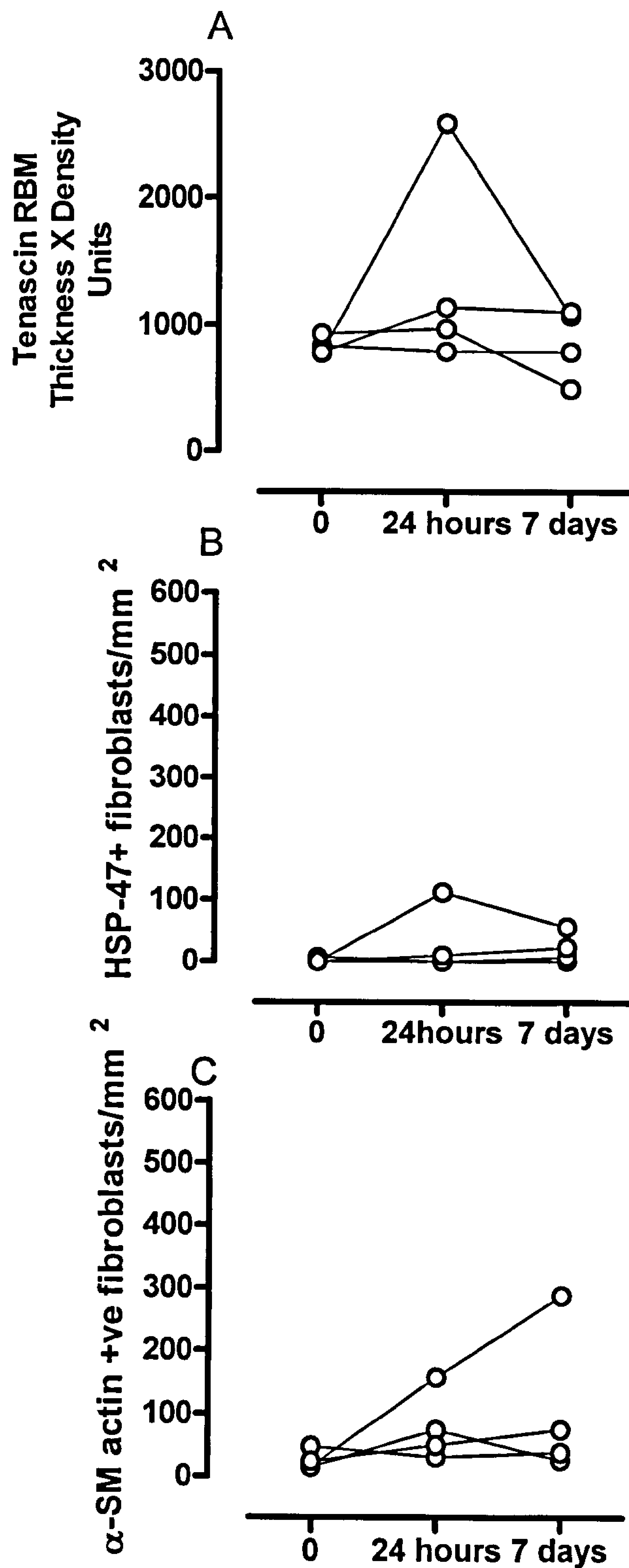


Figure 4.8: Illustration of the expression trend of remodelling markers in SER volunteers

Tenascin (A), HSP-47 (B) and α-SMA expression is shown. Other than one volunteer the general trend is that there is very little up-regulation of remodelling markers in the SER group.

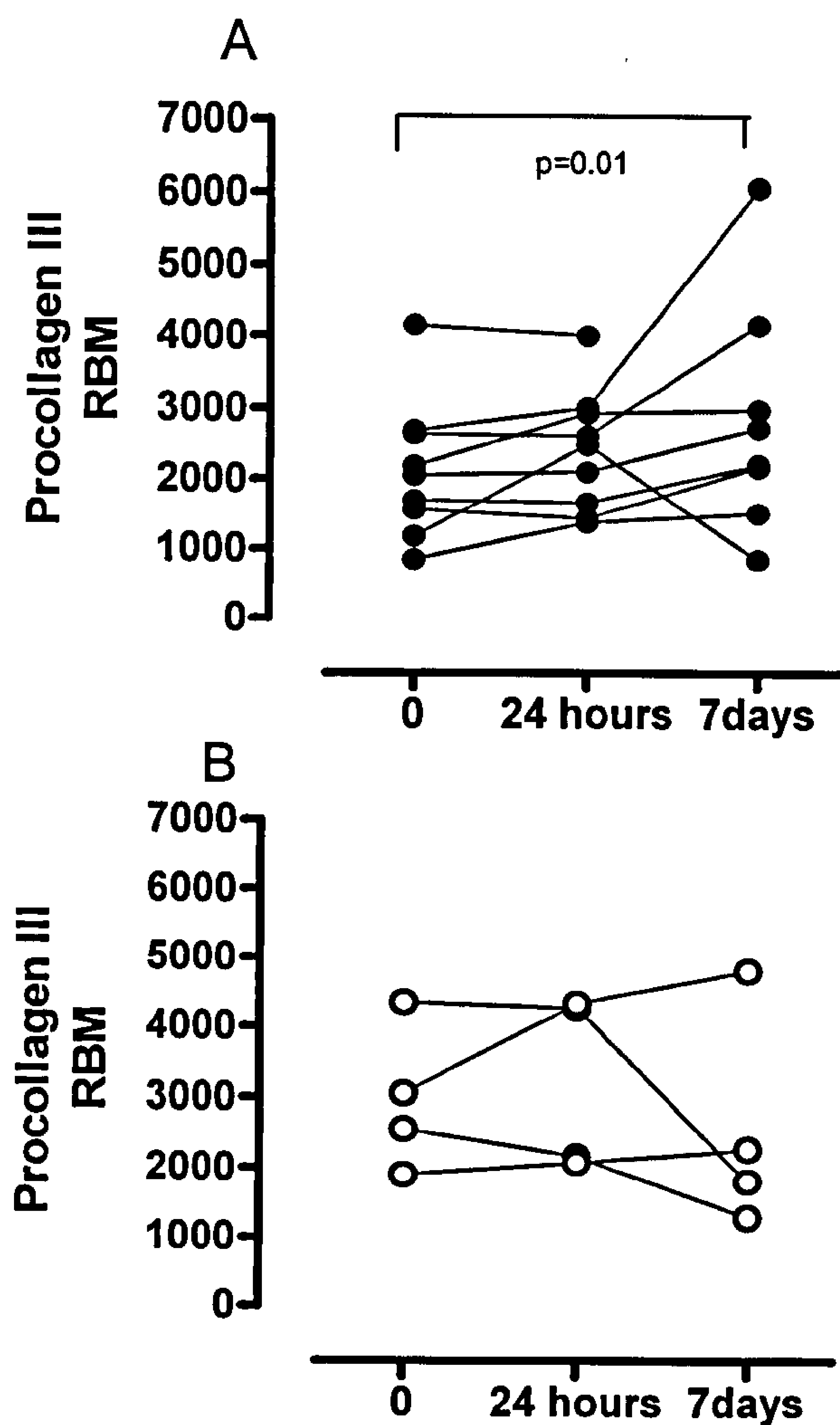


Figure 4.9: Procollagen III immunoreactivity at the RBM in the DAR group (A) and the SER group (B)

Using Scion image software, the immunoreactivity of RBM is expressed as the product of thickness of the RBM (measured as the distance of a line drawn perpendicular to the band of immunoreactivity in the RBM) and average density of this line, averaged over the whole thickness of the RBM. There was an significantly increased RBM procollagen III expression.

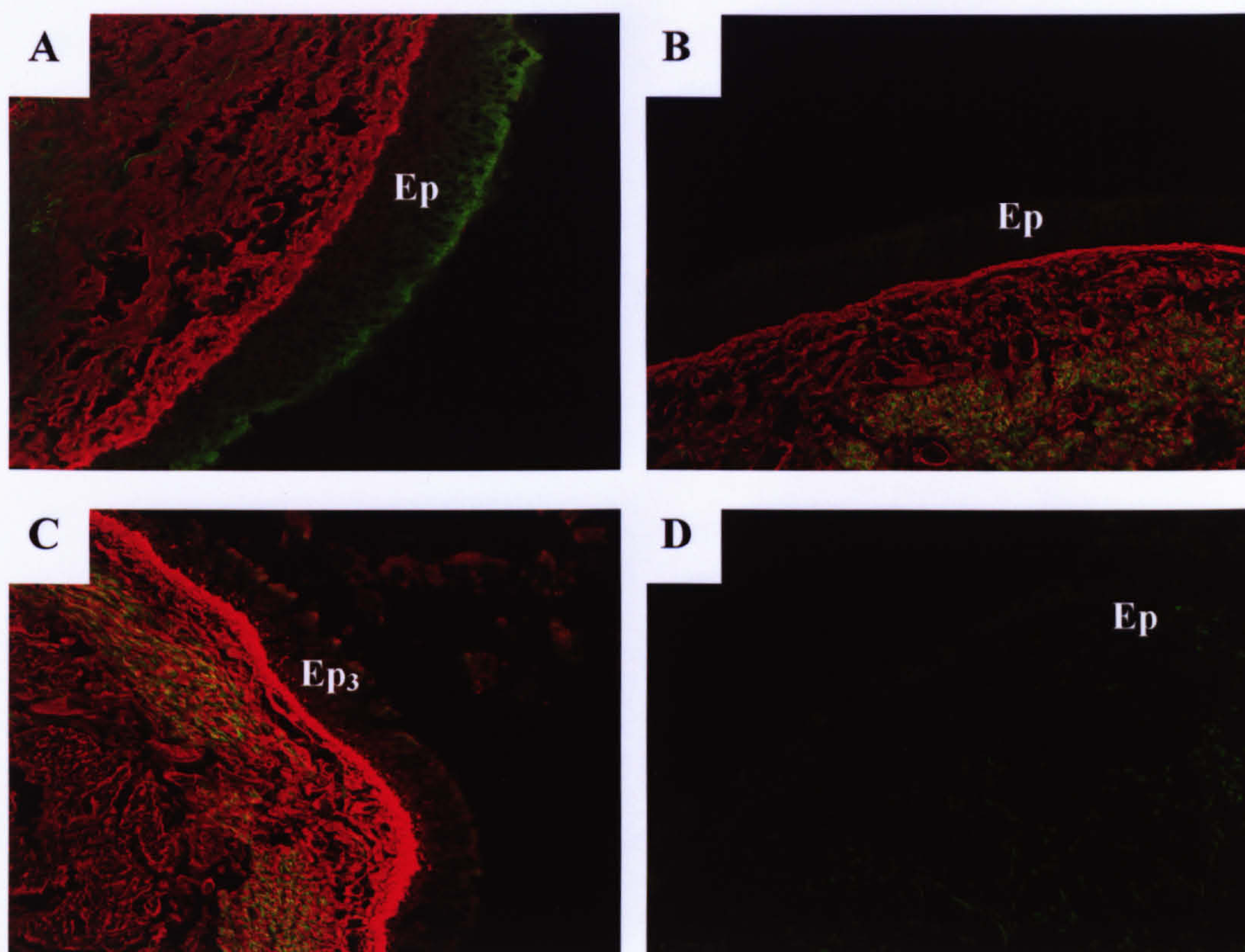


Figure 4.10: Procollagen III immunoreactivity in the reticular basement membrane (RBM)

The expression of Procollagen III in the RBM was not significantly altered from baseline (A) at 24 hours (B). There was a significant increase at 7 days (C) following allergen challenge as shown in a selected dual asthmatic volunteer. The confocal microscope settings were standardized to allow comparison of immunoreactivity intensity between different sections (density). Measurements were analysed using Scion Image Analysis software package. The thickness of immunoreactivity in the RBM area was calculated by taking multiple measurements over the length of the biopsy (more than 100 measurements) at 10 μm intervals. Briefly, at each measurement, a line was drawn perpendicular to, and across, the band of immunoreactivity in the RBM and image analysis software used to quantitate the length of the line (thickness) and its intensity (density). The values were averaged over the whole length of the RBM to give the mean product of thickness and density of immunoreactivity.

Procollagen III staining is less intense at baseline (A) as evidenced by the less distinct RBM borders. In contrast to the RBM at the day 7 time point is distinct in outline and the staining intensity for procollagen III is marked. Up-regulation of procollagen is also present in the epithelial cells as evidenced by the loss of green fluorescence and increase in redness leading to a mixed yellow-orange colour (labelled Ep_3). A negative control where the primary antibody was omitted is also presented (D).

Measure	Change per day	95% CI	p value
PC_{20}	-0.15	-0.26,-0.03	0.011
FEV_1	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

Table 4.3: Summary of data analysis using a mixed modelling approach

The data was also analysed 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. Analysis of data using a mixed modelling 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 together with the 95% confidence intervals (CI), based on the measurements at baseline, 24 hours and 7 days are summarised in Table 4.3 The change per day of only PC_{20} AHR, procollagen I, procollagen III and HSP-47 were significant. $p<0.05$ was taken as significant.

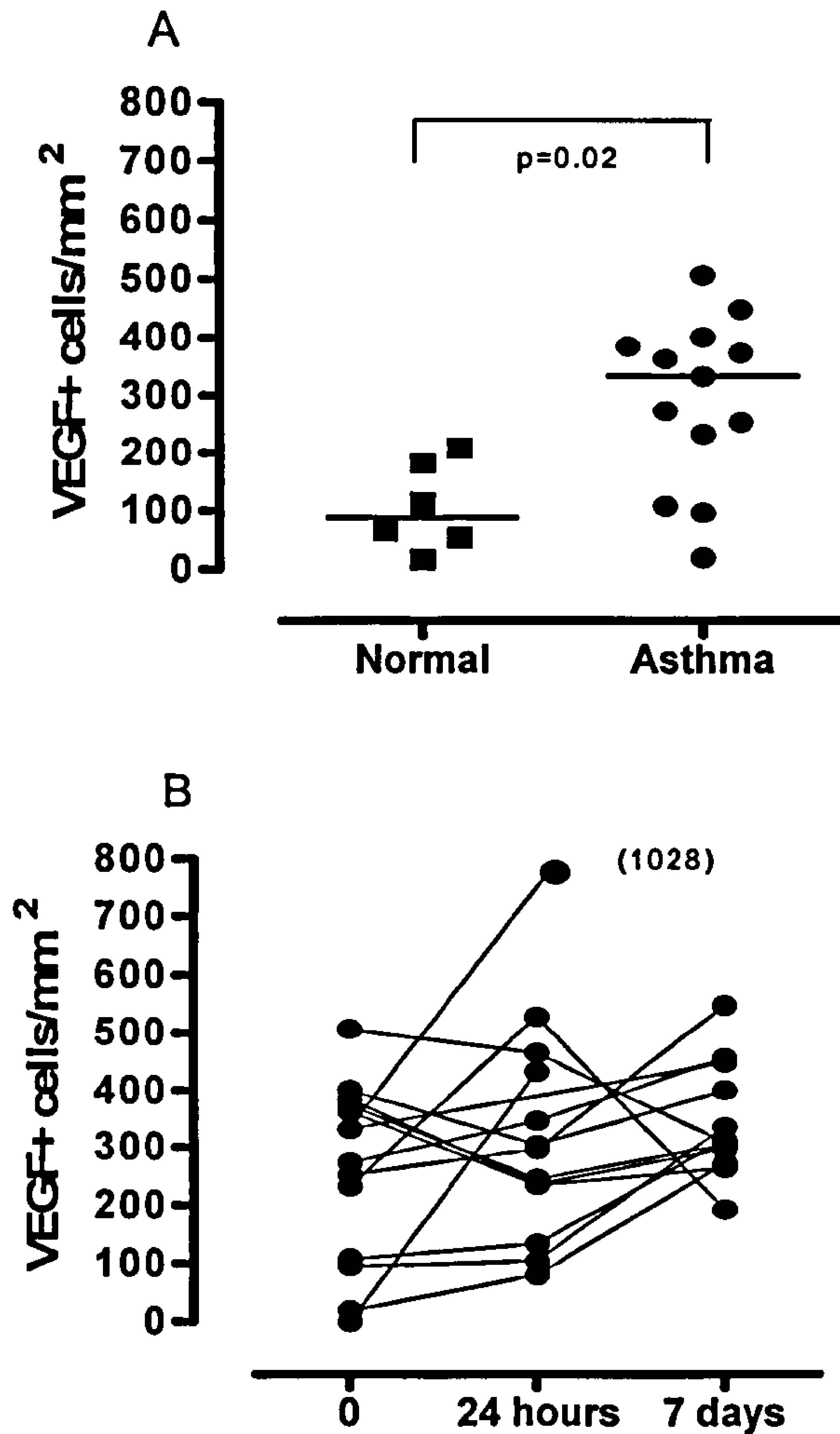


Figure 4.11: Summary of airway vascular endothelial growth factor (VEGF) expression

The expression of VEGF is increased in asthmatics at baseline compared to normal airways (p=0.02) (A). Following allergen challenge there was no significant increases in VEGF expression in the asthmatics (B).The Mann-Whitney test was used to compare non-paired data. All paired within-subject data was analysed using the Wilcoxon test. The results are expressed as the number of positive cells per mm^2.

4.3 Discussion

In this chapter it is shown that airway remodelling is an acute event in response to allergen induced airway injury and is up-regulated alongside airway inflammatory changes. Whereas the allergen-induced increases in airway inflammation in dual asthmatic responders was virtually resolved by seven days, increases in AHR and the expression of collagen markers of airway remodelling persisted. It is therefore demonstrated that AHR can be dissociated from cellular inflammation whilst remaining associated with sustained airway remodelling.

Inflammation can be considered as a response to tissue injury. With acute injury, inflammation occurs aiming to carry out restoration of the tissue to its normal state. In tissue that is in a state of chronic injury, inflammation must adapt to repair the tissue still with the aim of restoring tissue architecture, but in the context of ongoing tissue injury and activation that may lead to exaggerated and abnormal tissue repair. It therefore makes biological sense that induction of airway remodelling is an acute event and is in association with inflammatory cell recruitment, particularly eosinophils and macrophages which are both cells implicated in tissue repair. Interestingly, tenascin expression returned to baseline levels in parallel to inflammatory resolution. Some aspects of airway remodelling are therefore related to cellular inflammation. Several studies, including this one, have correlated the production of tenascin with eosinophil infiltration (Karjalainen *et al.* 2003). As eosinophilic inflammation resolves at 7 days, similarly RBM tenascin levels return to baseline values. This is in contrast to collagen expression.

The significant correlation of HSP-47 with AHR at the 24 hour time point and the association of procollagen I expression at day 7 is important as it suggests that individuals with increased AHR early on 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 such as the IgE response and degree of sensitisation being operative. Nevertheless the presence of sustained AHR at a time when remodelling is increased allows further hypotheses to be constructed and identifies remodelling markers that need further focus. The overall suggestion is that that selected features of airway remodelling might be related to inflammatory cell sources of cytokine and growth factors whilst other aspects maybe initiated by inflammation but can later proceed independently of inflammation, possibly through

structural cell activation. For example IL-13 and IL-4 can lead to epithelial release of TGF-β_2 that will subsequently activate down-stream mesenchymal cells (Richter *et al.* 2001). This suggests that some aspects of airway remodelling will be related to inflammatory cell sources of cytokine and growth factors whilst other aspects can proceed independently of inflammation, possibly through structural cell activation.

The mechanisms by which remodelling may contribute to AHR remains an area of important discussion. Mathematical modelling predicts that any increase in airway wall thickness internal to the airway smooth muscle (ASM) layer will amplify the airway narrowing at the time of ASM contraction and that 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 (Moreno *et al.* 1986). Such predictions are important given that increased ASM mass is the only structural feature that distinguishes severe asthma from moderate disease (Benayoun *et al.* 2003). Greater ASM mass will not only lead to an excessive degree of muscle shortening (Wiggs *et al.* 1990) but also greater force generation leading to a disproportionate reduction in airway patency for a given degree of ASM contraction (Lambert *et al.* 1993). ASM has been found to encroach onto the RBM and epithelium in severe asthma (Madison 2003), so that even minor contraction will affect airway narrowing. Such findings may explain the persistent AHR seen in asthma under basal conditions.

Several studies have documented that increases in AHR after allergen are associated with inflammatory cell infiltration in DARs (Wardlaw *et al.* 1988). The observation here that the DAR group also demonstrate both acute and sustained increases in airway remodelling is relevant. An obvious and important question is what component of the increased AHR is a result of cellular inflammation and what aspect is the consequence of any early remodelling process. It is possible that inflammation may lead to priming of neurogenic mechanisms with consequently enhanced neural reflexes on ASM which must have a contributory factor. Whilst such 'neurogenic' inflammatory mechanisms are an area of research focus at present there is little or no mechanistic understanding of such processes.

Airway obstruction defines the LAR response in the DAR group as stated earlier. Airway obstruction can be explained to some extent as a consequence of

inflammatory cell infiltration with subsequent mediator and cytokine release. The late phase response, in response to specific allergen injection into the dermis of atopic subjects, is seen as an oedematous, red and indurated area which peaks at 6-9 hours and resolves within 24-48 hours. In the airway, similar oedema and increased vascularity may occur during the LAR and this may be manifested as increasing airway obstruction as shown by the fall in FEV_1. An early study (with only n=5 asthmatics) could not demonstrate any relationship between increases in epithelial permeability and increases in AHR (O'Byrne *et al.* 1984), suggesting that increased permeability may not be a dominant pathway that leads to transient increases in AHR. Excessive mucus production will also contribute to airway obstruction and may be one mechanism by which IL-4 and IL-13 can contribute to acute increases in allergen-induced AHR (Perkins *et al.* 2006). Any airway narrowing will at least in geometric terms contribute to AHR, in that a narrowed airway will tend to narrow further in response to a lower dose of stimulus (a leftward shift in the bronchoconstrictor dose-response curve). IL-13 may also directly impair ASM relaxation (Laporte *et al.* 2001).

We and others have previously shown that asthmatics at baseline express significantly more tenascin in the RBM of the airway which is correlated to the degree of tissue eosinophilia at the time (Flood-Page *et al.* 2003a; Laitinen *et al.* 1997). This is in contrast to the RBM in normals where there is minimal or no expression of tenascin. The important observation is that there is further up-regulation in response to acute airway injury, again correlated to airway eosinophila. In this study we have confirmed these observations further to demonstrate that expression is significant in the DAR group with marked increases in AHR. There is a strong correlation between RBM tenascin expression and acute inflammation (Karjalainen *et al.* 2003). In asthma tenascin deposition in the airway correlates with both mast cell (Amin *et al.* 2000) and eosinophil, T cell and macrophage (Laitinen *et al.* 1997) numbers respectively. In both skin and asthma this deposition is rapid (Phipps *et al.* 2002; Phipps *et al.* 2004a).

Tenascin has been demonstrated to be a permissive substrate to regulate entry or exit of cells (Treasurywala & Berens 1998). The tenascin KO mouse exhibits prolonged influx and retention of leukocytes (Koyama *et al.* 1998) and tenascin can suppress $CD3^+$ T cell proliferation and cytokine production (Hibino *et al.* 1998). It is therefore probable that the up-regulation of tenascin alongside cellular inflammation is a functional response to co-ordinate inflammatory cell migration (via cell-matrix

interactions) and inflammatory responses. The first growth factor that was found to up-regulate tenascin expression was TGF-β_1 in chick embryo fibroblasts (Pearson *et al.* 1988) .The correlation of tenascin to eosinopils is probably related to eosinophils being the most significant cellular source of TGF-β_1 in asthma (Minshall *et al.* 1997).

It is also possible that tenascin may contribute to the acute increases in AHR at the 24 hour post-allergen time point. Even at this stage the increased deposition of ECM components may contribute to increased airway narrowing and effect airway wall compliance, both of which may contribute 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 (Kasahara *et al.* 2002). This is an important concept to consider. Despite the association of RBM thickening with asthma severity the presence of RBM thickening in itself does not lead to AHR as evidenced by studies in eosinophilic bronchitis (Brightling *et al.* 2003a). One can speculate that the exact composition of ECM components in the RBM differs between asthma and eosinophilic bronchitis or alternatively that RBM thickness must be in association with remodelling changes in the submucosa, particularly ASM changes for AHR to manifest.

The expression of collagen was evaluated in this study. Procollagen I carboxyterminal propeptide reflects the synthesis of Type I collagen rather than the degradation (Risteli & Risteli 1995) and is therefore a marker of intracellular Type I collagen synthesis. The RBM stains strongly for Type III collagen but only weakly for Type I collagen (Roche *et al.* 1989). If increased RBM collagen deposition is present as a result of activation of the EMTU, then it can be hypothesised that RBM Type III procollagen deposition will be increased in response to allergen challenge. Collagen III is the predominant collagen of the RBM (Wilson & Li 1997) and more recently it has been demonstrated that Type III collagen is increased in the RBM of children with asthma (Fedorov *et al.* 2005). It has previously been demonstrated that there is no significant increase in RBM expression of procollagen III at the 24 hour time point (Phipps *et al.* 2004a) and this again was the finding in this 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 submusoca. Again it is possible to hypothesise that such RBM increases may

contribute to AHR via enhanced luminal narrowing and compliance-dependent effects.

Whilst procollaen III expression was statistically significant the increases were not as marked as for tenascin deposition at 24 hours. It maybe that the increased thickening of the RBM observed in asthma is not predominantly dependent on excess collagen deposition. A recent study (Saglani *et al.* 2006) suggests that this may in fact be the case. Using electron microscopy (EM) the authors show that RBM structure does not resemble the excess fibrils that make up the interstitial submucosal collagen in the asthmatic airway and argue that interstitial collagens do not make any significant contribution to the excessive RBM thickening observed in asthma. Excessive RBM thickening may rather be a result of excessive other ECM component deposition.

Increased collagen synthesis is also probably initiated in response to inflammatory cell release of mediators, particularly TGF-β_1. The synthesis is in fact predominantly dependent on TGF-β_1 (King *et al.* 1994). However the binding of several other transcription factors such as SP-1, AP-1 and Ets-1 influence pro-peptide synthesis and may account for cell-lineage specific expression of collagens (Cutroneo 2000; Cutroneo 2003). Airway oedema could also activate fibroblasts as shown with *in-vitro* observations of induction of ECM production in fibroblasts by mechanical stretch (Chiquet *et al.* 2003; Ludwig *et al.* 2004). Such induction may then be further amplified and sustained in structural cells as evidenced by the predominant immunostaining of HSP-47 in fibroblasts at the 7 day time point when inflammation and airway obstruction (FEV_1) have returned to baseline levels. Structural cell activation will lead to the release of further TGF-β_1 and other growth factors which will have both autocrine and paracrine effects. Such a mechanism may explain the sustained expression of collagen at a time point when inflammation has returned to baseline values in this model.

Structural cell activation may also contribute to the sustenance of chronic airway inflammation. Until recently the role of fibroblast activation in immune regulation has not been considered in detail with the predominant focus being on the role of Th2 induced inflammation in asthma. The importance of the extended immune role of activated stromal cells is only now gaining relevance. Activated fibroblasts are a rapid and important source of cytokines, chemokines and growth factors (Jordana *et al.*

1994). Not only can fibroblasts contribute to the recruitment and activation of inflammatory cells and structural cells but also have autocrine feedback effect on the fibroblast itself. Moreover there is important cross talk between inflammatory cells and fibroblasts, with Th2 derived mediators influencing fibroblast proliferation as well as the secretory repertoire of the cell (Ingram *et al.* 2003). In fact there is growing evidence that activated fibroblast populations may sustain chronic inflammation as a result of mediator production and ECM deposition. This may lead not only to further inflammatory cell recruitment and activation but also to their inappropriate retention and survival.

Whilst this study has quantitated the number of inflammatory cells in airway biopsies at specific time points following allergen challenge, the state of activation or release of cytokines or mediators was not measured. This would be an important area to address. It is possible that persistent activation of recruited inflammatory cells or a change in the number of allergen responsive cells such as allergen specific T-cells that contribute to persistent changes in airway remodelling contribute to remodelling and AHR at the 7 day post-allergen time point and should be addressed in any future study. Whilst the presence of a particular cell type or increased numbers is an important measure of airway inflammation, cytokine production and altered cellular function are important inflammatory responses. Further directed focus is now required to help define to what extent such mechanisms contribute to the parameters of airway remodelling or enhanced AHR described in this study.

The concept that airway physiological responses are a result of separate inflammatory cell dependent and structural cell dependent remodelling components interacting may explain several clinical observations in terms of therapeutic response. 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 anti-inflammatory inhaled steroid therapy have failed to abolish AHR despite improvements in cellular inflammation (Lundgren *et al.* 1988; Duddridge *et al.* 1993; Adelroth *et al.* 1990; Juniper *et al.* 1990). Our observations may also explain several clinical observations in terms of therapeutic response. For example in the Childhood Asthma Management Program (CAMP) research group study (2000) the benefits of inhaled corticosteroid therapy on post-bronchodilator improvements are seen in the first three years. Similar lack of effect of inhaled steroids on the natural

history of asthma measured in terms of post-bronchodilator FEV_1 despite initial improvements in asthma outcome was observed in the START (inhaled Steroid Treatment as Regular Therapy in Early Asthma Study) (Pauwels *et al.* 2003). These findings maybe explained by the efficacy of steroids in decreasing inflammation related remodelling that lead to initial improvement in FEV_1. The observation this improvement is lost in the next three years maybe explained by epithelial-mesenchymal driven remodelling events that are considered refractory to corticosteroids. The persistence of troublesome AHR despite high dose inhaled corticosteroid in moderate-severe asthmatics is an important clinical problem and is currently being addressed by the addition of long-acting β_2 agonists which effectively targets airway smooth muscle, a key aspect of airway remodelling.

Eosinophils and macrophages are important sources of TGF-β_1 (Aubert *et al.* 1994; Minshall *et al.* 1997; Vignola *et al.* 1997) in asthma and probably accounts for the increased amounts of BAL TGF-β_1 found in the asthmatic airway at baseline (Minshall *et al.* 1997; Redington *et al.* 1997; Vignola *et al.* 1997) with further increases in response to allergen challenge (Redington *et al.* 1997). These cells were of an activated phenotype (Minshall *et al.* 1997; Chanez *et al.* 1991) and will therefore more readily release their stores of TGF-β_1 on tissue recruitment. The early increases in collagen markers in the DAR group may be related to such inflammatory sources of TGF-β_1 and other growth factors. The evidence for eosinopils, in this regard, is particularly strong. In mouse models of disease, over-expression of IL-5 in the induced lung was associated with peribronchial collagen deposition (Lee *et al.* 1997). Chronic allergen challenge in IL-5 knock-out mice was associated with decreased total lung collagen, decreased peribronchial collagen III and V, decreased thickness of the peribronchial smooth muscle layer and α-SMA immunostaining. This was all in association with a reduction in MBP^+ cells with parallel reduction in cells staining for TGF-β_1 (Cho *et al.* 2004). The eosinophil depleted GATA-1 knock-out mouse demonstrates reduction in airway remodelling (Humbles *et al.* 2004), although no reduction in TGF-β_1 was demonstrated here. The authors themselves could not explain this observation but may be related to inherent differences in the murine strains used. Mild atopic asthmatics treated with anti-IL-5 demonstrate reduced expression of tenascin, lumican and procollagen III in the RBM associated with the concomitant reduction in $TGF\beta_1^+$ eosinophils (Flood-Page *et al.* 2003a). Disappointingly, no reduction in basal AHR that defines chronic asthma was found.

These observations suggest that eosinophils, mainly through TGF-β_1, can contribute to the acute induction of collagen production and that this induction is then sustained in structural cells in the absence of any further eosinophil recruitment.

The finding that AHR and remodelling markers remain associated at the 7 day time point when inflammation has resolved to baseline levels is important. It is the first time the persistence of allergen increased AHR and remodelling markers have been shown at a time point when inflammation has returned to baseline levels in human asthma. This finding is consistent with the findings from animal models. It has been shown (Leigh *et al.* 2002) in a chronic setting of allergen-induced airway murine model of remodelling, that despite the resolution of inflammation early on after the cessation of allergen exposure, changes in AHR and remodelling remain associated for at least eight weeks after the final allergen exposure. Interestingly, whilst acute allergen exposure induced increases in AHR were associated with increased IL-13 levels, AHR that persisted beyond the inflammatory stages was not associated with increased IL-13 (Leigh *et al.* 2004a). This led the authors to conclude that early cellular inflammatory events with associated Th2 cytokines contributed to the initiation of remodelling events, and that sustained AHR was a consequence of increased airway contractile tissue. Their model is therefore in keeping with the observations here. IL-13 induction of TGF-β_1 and other growth factors predominantly occur in the airway epithelium rather than mesenchymal cells (Wen *et al.* 2002). Whether structural cell activation can occur in the absence of inflammation remains unanswered and it will be important to pursue this question may by using over-expression and knock-out experiments of inflammatory cytokines in murine models.

The late asthmatic response (LAR) that defines the dual asthmatic response (DAR) group is manifested by increased airway obstruction. Such obstruction may be mediated to a significant extent by increased vascular flow and tissue oedema if allergen-studies of atopic skin are considered (Phipps *et al.* 2002). Vascular endothelial growth factor (VEGF) is considered the key mediator for the induction of airway oedema and angiogenesis but transgenic over-expression in a mouse model suggests that VEGF also contributes to enhanced Th2 responses and increased collagen production with RBM thickening and ASM hyperplasia associated with the production and activation of TGF-β_1 as well (Lee *et al.* 2004). These changes were associated with increased AHR. Inflammatory cells are a predominant source of

preformed-VEGF. However, VEGF (like other growth factors) is ECM bound and ECM is an important reservoir. If it is presumed that airway oedema and increased vascularity is associated with DAR status together with significant inflammatory cell infiltration, it is possible that increased VEGF in this group will also contribute to the sustained remodelling observed in this study. As expected, the overall expression of VEGF was significantly higher in asthmatics versus normal volunteers. Following allergen challenge, no obvious modulation of VEGF protein expression was seen, suggesting that it maybe local release and activation of pre-stored VEGF that mediates vascular events and contributes to inflammation and remodelling. The detection of RNA rather protein would be required to detect exact expression kinetics. For example in allergic rhinitis there is induction of VEGF mRNA in response to allergen challenge (Benson *et al.* 2002). An important observation from *in-vitro* work is that the serum from asthmatics induce greater synthesis and release of ECM components compared to normal serum when added to ASM cells (Johnson *et al.* 2000) and this not effected by corticosteroids. The exact serum factors that induce such synthesis remain undefined. Given that growth factors such as theTGF-β Superfamily including potent ASM stimulatory factor such as activin-A (Cho *et al.* 2003) are found in high amounts in asthmatic serum (Karagiannidis *et al.* 2006), it is possible that the serum factors penetrate into airway tissue during the increased vascular leakage that is attributed to occur during late phase response and will lead to induction of airway remodelling processes.

It was Brewster *et al* that first showed a significant association between RBM thickness and the number of elongated fibroblast-like cells staining positive for α-SMA using light microscopy. Electron microscopy was further used to confirm the myofibroblast morphological features of the cells. It was subsequently shown (Gizycki *et al.* 1997) that this population of cells increased in number as early as 24 hours after allergen challenge, identified on the basis of morphology as spindle-like cells expressing α-SMA using light microscopy with further confirmation of myofibroblast morphology with electron microscopy. Only DAR volunteers were recruited into this study. In this current study both the single early response (SER) and DAR group combined demonstrated increased numbers of cells expressing α-SMA in response to allergen provocation at 24 hours and this was sustained to a week later when collagen synthesis was maximal. In normal tissue, at resolution of wound repair, myofibroblasts either differentiate back into a quiescent form or disappear by

the process of apoptosis. In the asthmatic airway and other disease states associated with excessive tissue fibrosis such myofibroblast down regulation is absent (Mountz *et al.* 1983; Pablos *et al.* 2004; Scaffidi *et al.* 2001).

It is important to comment on the observations made in the SER group. In the four SER individuals there was no demonstration of increased AHR or airway obstruction at either 24 hours or 7 days after allergen challenge as has been previously published (Dorman *et al.* 2004a). The numbers of SER in this study are too small and therefore no firm assumptions can be made. However the SER individuals did demonstrate non-significant 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 (Wood *et al.* 1998; Gauvreau *et al.* 1999). Three of the four SER volunteers failed to demonstrate any increase in RBM tenascin and procollagen expression whilst all four volunteers failed to demonstrate any increased expression of HSP-47 or procollagen in submucosal fibroblast-like cells. Such an observation is important and may support the concept that inflammatory cell recruitment and increased airway vascularity leading to airway wall oedema and therefore cellular traction must occur to a greater level for marked induction of remodelling to occur.

Myofibroblasts will contribute to airway remodelling not only by the rapid synthesis and secretion of ECM components but also through the production of TIMPs alongside down-regulation of MMP (Sasaki *et al.* 2000). Excessive collagen accumulation in asthma may then be explained by persistence of myofibroblasts leading to excessive collagen production together with an imbalance of collagen degradation. Myofibroblasts will also contribute to the chronic inflammatory state by the secretion of inflammatory mediators and growth factors (Evans *et al.* 1999).

The skin model of allergen induced inflammation has provided some interesting insight into the time course of remodelling markers in another system (Phipps *et al.* 2002). In this model all markers of remodelling measured (myofibroblast numbers, procollagen^{+} cells and tenascin) returned to baseline levels within 72 hours of allergen injection. Thus at least in atopic skin, the control mechanisms that are in place to regulate the repair process are functional.

Given that the excessive accumulation of ASM cells and myofibroblasts in asthma is fundamental to the remodelling process and severity of disease (Benayoun *et al.* 2003), understanding the origins and mechanisms that lead to the excessive accumulation of such mesenchymal cells is important. The origins of myofibroblasts remains controversial but they are considered to derive either from resident ASM and fibroblasts or from haemopoietic progenitor airway recruitment. TGF-β_1 and activin-A, from both inflammatory and epithelial sources, will potently induce the fibroblasts to undergo myofibroblast activation (Karagiannidis *et al.* 2006; Matsuse *et al.* 1996). It is also possible that myofibroblasts may be recruited into the airway from the bloodstream. The identification of such progenitors termed fibrocytes (CD34$^+$ Collagen I $^+$ cells) in the circulation with subsequent recruitment into sites of airway injury is an exciting and important finding. These progenitors undergo subsequent differentiation into myofibroblasts in the mucosa as evidenced by down-regulation of CD34 and up-regulation of α-SMA. Allergen exposure is associated with accumulation of such fibrocytes into the airways of mild asthmatics (Schmidt *et al.* 2003). It is possible that fibrocyte recruitment into the airway contributes to the smooth muscle mass increases in asthma. One can speculate that at a time point when the need for rapid and excessive collagen production ceases these cells may revert back to a smooth muscle phenotype and thus contribute to the increased airway smooth muscle mass observed in asthma.

In this study there was evidence of epithelial cell expression of mesenchymal markers in both the normal and asthmatic airway at baseline. There was no significant difference between the normals and asthmatics at baseline in the frequency of cells expressing vimentin, HSP-47 and procollagen I. This must indicate that epithelial plasticity is of functional significance in normal airway homeostasis. This suggests that either the differentiated state of the airway epithelium is not fully protected and can change its phenotypic properties or that there are basal cells within the mature epithelium with progenitor-like properties. What is important is to understand whether the plasticity of these cells is utilised in the epithelial response to injury and repair and also whether these cells significantly contribute to accumulation of activated fibroblasts below the RBM seen in asthma that drive ECM production.

There was no increase in the number of epithelial cells that stained positive for either vimentin or α-SMA at either time point after allergen challenge. There was also no change in epithelial morphology or cytokeratin positivity (data not presented) which one might expect in any transitory epithelial cell. However there was an increase in epithelial cell collagen as evidenced by HSP-47 production, both 24 hours and 7 days after allergen challenge, although these increases were not statistically significant. This is consistent with the concept that local epithelial collagen production is not the dominant source of airway collagen. The supposition from this is that the capacity of the epithelium to generate collagen can be up-regulated without the requirement for full adaptation of the activated myofibroblast phenotype. It is also possible that the sampling time points are not at a time when epithelial mesenchymal transition (EMT) is maximal and the EMT cells migrate out earlier or later. Given that we and others have noted increased myofibroblast numbers at 24 hours post-allergen in the submucosa, it is plausible that earlier migration can occur.

Thus in summary, in allergen-induced asthma sustained increases in AHR remain associated with increases in airway remodelling at a time point where cellular inflammation has returned to baseline levels. Activation of airway remodelling is a rapid and sustained event in response to allergen. This response is associated with acute inflammatory cell recruitment. In response to allergen, inflammation and remodelling are initiated as concurrent events but eventually dissociate. It is probable that inflammatory cell release of factors such as TGF-β_1 and IL-13 will initiate structural cell activation leading to sustained remodelling even after resolution of inflammatory cell recruitment. Some aspects of airway remodelling may be a consequence of inflammatory cell activation whilst other aspects may be related structural cell activation. It is also possible that both inflammation and remodelling events observed contribute separately to the increases in AHR and remains to be investigated whether induction of remodelling can occur independent of inflammation by direct activation of structural cells.

4.4 Summary of chapter

Allergen inhalational challenge of asthmatics with a late phase asthmatic reaction (dual asthmatic responders) leads to sustained increases in AHR. Both cellular inflammation and markers of airway remodelling were increased 24 hours after allergen challenge. It was shown in the previous chapter that although there was a sustained increase in AHR at the 7 day post-allergen time point cellular inflammation at this time returned to baseline levels. The aim of this chapter was to evaluate whether persistence of increased AHR was associated with persistent activation of remodelling markers.

Using bronchial biopsies obtained at baseline, at 24 hours and 7 days following allergen inhalational challenge in dual asthmatic response (DAR) group, expression of RBM tenascin, RBM procollagen III, cellular procollagen I, HSP-47 collagen and α-smooth muscle actin (myofibroblasts) was evaluated as markers of activation of airway remodelling,

RBM tenascin expression was significantly elevated at 24 hours but returned to baseline levels at 7 days. RBM procollagen III, cells expressing procollagen I, HSP-47 and α-smooth muscle actin expression all increased at 7 days compared to 24 hours.

In DARs allergen inhalation lead to rapid induction of remodelling markers which was sustained at the 7 day post-allergen time point. Airway remodelling remained associated with AHR at a time point when when cellular inflammation had returned to baseline levels.

Chapter 5

Transforming Growth Factor (TGF)-β Superfamily

5.1 Introduction

This chapter examines the dynamic process of TGF-β Superfamily signalling using the samples from asthmatic airways obtained at baseline and 24 hours and 7 days after allergen challenge. The pattern of activation and signalling via the TGF-β Superfamily in the normal airway and that what is specific to asthma is defined. The relationship to the activation and resolution of inflammatory and remodelling events are evaluated. Whilst it is appreciated that balanced and co-ordinated signalling of TGF-β Superfamily members is essential to maintain cellular homeostasis in health and imbalance of such factors are implicated in disease states, the exact expression pattern in the human airway in health and asthma remain undefined. In addition, it is not known whether there is further modulation of expression in response to asthma activation.

It has recently been shown that TGF-β signalling and airway remodelling are acute events in the airway in asthma following inhalaed allergen challenge (Phipps *et al.* 2004). Bronchial biopsies obtained 24 hours after either allergen from mild atopic asthmatics showed significant increases in RBM tenascin deposition, HSP-47 expression (a marker of induction of collagen synthesis) and pSmad2 expression. These data support the hypothesis of allergen-induced remodelling and TGF-β Superfamily signalling and is consistent with the concept of allergen-induced activation of the epithelial-mesenchymal trophic unit (EMTU), the embryological unit driving airway development. The work presented in this chapter is an extension of the observation that there is rapid up-regulation of TGF-β ligand signalling in response to allergen-induced airway injury.

The principles of TGF-β Superfamily signalling is summarised in Figure 5.1 and briefly introduced again in order to orientate the reader to the contents of this chapter. The TGF-β Superfamily of ligands that includes TGF-β_{1-3}, activins and bone morphogenetic proteins (BMPs) are pluripotent cytokines with an array of biological effects on a variety of cell types. These ligands, upon activation, signal via a constitutively active serine-threonine kinase specific Type II receptor that complexes with a Type I receptor which subsequently propagates the signal downstream by phosphorylating receptor-regulated Smads that translocate to the nucleus to initiate gene transcription. TGF-β_1 signals exclusively through the Type II receptor TβRII which subsequently recruits the Type I receptor ALK-5. The activins signal

predominanlty through ActRIIA leading to recruitment of ALK-4, although the activins can also signal via ActRIIB. BMP signalling is predominantly through BMPRII leading to recruitment of ALK2, ALK3 or ALK-6. Versatility of the signalling system is such that the BMPs can also signal via ActRIIA or ActRIIB. TGF-β_{1-3} and activin nuclear signalling is via phosphorylated (p)Smads2 or pSmad3 whilst pSmads1, 5, and 8 mediate BMP signals. These R-Smads associate with the common Smad 4. TβRII can also interact with ALK-1 leading to phosphorylation of Smad1 and Smad5. Smad6 and Smad7 introduce regulation of TGF-Superfamily signalling by interacting with the Type I receptor. Follistatin is a natural inhibitor of activin signalling.

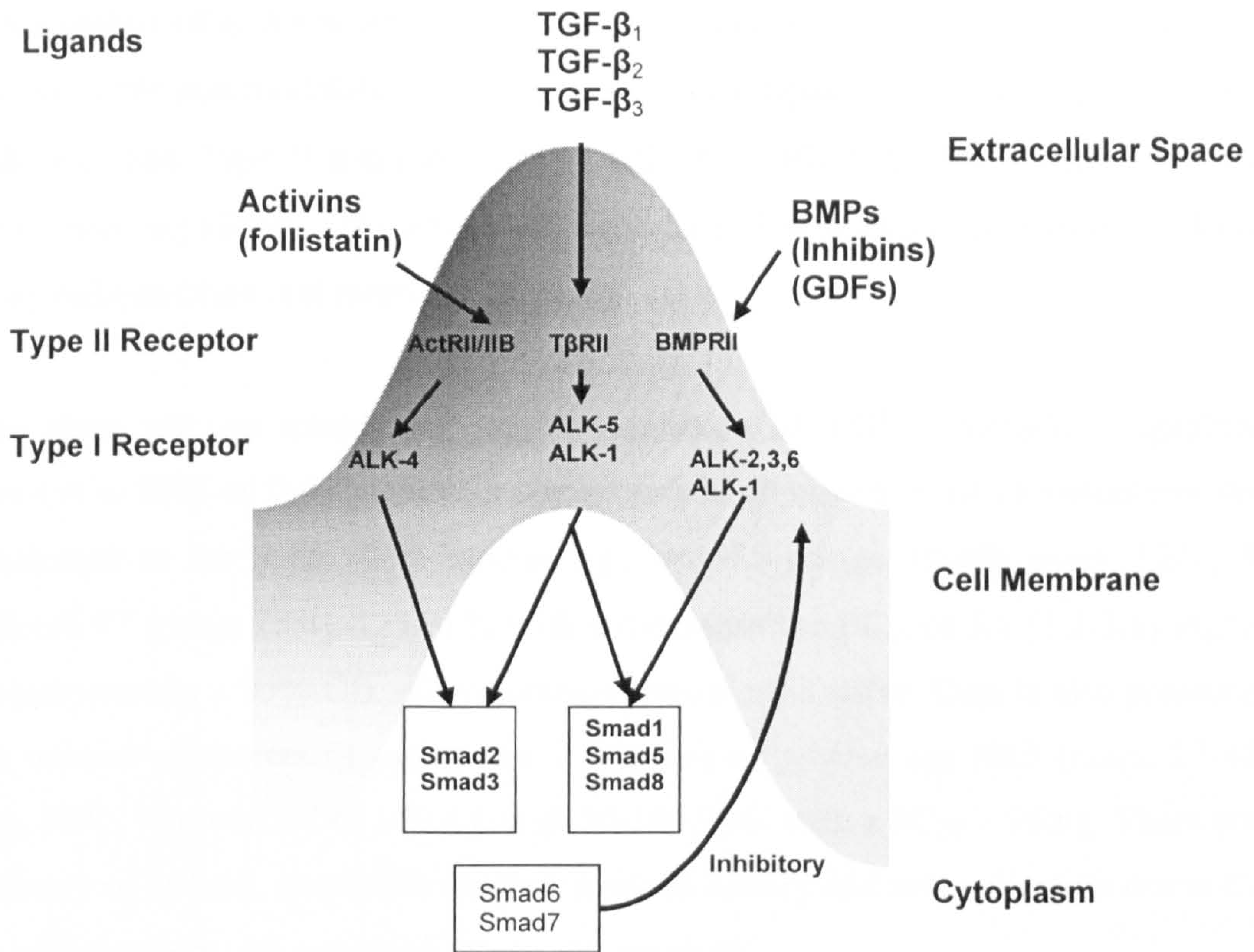


Figure 5.1: Summary of TGF-β Superfamily signalling pathways

Ligands, upon activation, signal via a constitutively active serine-threonine kinase specific Type II receptor that complexes with a Type I receptor which subsequently propagates the signal downstream by phosphorylating receptor-regulated Smads that translocate to the nucleus to initiate gene transcription. TGF-β_1 and activin signalling is via phosphorylated (p) Smad2 and Smad3 whilst pSmads1, 5, and 8 mediate BMP signals. These R-Smads associate with the common Smad 4. Smad6 and Smad7 inhibit further signalling by interacting with the Type I receptor.

It is hypothesised that imbalance of TGF-β Superfamily signalling is present in asthma compared to the normal airway. Allergen challenge of asthmatics is associated with activation and modulation of TGF-Superfamily signalling with changes in Type I (ALK 1-6) and Type II receptor (TβRII, ActRIIB, ActRIIA, BMPRII) expression and active Smad signalling. This activation will be rapid in response to allergen-induced airway inflammation and remodelling events.

Since there was no relationship in the activation of TGF-Superfamily signalling pathways to SER or DAR volunteer status, data is presented in all 15 volunteers that participated in the study. The median age was 25 (range 19-46) years, FEV_1 % predicted 97 (range 75.41-125.7) % with a methacholine PC_{20} of 2.1 (1.2-3.6) mg/ml (geometric mean ± 95% CI). All volunteers were steroid naïve. Data is also presented on 6 normal volunteers (4 males and 2 females) of median age 30.5 (range 27-42) years, FEV_1 % predicted of 100.4 (range 80-104.3)% with a PC_{20} > 16mg. There was no history of asthma, no significant past medical history and no medication use in the normal volunteers. All volunteers were non-smokers.

The chapter is subdived into specific sections that evaluate the expression of the different layers of the TGF-β Superfamily signalling cascade in the asthmatic airway compared to the normal airway and then define the modulation of expression in response to allergen challenge in asthma.

5.2 Section A: TGF-β Superfamily ligand expression

5.2.1 Introduction

This section presents the expression patterns of the TGF-$\beta_{1\text{-}3}$ mRNA isoforms and activin-A, follistatin, BMP-2, BMP-4 and BMP-7 ligand expression. The expression data in the normal volunteer airway is compared to the asthmatic airway at baseline. The modulation of expression in response to allergen challenge in asthma is presented. Ligands that demonstrate modulation of expression in inflammatory cells are co-localised to cell types.

The group's previous experience of using IHC for TGF-β_1 protein expression (Phipps *et al.* 2002) was of wide-spread ECM staining of TGF-β_1 reservoirs making cellular quantification difficult. The kinetics of TGF-$\beta_{1\text{-}3}$ isoform mRNA expression was therefore investigated using *in-situ* hybridisation (*ISH*).

5.2.2 Results

Normal vs asthmatic airway

TGF-$\beta_{1\text{-}3}$ mRNA isoform expression

There was no significant difference between baseline expression in normal volunteers versus asthma for either TGF-β_1 or TGF-β_2 or TGF-β_3 mRNA isoforms in airway epithelium (Figure 5.2). Significantly increased numbers of submucosal inflammatory-like cells expressing TGF-β_3 mRNA ($p=0.03$) were present in the asthmatic airways. Cells with fibroblast-like morphology expressing TGF-β_1 only were present in low numbers. No expression could be convincingly detected in airway smooth muscle. The predominant isoform expressed in submucosal inflammatory-like cells of the asthmatic airway was TGF-β_3 (Figure 5.2F and Figure 5.3C).

Activin-A and follistatin

IHC confirmed that activin-A is expressed in the airways of both normal volunteers and asthmatics. The asthmatic group demonstrated increased amounts of activin-A expression, with epithelium and infiltrating inflammatory cells identified as significant sources ($p=0.04$ and $p=0.005$ respectively) (Figure 5.4A and 5.4B). Images of activin-A expression are presented in Figure 5.5. Expression of follistatin was predominantly confined to the airway epithelium although there were randomly located inflammatory cells in the submucosa (low in number) that also expressed

follistatin. There was a trend only for increased expression of epithelial follistatin in the normals compared to asthmatics (p=0.09) (Figure 5.4C).

BMP-2, BMP-4 and BMP-7

The expression of BMP-2, BMP-4 and BMP-7 in normal airways was equivalent to that of asthmatics at baseline, with airway epithelium identified as the predominant source. Inflammatory-like cells were identified as sources of BMP-4 and BMP-7 (Figure 5.6, 5.7 and 5.8 respectively). As with TGF-β_{1-3} and activin-A expression, the BMPs also demonstrate heterogeneity of expression indicating dynamic regulatory mechanisms of expression are in operation.

Post-allergen challenge in asthma

TGF-β_{1-3} mRNA isoform expression

There was no significant up-regulation of any of the TGF-β_{1-3} isoform mRNAs at either 24 hours or 7 days after allergen challenge (Figure 5.9) in either the airway epithelium or inflammatory cells.

Activin-A and follistatin

There was no significant change in activin-A or follistatin expression in response to allergen challenge in either the airway epithelium or inflammatory cells (Figure 5.10). Neutrophils were identified as the most significant inflammatory cell source of activin-A (Figure 5.13 and Figure 5.14A), although mast cells, macrophages and $CD4^+$ T cells were also sources. Eosinophils did not stain for activin-A.

BMP-2, BMP-4 and BMP-7

No modulation of BMP-2 or BMP-4 expression in response to allergen challenge in asthma was seen (Figure 5.11). Increased numbers of epithelial cells stained for BMP-7 at the 7 day time point (p=0.01) (Figure 5.11E and Figure 5.12B-D). In addition there were significant increases in the number of inflammatory cells expressing BMP-7 both 24 hours and 7 days after allergen (p=0.01 and p=0.03 respectively, Figure 5.11F). Approximatley 50% of post-allergen inflammatory cells expressing BMP-7 were identified as MBP^+ eosinophils. $CD3^{+,}$ $CD4^+$ T cells, $CD68^+$ macrophages and $tryptase^+$ mast cells also expressed BMP-7 (Figure 5.13 and Figure 5.14B). Neutrophils were not found to express BMP-7.

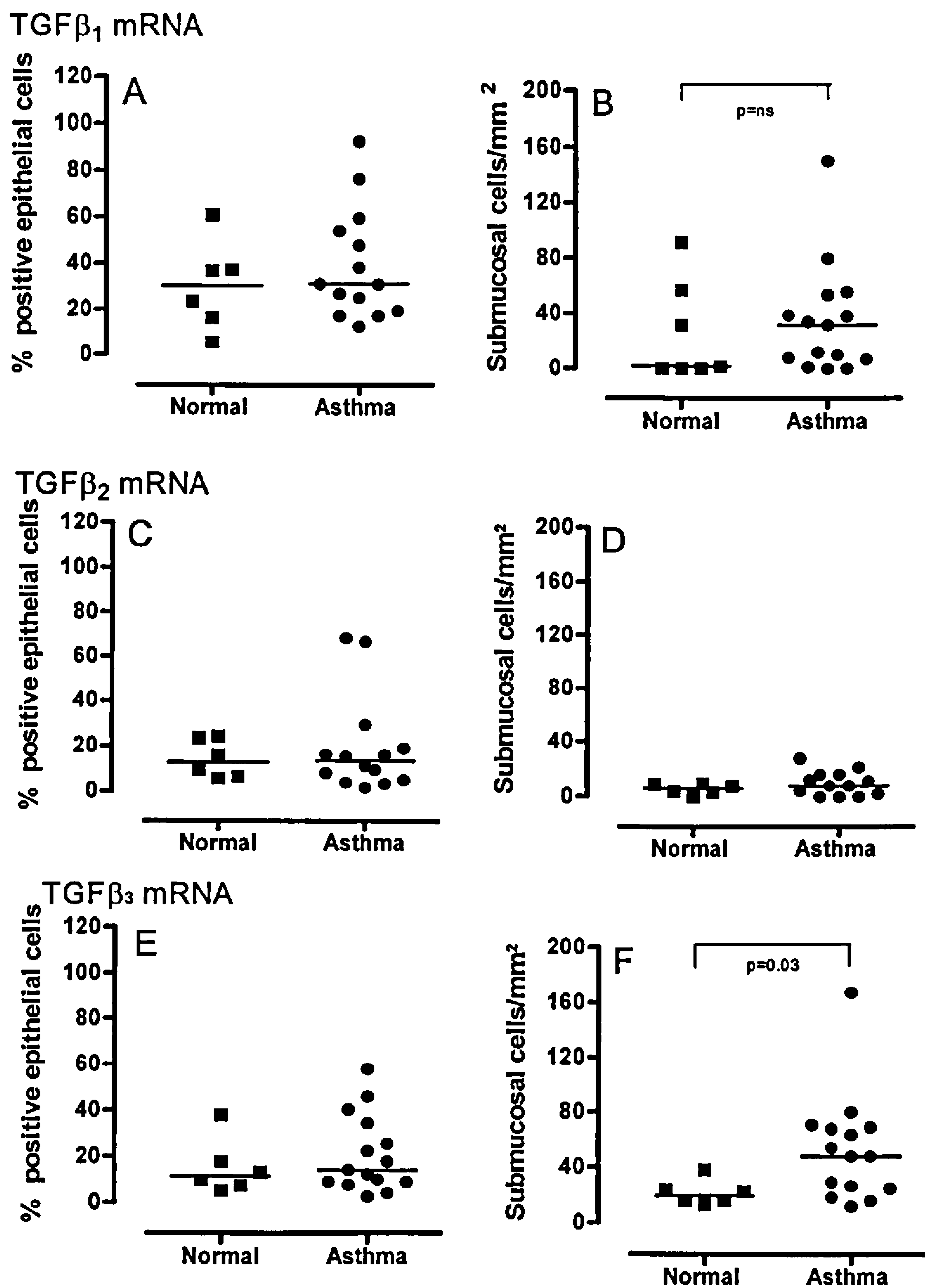


Figure 5.2: The expression patterns of TGF-$\beta_{1\text{-}3}$ isoform mRNA in the normal airway compared to asthma

The number of epithelial cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Positive submucosal cells present are expressed as cells/mm^2. Significant differences between the groups were analysed using the Mann-Whitney Test. $p < 0.05$ was taken as significant.

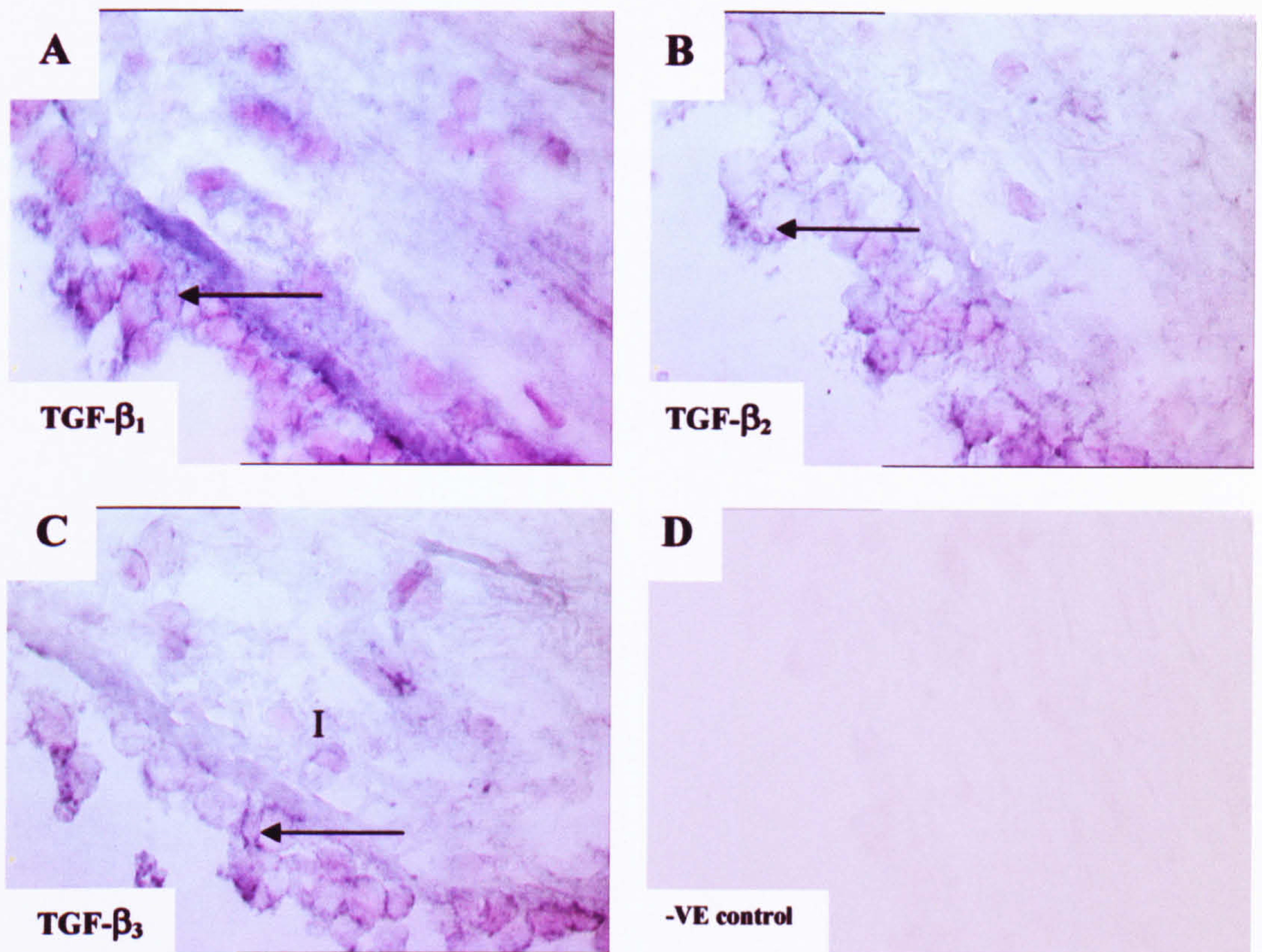


Figure 5.3: TGF-β isoform mRNA expression in asthmatic bronchial tissue

TGF-β_1 (A), TGF-β_2 (B) and TGF-β_3(C) expression is shown as dark-blue pigment-like colour around the nucleus counterstained with nuclear red. An immunogenic detection procedure using non-radioactive FITC-labelled probes was used. Three FITC-labelled probes, each designed with a sequence directed against a different region of the same target mRNA was used for TGF-β_1, TGF-β_2 and TGF-β_3 detection. Simultaneous use of three different probes for each isoform target mRNA allowed enhanced signal intensity. Following incubation with an anti-FITC-alkaline phosphatase antibody in conjunction with NBT/BCIP substrate, mRNA is indicated as a dark blue insoluble product seen (arrowed). A TGF-β_3 positive submucosal cell is indicated (Figure C) by I. A negative control where primers for TGF-β_1 were omitted is also presented (Figure D). Selected slides were also counter-stained with H and E and counted in order to confirm the ISH staining protocol was effective and the data consistent. Dark-blue pigment perinuclear staining is seen in positive cells (E) but absent in the negative control (F).

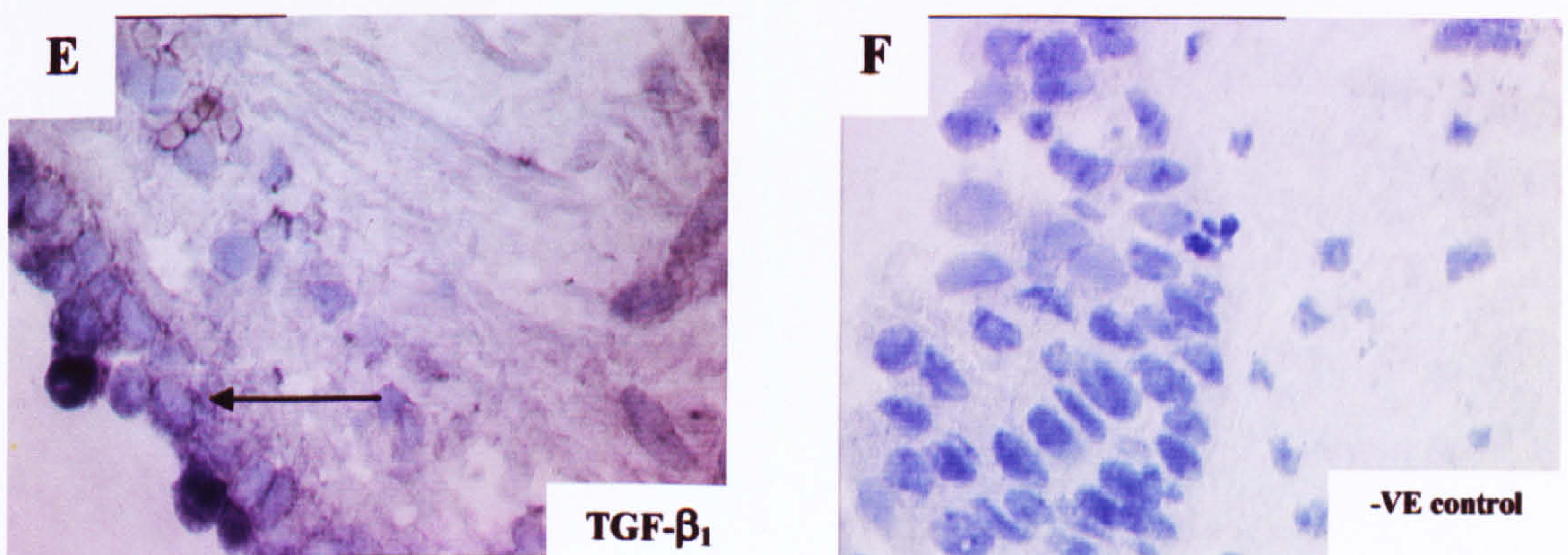


Figure 5.4: The expression patterns of activin-A and follistatin in the normal airway compared to asthma

The number of epithelial cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Positive submucosal cells present are expressed as cells/mm^2. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

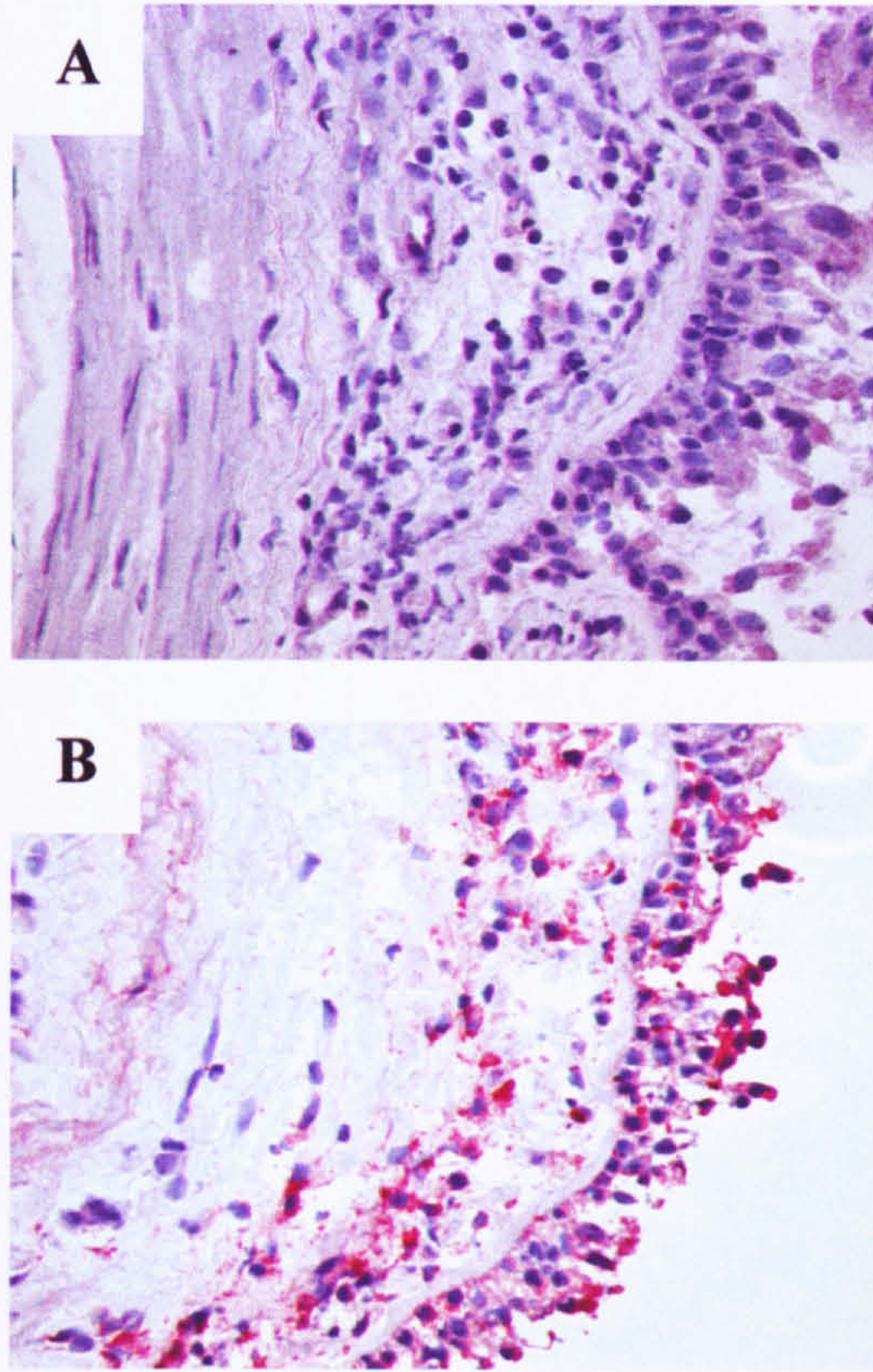


Figure 5.5 Photomicrographs of activin-A expression in normal and asthmatic bronchial tissue

Sections were stained with a goat anti-human antibody to activin-Aand developed using the APAAP system. Positive immunoreactivity was localised predominantly to epithelium and inflammatory-like cells. The normal airway (A) demonstrates considerably less activin-A immunoreactivity compared to the asthmatic airway (B).

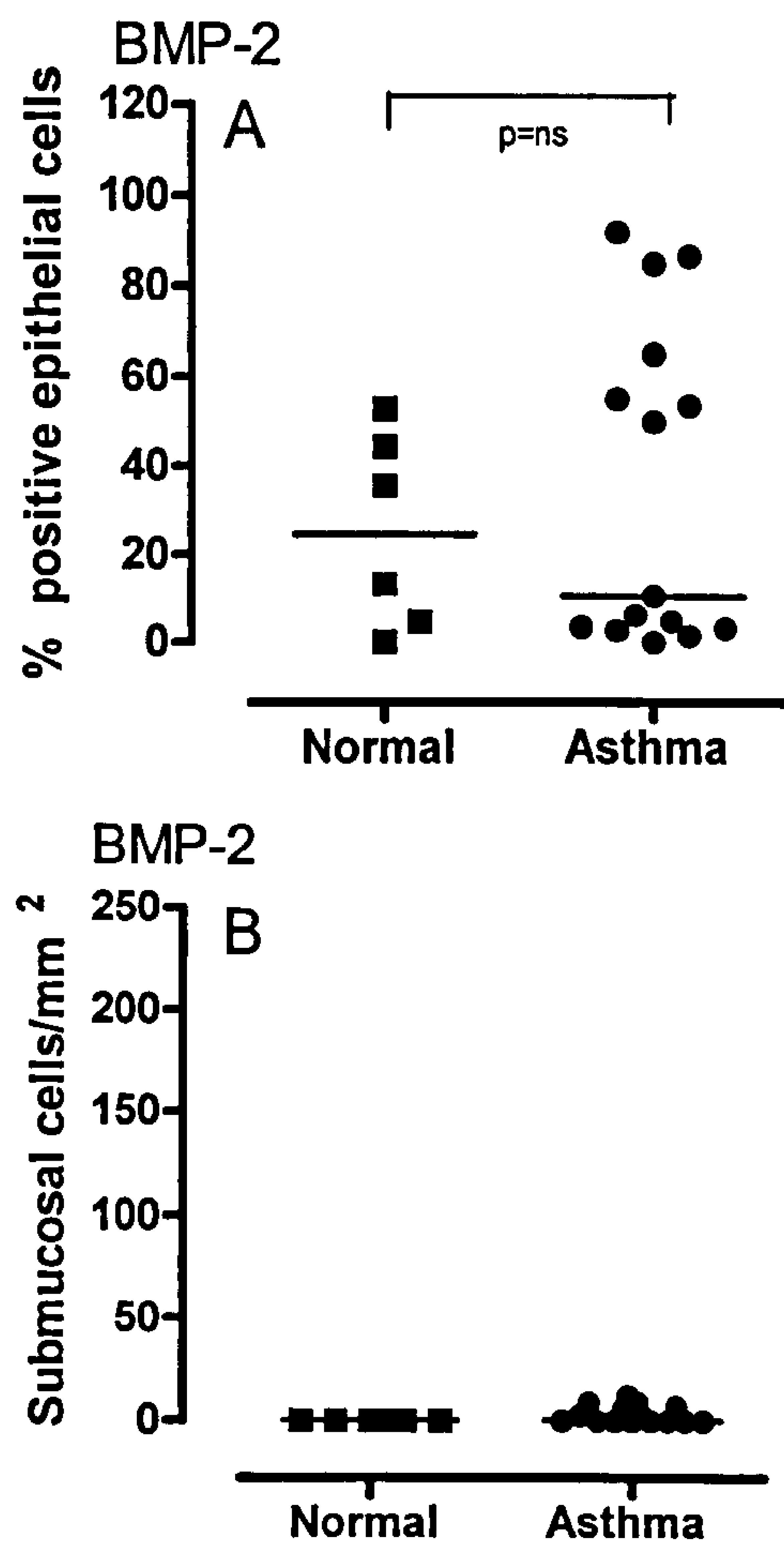


Figure 5.6: The expression patterns of BMP-2 in the normal airway compared to asthma

The number of epithelial cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present (A). Positive submucosal cells present are expressed as cells/mm^2 (B).Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

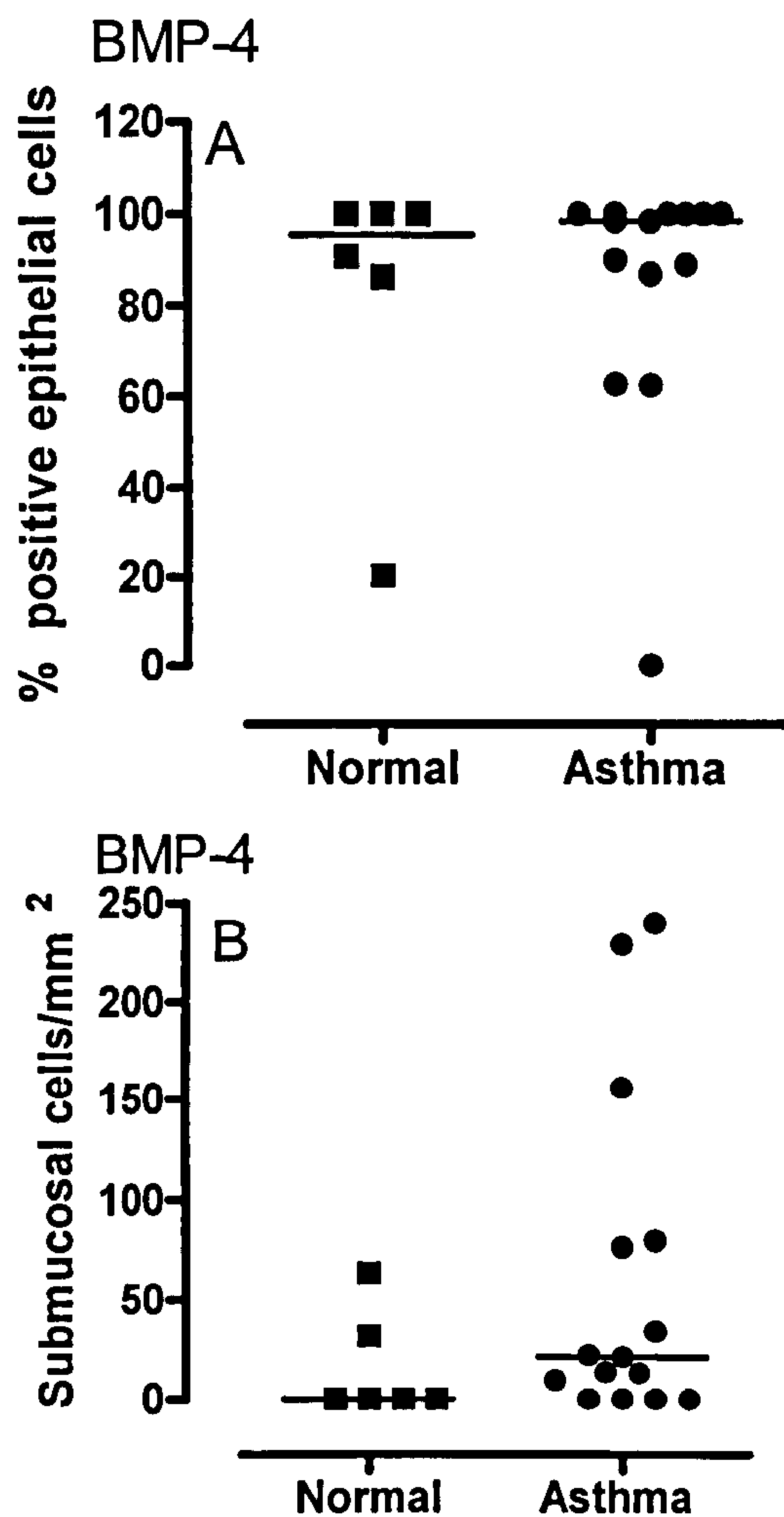


Figure 5.7: The expression patterns of BMP-4 in the normal airway compared to asthma

The number of epithelial cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Positive submucosal cells present are expressed as cells/mm^2. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

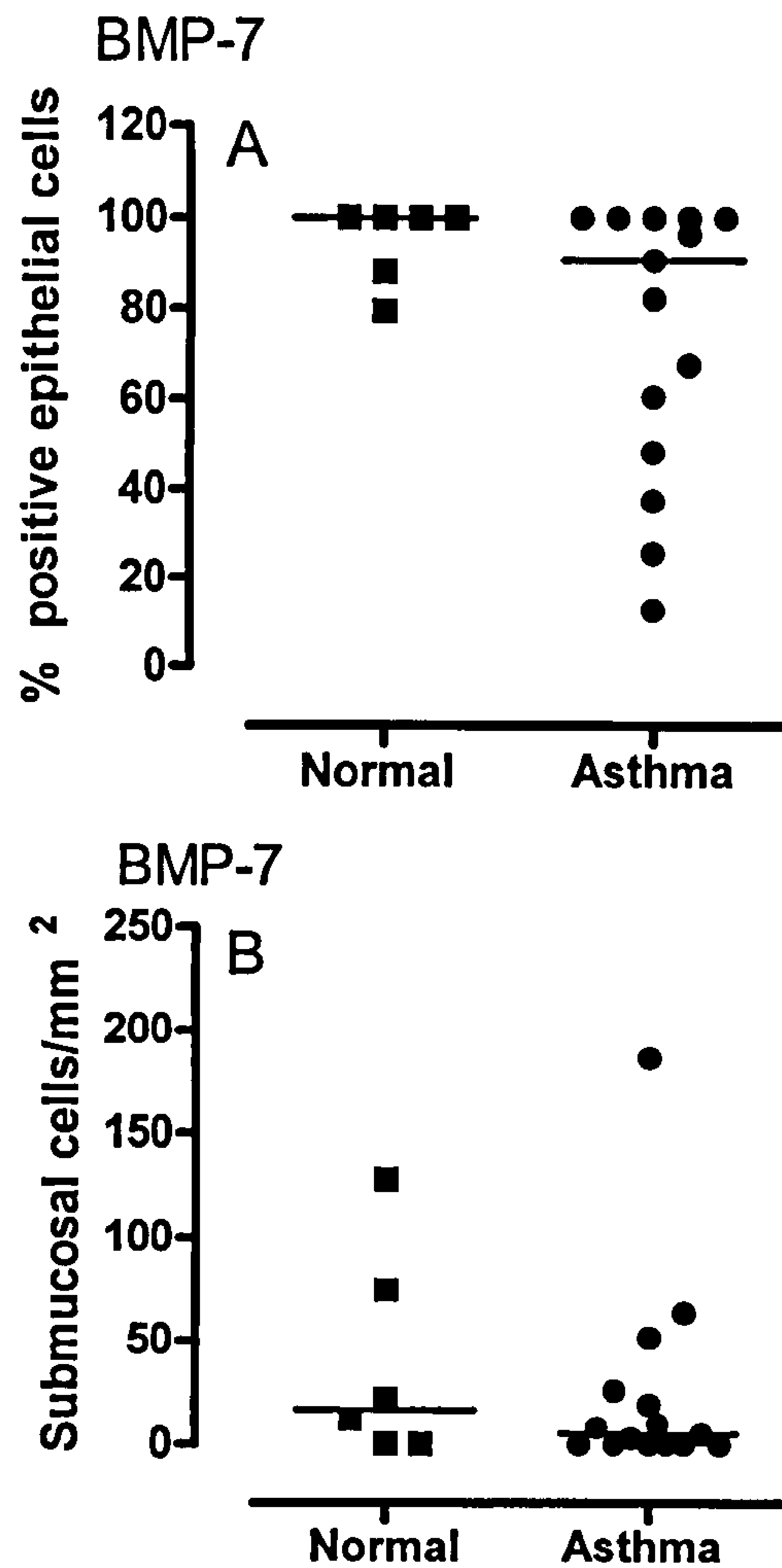


Figure 5.8: The expression patterns of BMP-7 in the normal airway compared to asthma

The number of epithelial cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present (A). Positive submucosal cells present are expressed as cells/mm^2 (B). Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

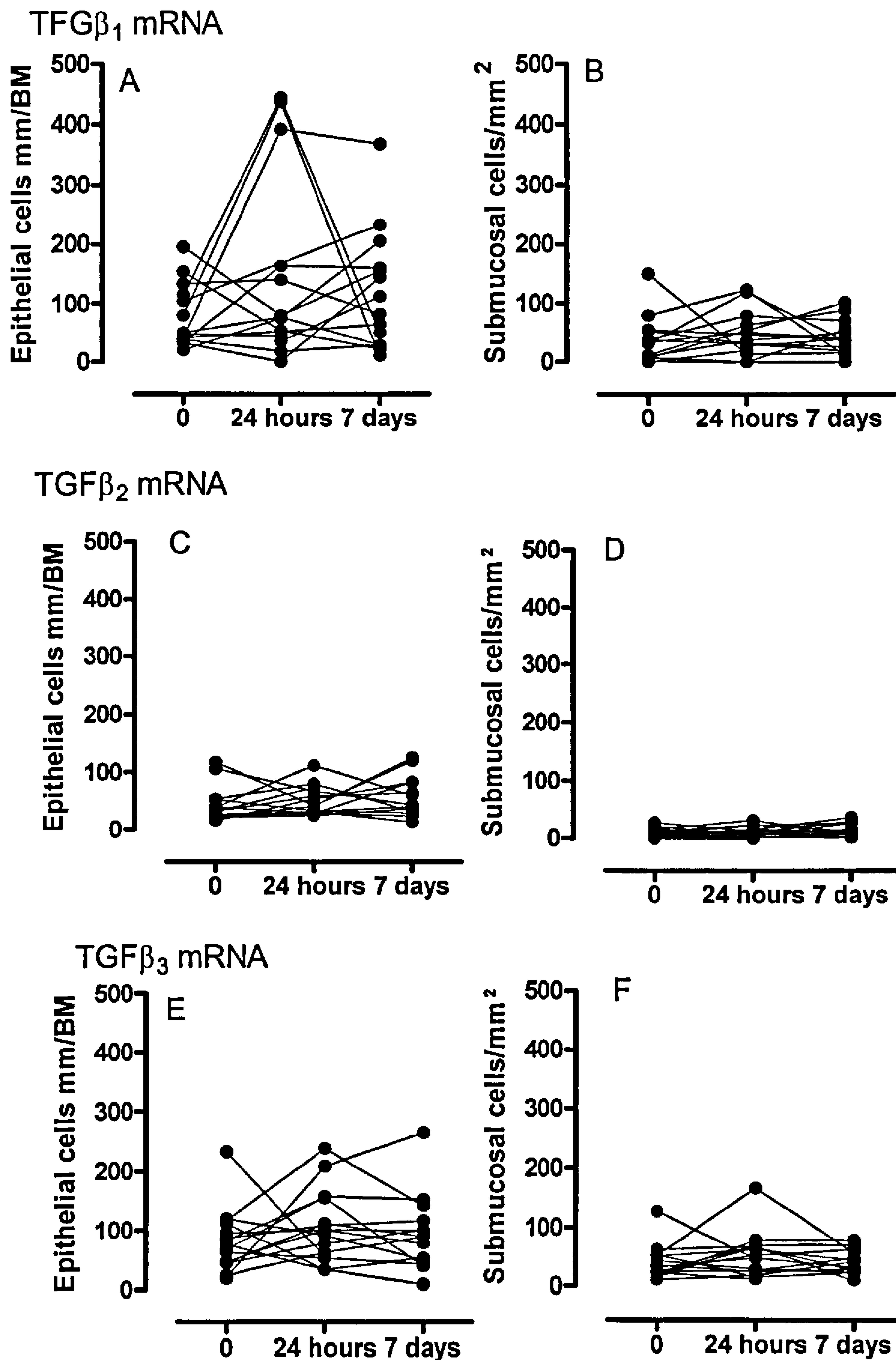


Figure 5.9: The expression kinetics of TGFβ1-3 (mRNA) in the asthmatic airway 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/mm BM) (A, C and E for TGF-β_1, β_2 and β_3 respectively). Positive submucosal cells present are expressed as cells/mm^2 (B, D and E respectively). Significant differences between time points were analysed using the Wilcoxon signed-rank test. $p<0.05$ was taken as significant.

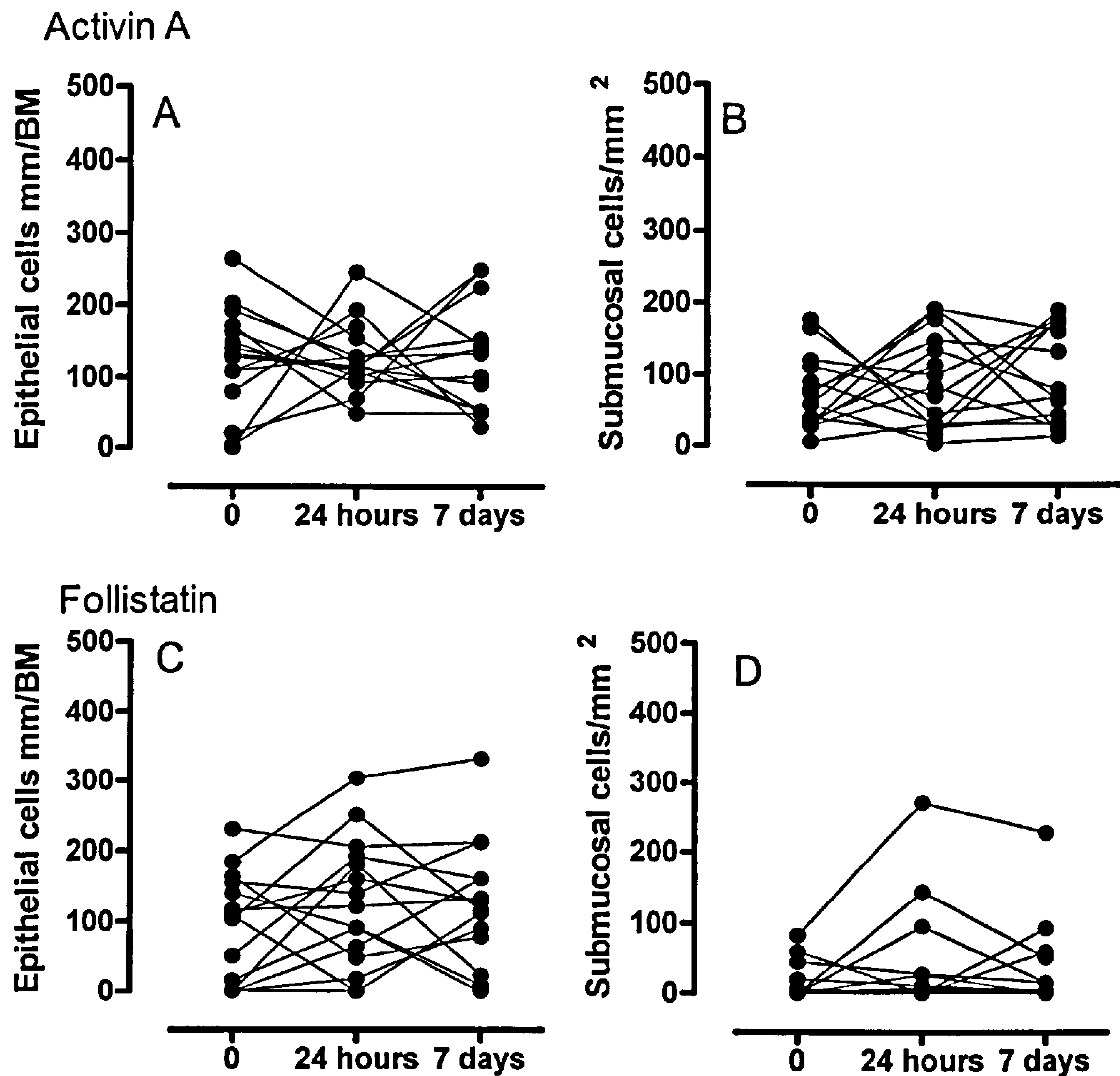


Figure 5.10: The expression kinetics of activin-A and follistatin in the asthmatic airway 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/mm BM). Positive inflammatory cells present are expressed as cells/mm^2. Significant differences between time points were analysed using the Wilcoxon signed-rank test. $p<0.05$ was taken as significant.

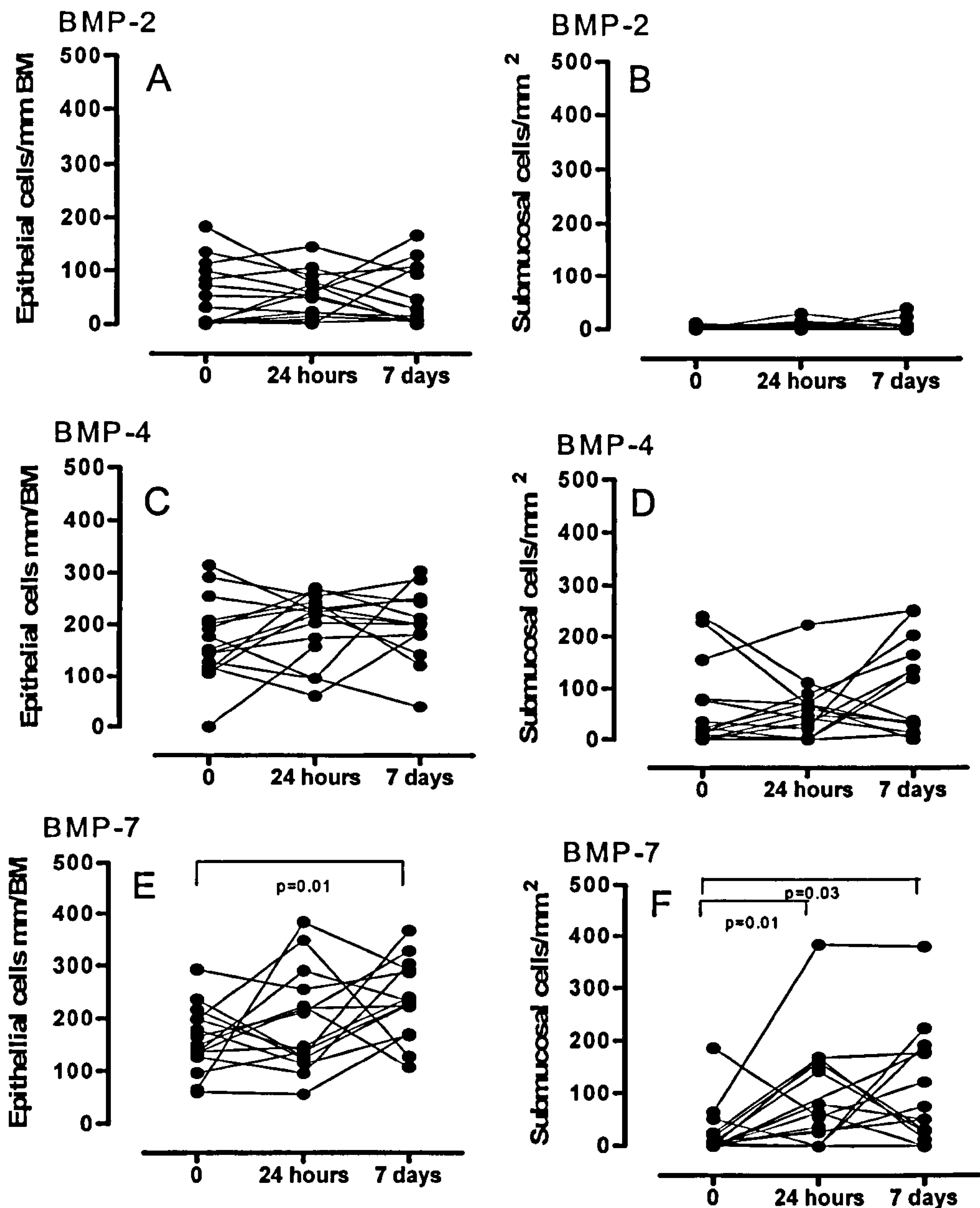


Figure 5.11: The expression kinetics of BMP-2, BMP-4 and BMP-7 in the asthmatic airway 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/mm BM). Positive inflammatory cells present are expressed as cells/mm^2 (B, D and E respectively. BMP-7 expression was up-regulated in epithelium at 7 days post-allergen whilst increased numbers of inflammatory cells were seen at both 24 hours (p=0.01) and 7 days (p=0.03). Significant differences between time points were analysed using the Wilcoxon signed-rank test. p<0.05 was taken as significant.

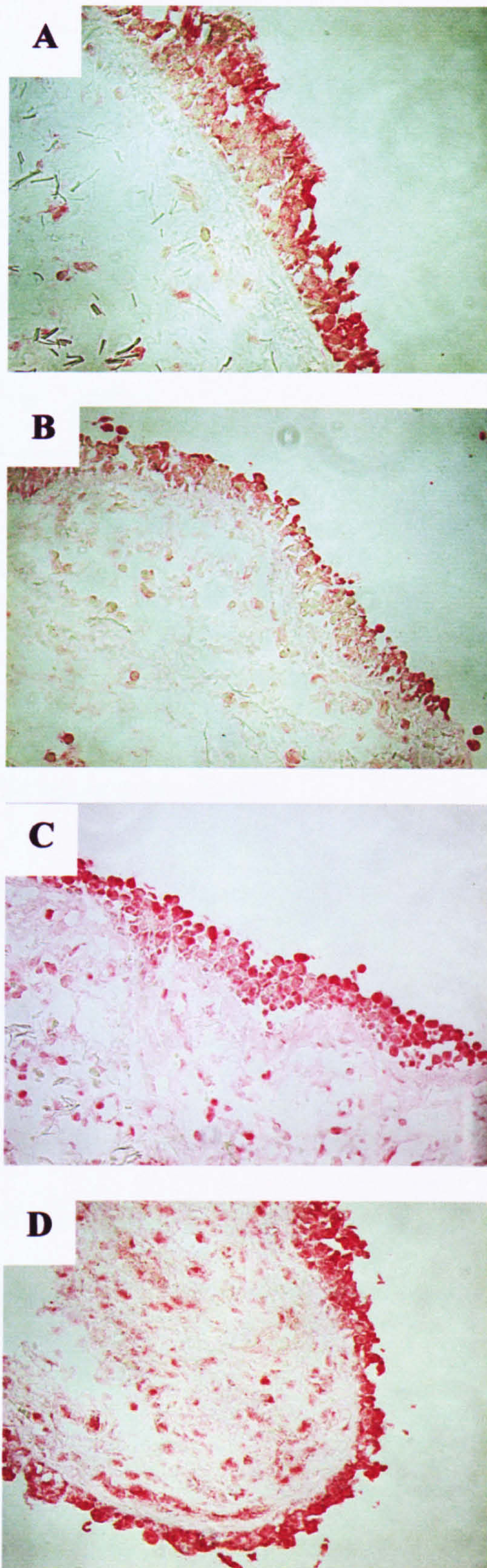
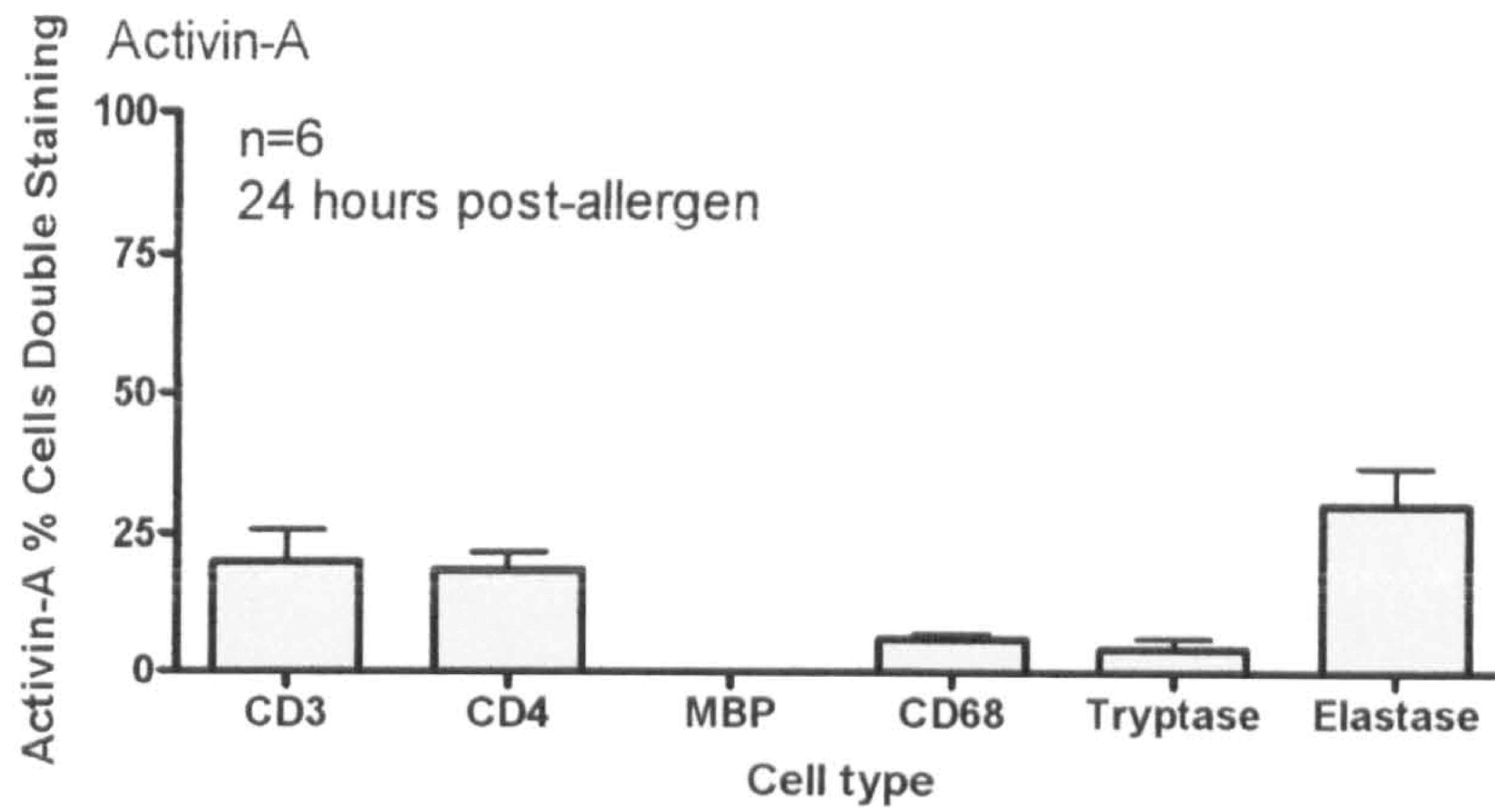


Figure 5.12: Photomicropgraphs of BMP-7 immunoreactivity

Sections were stained with a mouse anti-BMP-7 antibody and developed using the APAAP system. The expression in the normal airway (A) was similar to that in baseline asthma (B). Allergen challenge was associated with increased numbers of epithelium and inflammatory-like cells staining for BMP-7.



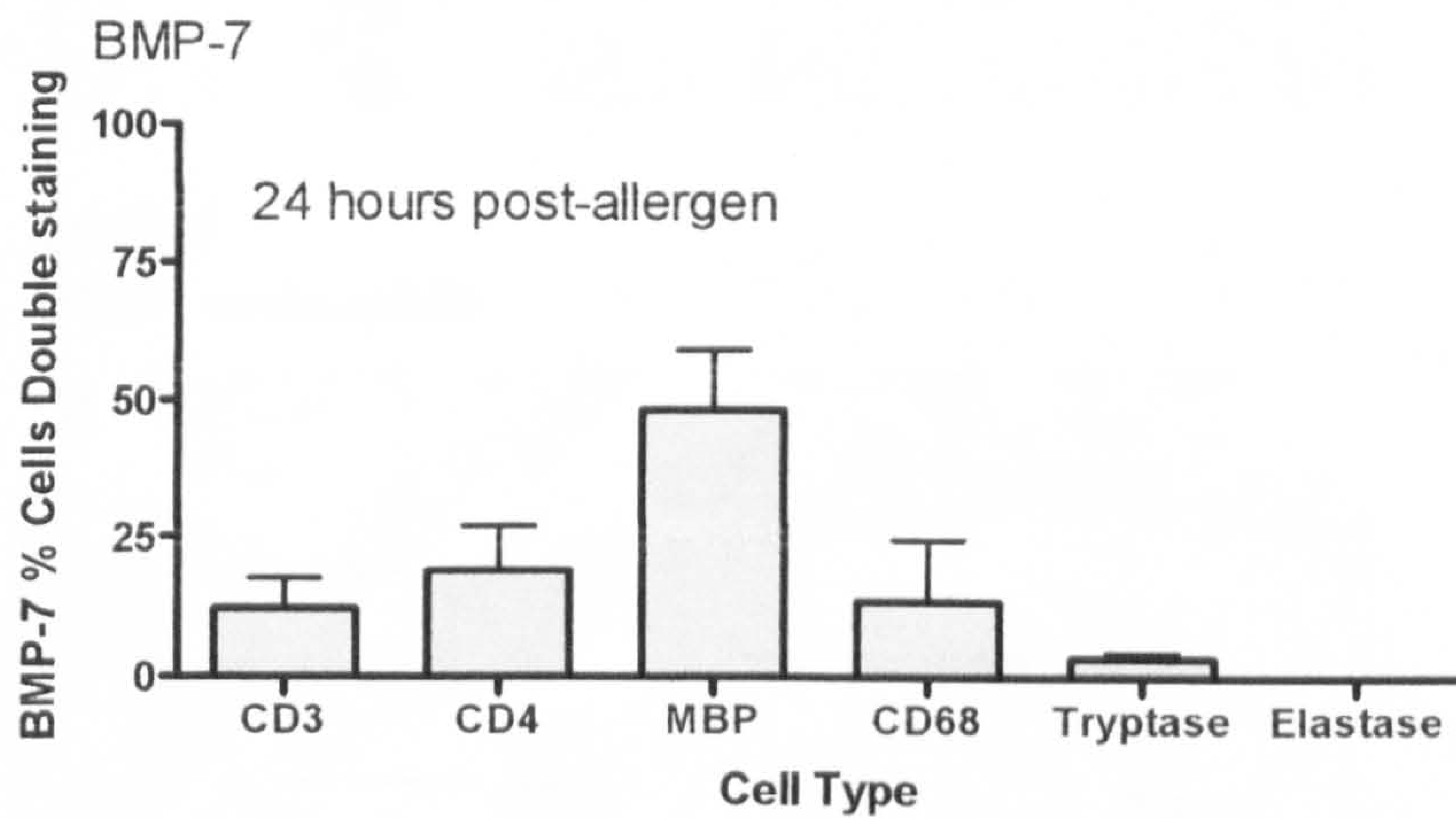
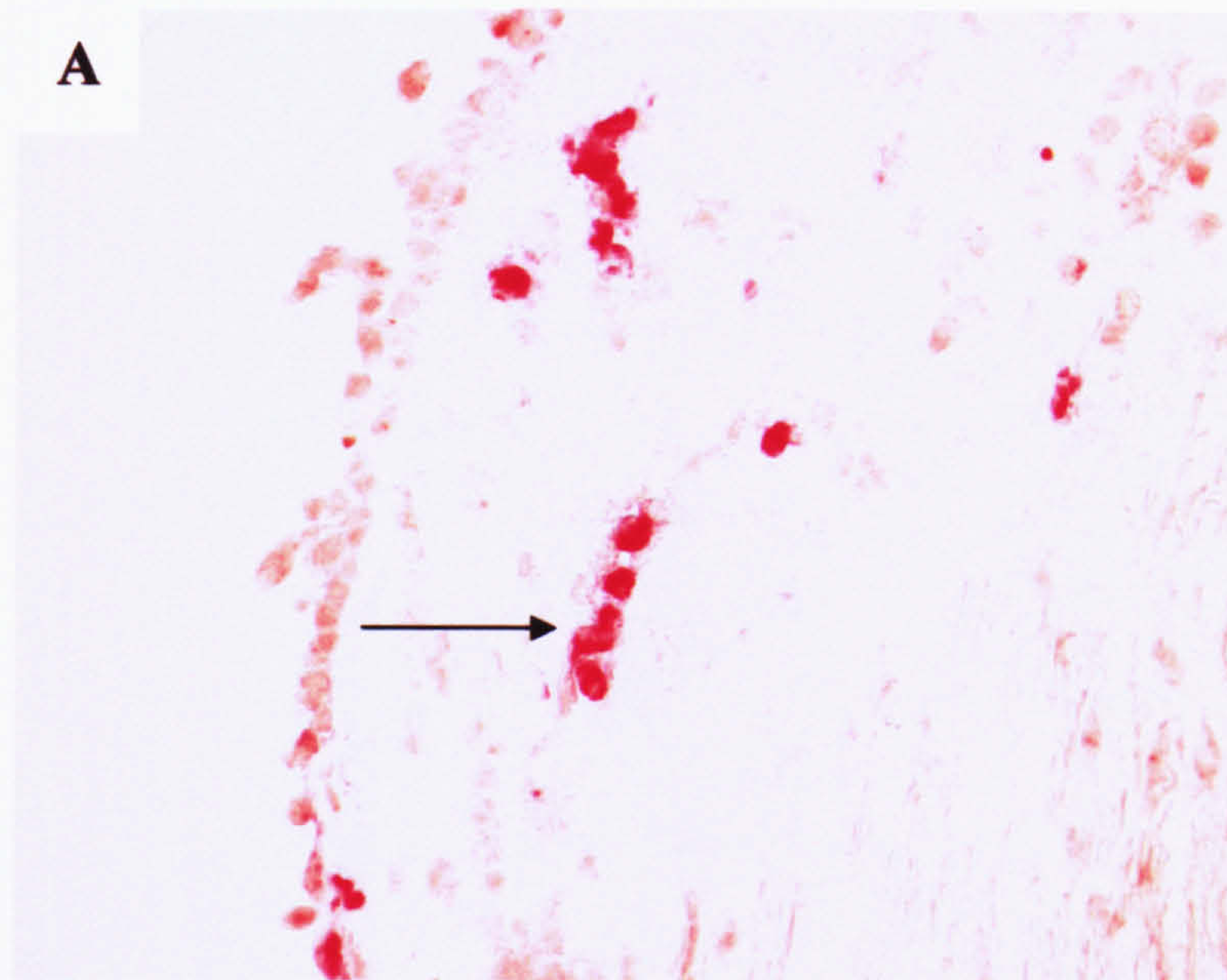


Figure 5.13: Summary of the cell types expressing activin-A and BMP-7

Using double staining technique immunohistochemistry activin-A (5.14A) and BMP-7 (5.14B) expression was localised to inflammatory cells phenotypes in tissue sections obtained at the 24 post-allergen time point. Cells counts are expressed as the percentage of double positive cells.

Activin-A
Elastase$^+$ neutrophils



BMP-7
MBP$^+$ eosinophils

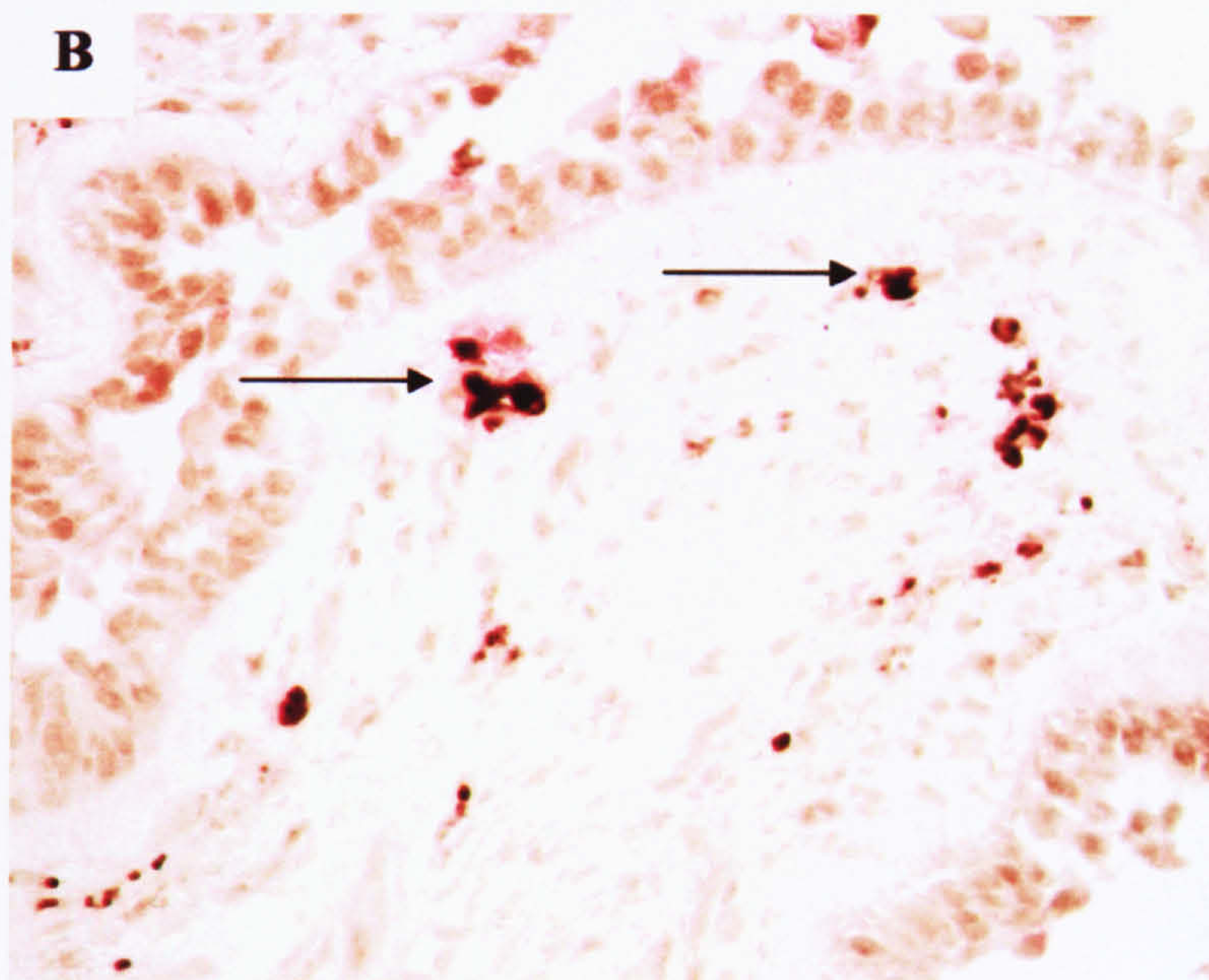


Figure 5.14: Photomicrograph of immunohistochemical co-localisation of activin-A to the dominant cell type elastase$^+$ neutrophils (A) and BMP-7 to the dominant cell type MBP$^+$ eosinophils (B)

The TGF ligand is stained brown via DAB and the cell phenotyped using chromogen Fast Red staining red. Co-localisation is seen as a darker red-brown colour in cells (arrowed).

5.2.3 Discussion

Several studies have confirmed the differential expression of TGF-β_{1-3} isoforms through immunohistochemistry (IHC) and *in-situ* hybridisation (ISH) in the lungs of normal individuals and asthmatics. Immunolocalisation of TGF-β can be difficult to interpret with a pure immunohistochemical approach as such given that the translational gene product is present in so many different forms (unprocessed, latent and degraded). It is impossible to differentiate between these states through IHC. Further it has been confirmed that mature TGF-β_1 is predominantly extracellular (Magnan *et al.* 1997) with localisation to ECM components such as decorin (Aubert *et al.* 1994) (Redington *et al.* 1998). Thus the expression kinetics and tissue sources are best determined by localising mRNA through *in-situ* hybridisation (ISH).

In this study the expression of TGF-β_{1-3} isoform mRNA was predominantly localised to the airway epithelium although submucosal cells with inflammatory-cell like morphology were also present. The airway epithelium and macrophages have been previously identified via ISH (using digoxgenin labelled ribroprobes) as the predominant source of TGF-β_1, TGF-β_2 and TGF-β_3 (Coker *et al.* 1996; Coker *et al.* 1998). It has also been reported that macrophages (Aubert *et al.* 1994) and eosinophils (Minshall *et al.* 1997) are the dominant cellular sources of TGF-β_1 in the asthmatic airway. It is confirmed here that the predominant isoform expressed by the submucosal cells in our population of asthmatics is TGF-β_3. Airway smooth muscle does not appear to be a significant source of any of the TGF β_{1-3} isoforms which is consistent with other studies (Joubert & Hamid 2005; Balzar *et al.* 2005; Aubert *et al.* 1994). There was no significant difference between the number of epithelial cells staining positive for TGF-β_{1-3} in asthma at baseline versus normal volunteers, concurring with previous studies on human airway biopsies in mild asthma (Aubert *et al.* 1994; Redington *et al.* 1998; Minshall *et al.* 1997). Given the critical role of TGF-β_1 in the regulation of airway inflammatory homeostasis it is expected that normal tissue will express similar levels. Inflammatory cells expressing TGF-β_1 and TGF-β_2 mRNA did not differ between normal and asthmatic airway as previously shown (Balzar *et al.* 2005). The finding of increased numbers of TGF-β_3 expressing submucosal cells in baseline asthma compared to the normal group in this study is novel and it will be important to determine the exact cellular types that express it and the extent to which this specific isoform is able to contribute to tissue repair. *In-vitro* studies indicate that TGF-β_3 may be more potent than the other isoforms in the

induction of procollagen and the inhibition of subsequent procollagen degradation (Coker *et al.* 1997). At present the exact roles of the individual isoforms in health and disease is unknown. Understanding the functional significance of such expression will have therapeutic implications.

Following allergen challenge no significant increase in the number of TGF-β isoform positive epithelial and inflammatory cells was seen. In contrast, asthmatics with severe and moderate disease demonstrate increases in TGF-β_1 and TGF-β_2 expression (Minshall *et al.* 1997; Balzar *et al.* 2005). It is therefore possible that other disease modifying factors other than allergen-provocation can regulate TGF-β isoform expression.

Asthmatics have significantly more TGF-β_1 protein levels compared with normal volunteers in BAL fluid at baseline with further increases after allergen (Redington *et al.* 1997). Concentrations in BAL and tissue probably represent reservoirs of TGF-β_1 that is present in ECM (Redington *et al.* 1998), epithelium and inflammatory cells. Allergen challenge is associated with inflammatory cell influx in an activated state with increased propensity for the release of TGF- β_1 (Vignola *et al.* 1997). It may be that the main level of control in TGF-β isoform signalling is not in the regulation of expression of the mRNA that encodes the TGF-β isoforms but in the activation of locally stored latent TGF-β that is present in excess in asthma. There is therefore a temporal discontinuity between TGF-β synthesis and action. In support of this view *in-vitro* experiments of epithelium and fibroblast co-culture on an amnion membrane (i.e the matrix) suggests that basal TGF β_1 mRNA production does not change with epithelial mechanical injury but leads to decreased matrix TGF-β immunoreactivity associated with myofibroblast transformation (Morishima *et al.* 2001). Thus epithelial injury may release TGF-β ligands from local ECM stores and this now undergoes activation. Epithelial integrin αvβ6 is rapidly up-regulated in response to airway injury. It is critical for TGF-β_1 activation as shown by β_6 knock-out mice in models of pulmonary fibrosis where the mice are essentially protected against fibrosis. Epithelium and inflammatory cells will continue to synthesise TGF-β but at a level which serves to replenish stores in ECM such as that bound to decorin.

In mouse models of allergen-induced airway injury, TGF-β mRNA quantification in whole tissue blocks using real-time polymerase chain reaction (RT-PCR) showed only

fold induction of only 1.1 for TGF-β_1, 1.2 for TGF-β_2 and 2.2 for TGF-β_3 (Rosendahl *et al.* 2001). Mouse models of TGF-β isoform expression need to be interpreted with caution as they cannot be interpreted in the context of disease severity as with the human disease but again suggests that it is not rapid local synthesis but rapid local activation of TGF-isoform reservoirs that is important in response to allergen-induced airway injury (Rosendahl *et al.* 2001).

Increased activin-A levels have been identified in asthmatic serum and $CD4^+$ T cells (3.6 fold) (Karagiannidis *et al.* 2006) with several mouse models also confirming increased activin-A mRNA expression (Rosendahl *et al.* 2001; Karagiannidis *et al.* 2006) in response to allergen challenge. IHC in this study confirmed that activin-A expression is present at baseline in the airways of both normal volunteers and asthmatics. The intensity and distribution of activin-A expression was significantly increased in the airways of asthmatics compared to normal airways with both epithelium and inflammatory cells identified as important sources. Given the potent fibrotic properties of activin-A this finding may indicate that asthmatic airways have an increased propensity for induction of fibrosis. Epithelium was a predominant source of activin-A with inflammatory cells also identified as significant sources as such. There was no significant modulation of staining in response to allergen challenge. This maybe because like TGF-β_1, activin-A is present pre-stored in cells with local release and activation occurring in response to injury in asthma (Jones *et al.* 2004b). Quantitative mRNA by RT-PCR, if possible of individual cellular populations, would be needed to fully evaluate the expression kinetics of such growth factors. Although activin-A mRNA expression was not looked at in our study, it is important that activin-A mRNA expression is rapidly induced unlike that of TGF-β_1 in asthma (Karagiannidis *et al.* 2006).

One of the striking observations in the asthmatic airways was the strong expression of activin-A in infiltrating inflammatory cells. It has been noted before that mast cells (Cho *et al.* 2003), macrophages (Abe *et al.* 2002) and $CD4^+$ T cells (Karagiannidis *et al.* 2006) are important sources of activin-A and this has again been confirmed in this study. The important finding in this study is that neutrophils were identified as the predominant source of activin-A in the post-allergen tissue sections.

Recently there has been increasing focus on what role the neutrophil may play in the pathogenesis in asthma. Such focus has been driven by the findings that neutrophils

are increased in number (more than eosinophils) in asthma death airway specimens (Sur *et al.* 1993), in patients ventilated for status asthmaticus (Lamblin *et al.* 1998) and in chronic severe disease as evidenced by studies in BAL and endobronchial biopsy (Wenzel *et al.* 1997). Whether neutrophils are just bystander cells or active in disease pathogenesis remains unknown. The mediator profile of neutrophils (predominantly IL-8, LTB_4) does not directly suggest an important role in the induction of acute bronchconstriction. The identification that neutrophils are an important source of TGF-β_1 in asthmatic airways (Chu *et al.* 2000) have implicated a role for these cells in repair and remodelling. Finding that neutrophils are the dominant cellular source of activin-A in this study further suggests an important role for neutrophils in the remodelling processes. For example activin-A is a potent inducer of smooth muscle cell proliferation (Cho *et al.* 2003). In fatal asthma there is excessive accumulation of ASM and it is ASM hypertrophy, not airway inflammation, that are the only selective determinants of severe persistent symptoms (Benayoun *et al.* 2003). It is therefore possible that neutrophil derived growth factors such as activin-A will contribute to the ASM increases and other remodelling features present in the dfficult asthmatic airway. It has also been shown that neutrophil elastase is an activator of TGF-β_1 (Chua *et al.* 2007). An important clinical point is that steroids, the mainstay of asthma anti-inflammatory therapy, prolong neutrophil survival through inhibition of apoptosis (Cox 1995) thus leading to augmentation of neutrophil function that may lead to detrimental effects in asthma. In $CD4^+$ T cells at least actvin-A expression is attenuated by glucocorticoids (Karagiannidis *et al.* 2006) and it would be important to investigate whether there is such an effect on neutrophil activin-A expression.

The number of intact mast cells with activin-A were low in this study. This maybe because the sections studied were all post-allergen provocation leading to mast cell degranulation and therefore low numbers of intact cells that can be double stained were present. The finding that $CD4^+$ T cells are also an important inflammatory source of activin-A in this study is important in that asthma is a Th2 driven disease.

Until now the expression of BMP ligands has remained mostly undefined in the normal and diseased lung. This is despite increasing awareness that the BMP ligand system represents a major developmental signalling pathway critical for organ and tissue generation in early development. In adult tissue and organ systems it may be

reactivated to promote tissue regeneration, repair and maintenance. At present there is no data on the expression patterns of BMP ligands in asthma. Given the diversity of BMP ligands that may be present in the lung, the study analysed the expression of BMP-2, BMP-4 and BMP-7 as they have an important role in lung branching morphogenesis. BMP-7 expression was particularly important to evaluate in that it has the therapeutic potential to modulate TGF-β_1 induced fibrosis. The expression levels of epithelial BMP-2 and BMP-4 did not change. Again this may suggest that local activation of stored BMP ligand may be important. The significant and sustained increase in BMP-7 expression in both epithelium and inflammatory cells may be related to the role of BMP-7 in epithelial-mesenchymal tissue interactions (Vukicevic *et al.* 1994b) required for branching morphogenesis which is reactivated in the process of tissue repair as well as the ability of BMP-7 to down-regulate inflammation (Maric *et al.* 2003). The important finding of BMP-7 being expressed in nearly 50% of infiltrating eosinophils is consistent with the evolving view that these cells are important tissue-repair cells (Kay *et al.* 2004). It is therefore possible that BMP-7 expression may therefore be an attempt to regulate both the inflammatory and repair response in asthma.

Several lines of evidence have delineated a role for eosinophil-derived TGF-β_1 in airway remodelling. *In-vitro* co-culture of fibroblasts with eosinophils lead to phenotypic change to myofibroblasts and synthesis of ECM proteins tenascin and procollagen III: this was dependent on eosinophil-derived TGF-β_1. Allergen challenge in the skin was associated with eosinophil infiltration into the site of late cutaneous reaction, together with eosinophil TGF-β_1 expression, myofibroblast-like cells and tenascin deposition (Phipps *et al.* 2002). Anti-IL-5 antibody treatment of asthmatics led to reductions in airway eosinophils, TGF-β_1 expression and RBM staining for the ECM proteins tenascin, lumican and procollagen III (Flood-Page *et al.* 2003a). Whilst no appreciable improvements in clinical outcomes were seen the study was not powered to detect changes in lung function or AHR. The results do, nevertheless, provide evidence that there is a relationship between eosinophils and matrix deposition in the ECM. The functional significance of eosinophil derived BMP-7 remains to be determined but it may be predicted it is related to regulating repair through interaction with other TGF-β Superfamily signalling pathways.

5.2.4 Summary of ligand expression

1. The baseline expression of TGF$\beta_{1\text{-}3}$ mRNA isoforms in asthmatic airway epithelium does not differ from baseline expression in airway epithelium from normal volunteers.

2. There are increased numbers of submucosal inflammatory-like cells staining positive for both TGF-β_1 and TFG-β_3 mRNA in the asthmatic airway at baseline compared to normal volunteers. However, this is only significant for TGF-β_3 ($p=0.03$).

3. There are significantly increased amounts of activin–A protein expression in asthma, with both epithelial and submucosal inflammatory-like cells identified as significant sources.

4. Allergen challenge of mild asthmatics is not associated with any modulation of TGF-$\beta_{1\text{-}3}$ isoform mRNA or activin-A ligand expression in either the epithelium or inflammatory cell population.

5. Whilst the levels of BMP-2, BMP-4 and BMP-7 expression are similar in the normal airway and asthmatic airway, there is significant up-regulation of BMP-7 in epithelial and inflammatory-like cells upon allergen challenge at both 24 hours and 7 days in asthma.

5.3 Section B: Type II receptor expression

5.3.1 Introduction

This section evaluates the expression of the TGF-β Superfamily Type II receptors (TβRII, BMPRII, ActRIIA and ActRIIB) in the normal airway and the baseline asthmatic airway. Modulation of expression in response allergen challenge asthma is then examined.

5.3.2 Results

Normal vs asthmatic airway

TβRII expression was evident throughout the airway with localisation to epithelium, inflammatory cells, fibroblast-like cells, airway smooth muscle and vascular structures. There was no significant difference in TβRII expression in either the airway epithelium or inflammatory cells between asthmatic and normal volunteers. The data is presented in Figures 5.15A with representative photomicrographs in Figures 5.16A and 5.16B.

BMPRII expression was significantly decreased in the asthmatic airway epithelium compared to normal volunteers (p=0.009) (Figure 5.15B and Figure 5.16C and 5.16D). BMPRII expression was evident on infiltrating inflammatory cells as well as vascular smooth muscle of the normal airway. Few inflammatory cells stained for BMPRII in the asthmatic airway.

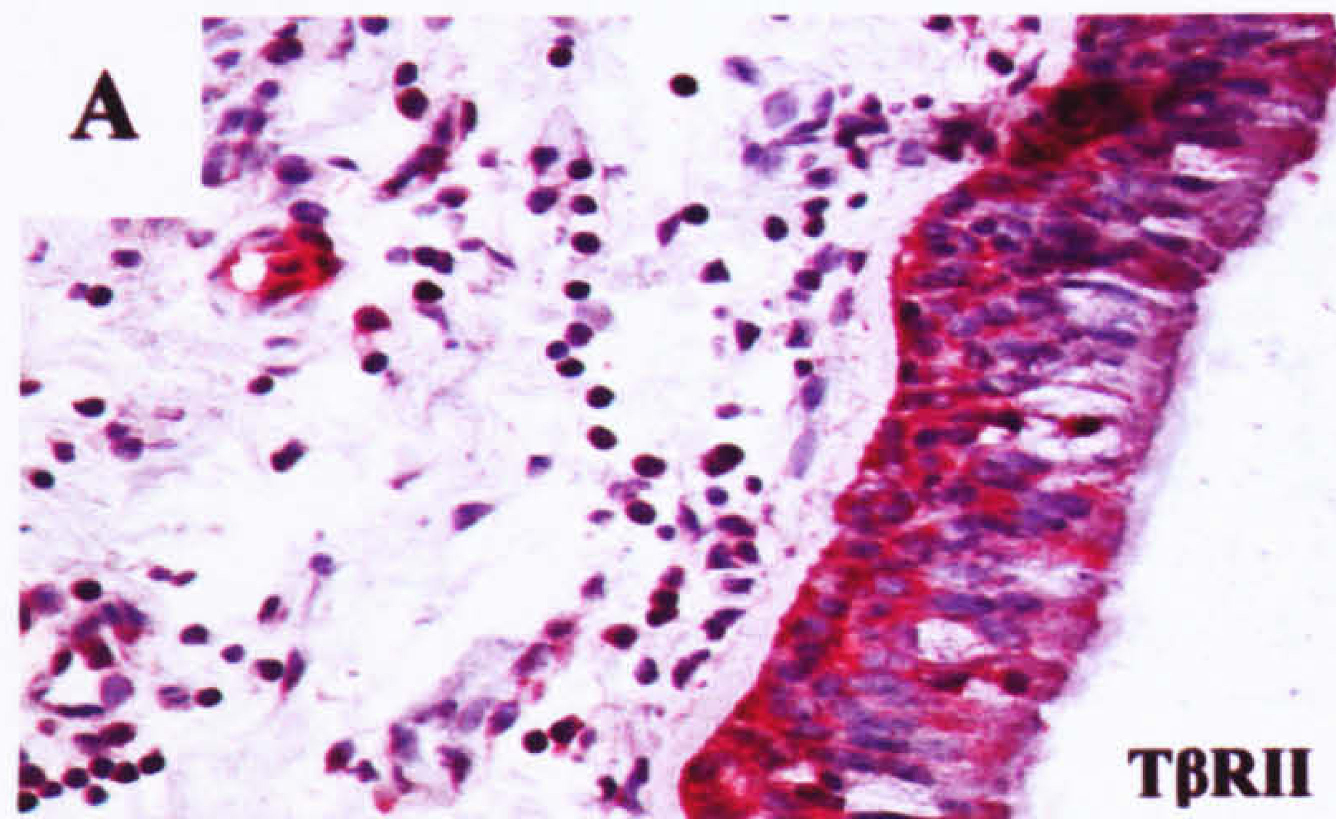
The expression of both ActRIIA and ActRIIB in epithelium was significantly less in asthmatics compared to normal volunteers (p=0.0008 and p=0.04 respectively) (Figure 5.15C and Figure 5.15D). In fact there was also most no detection of ActRIIA in the asthmatic group at baseline (Figure 5.16E and 5.16F). There were inflammatory and fibroblast-like cells expressing ActRIIA in the normal airway. Expression of ActRIIB was confined mostly to the airway epithelium in both the normal and asthmatic airway (Figure 5.16G and 5.16H).

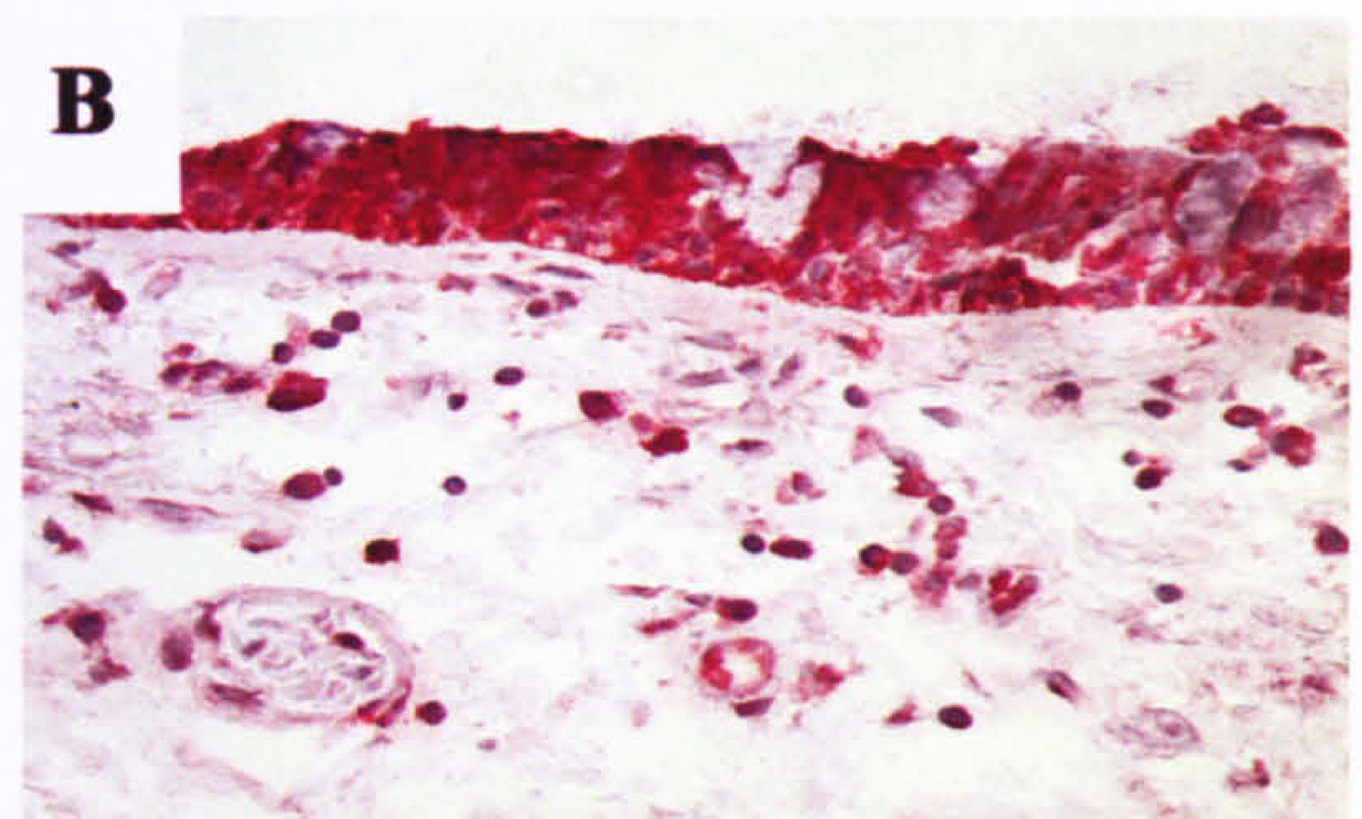
Figure 5.15: The expression patterns of Type II receptors in the normal airway epithelium compared to the asthmatic airway

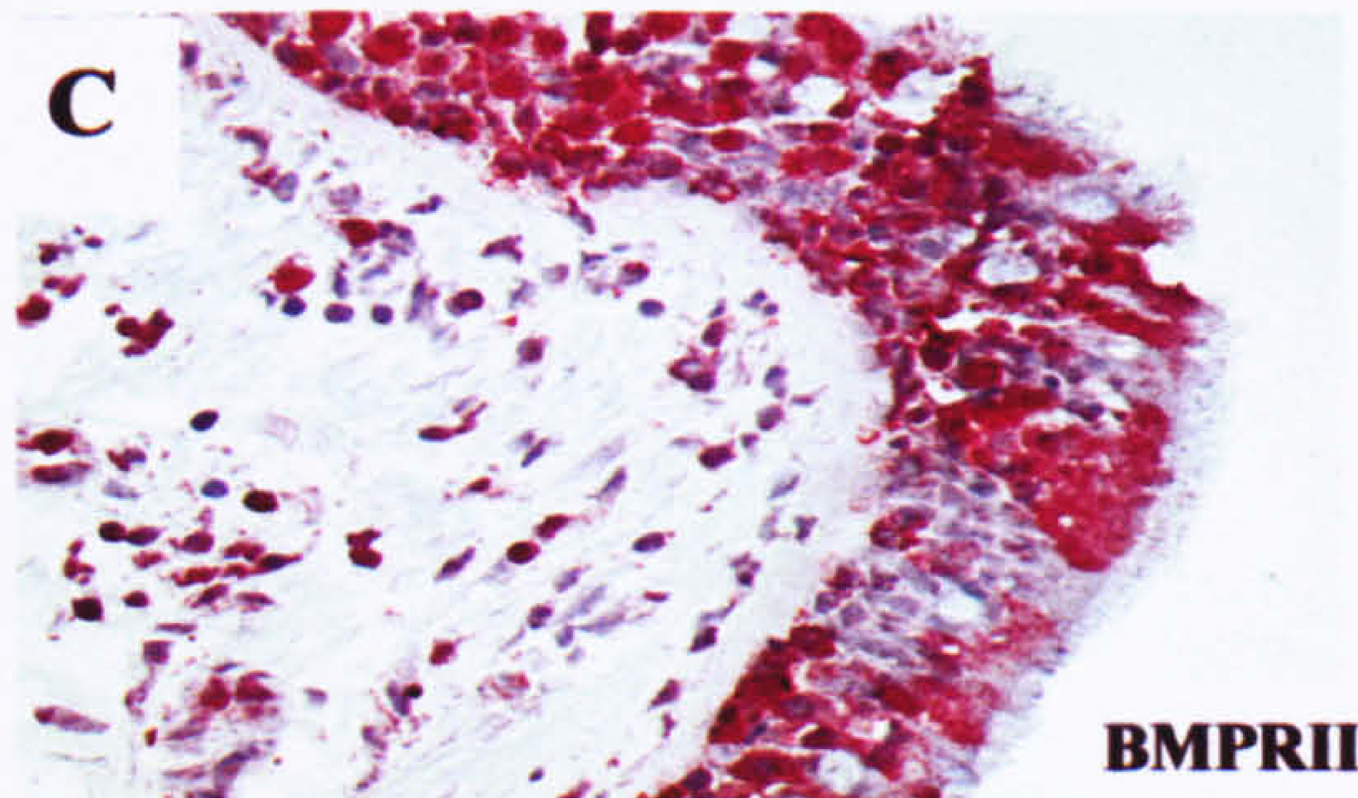
The number of cells expressing each Type II receptor is expressed as a percentage of the total number of epithelial cells present. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

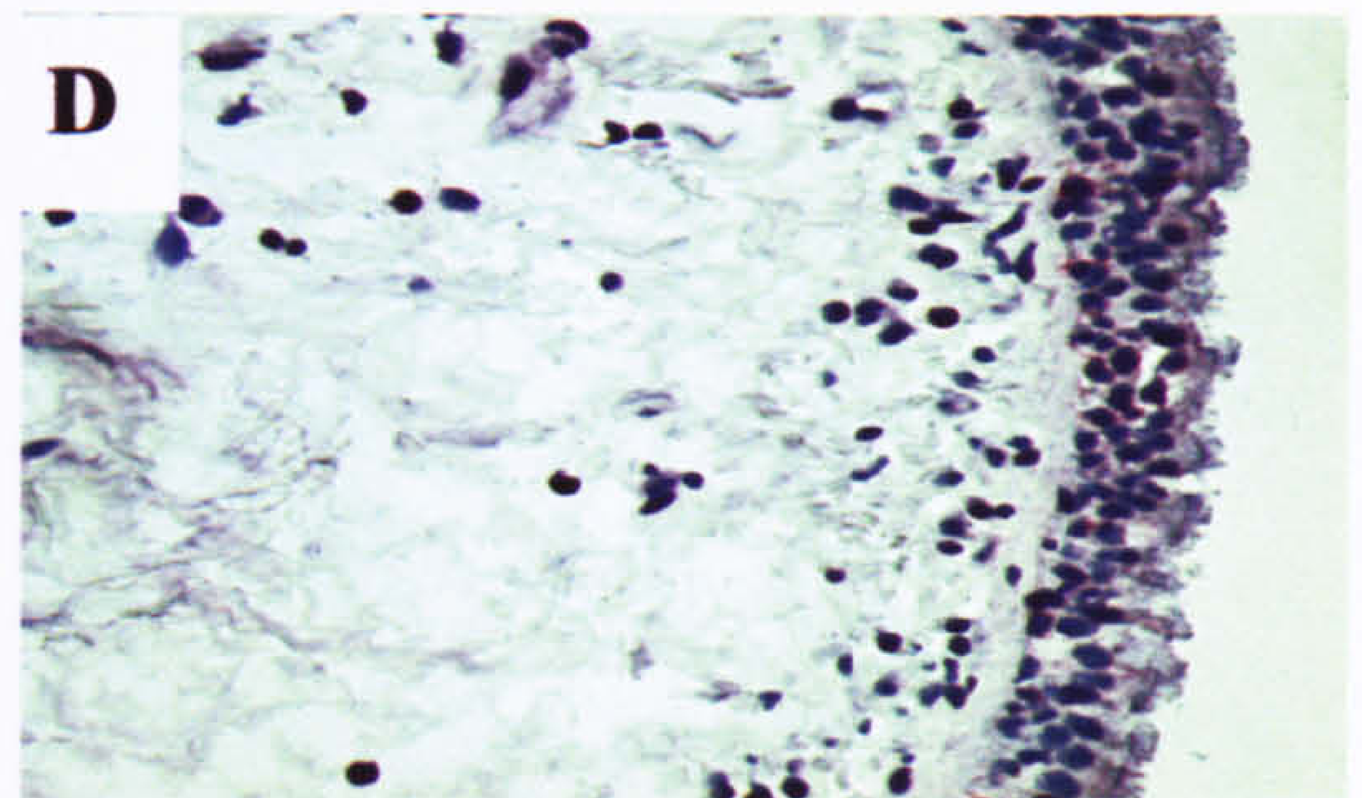
Type II receptors

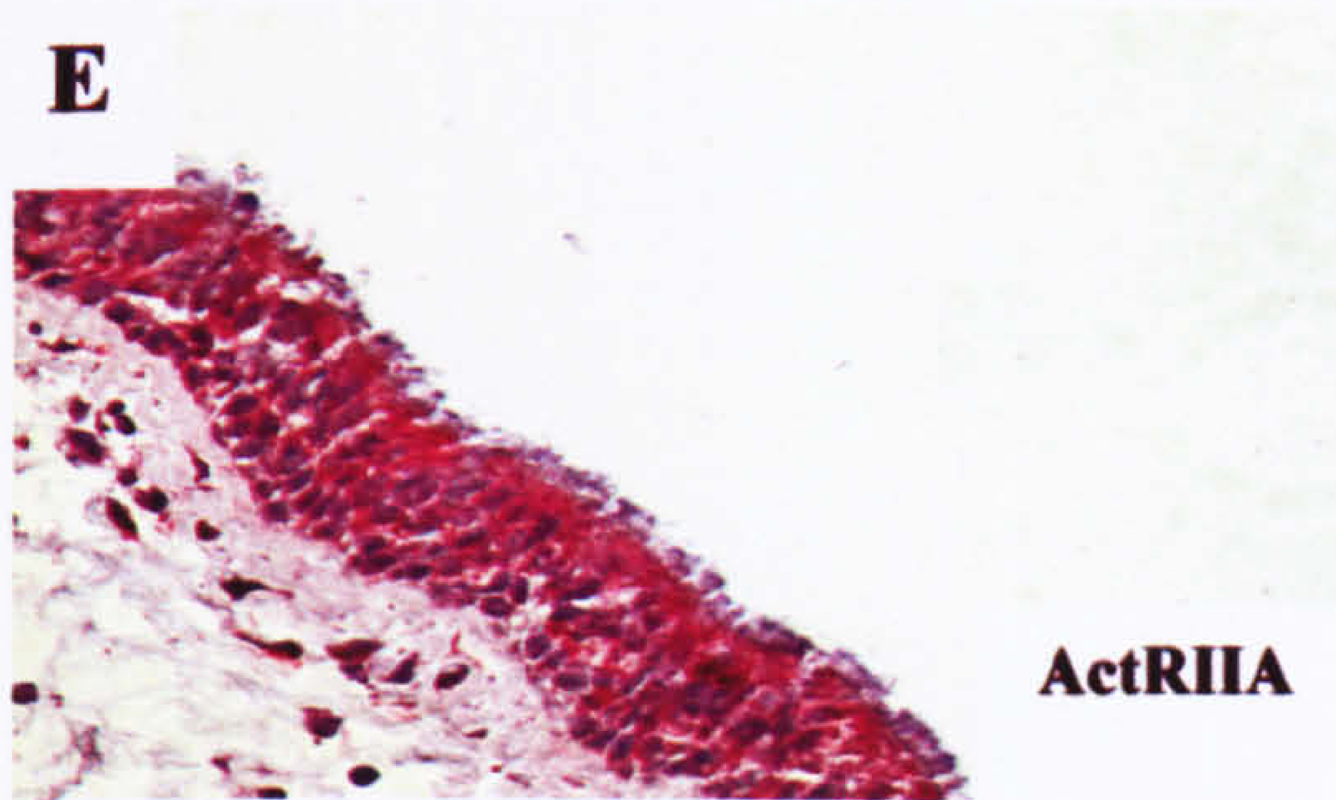
Normal **Asthma**

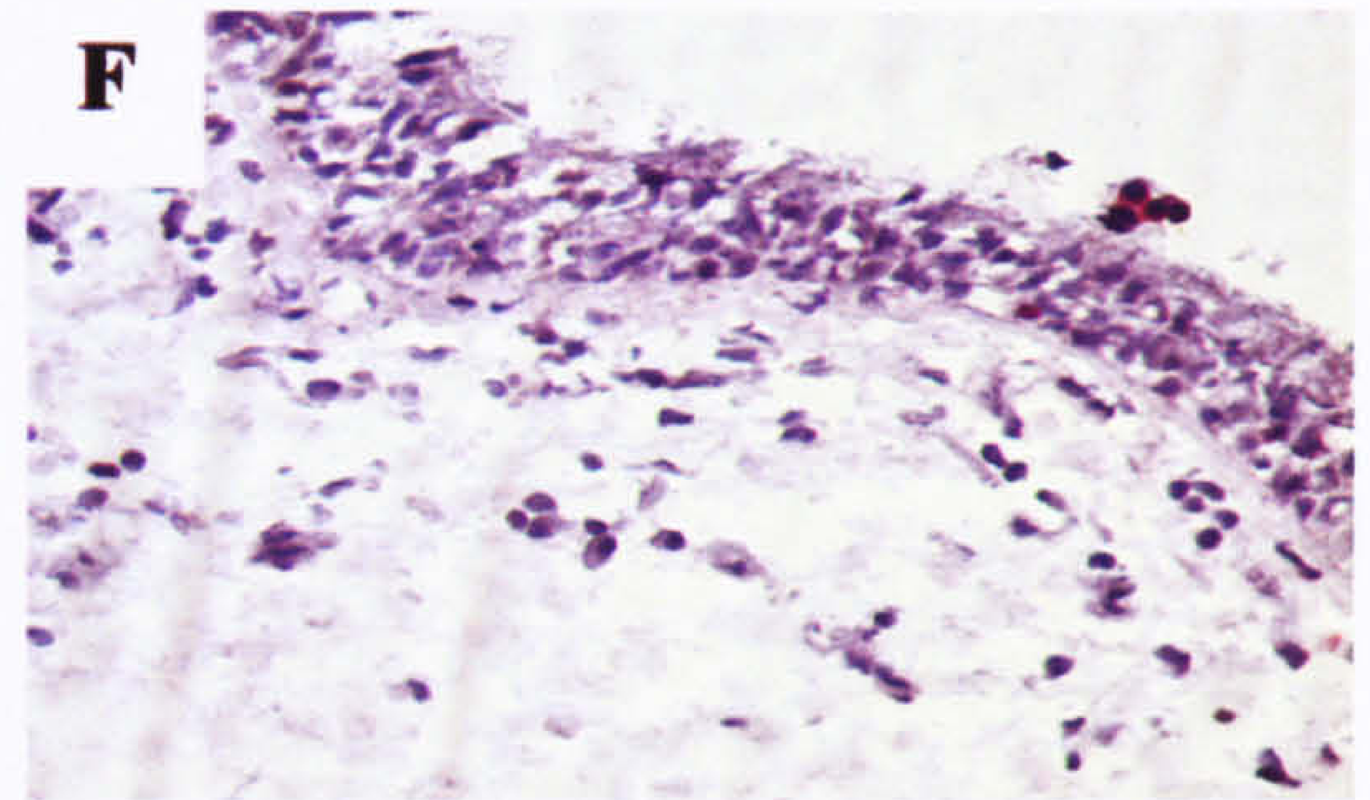


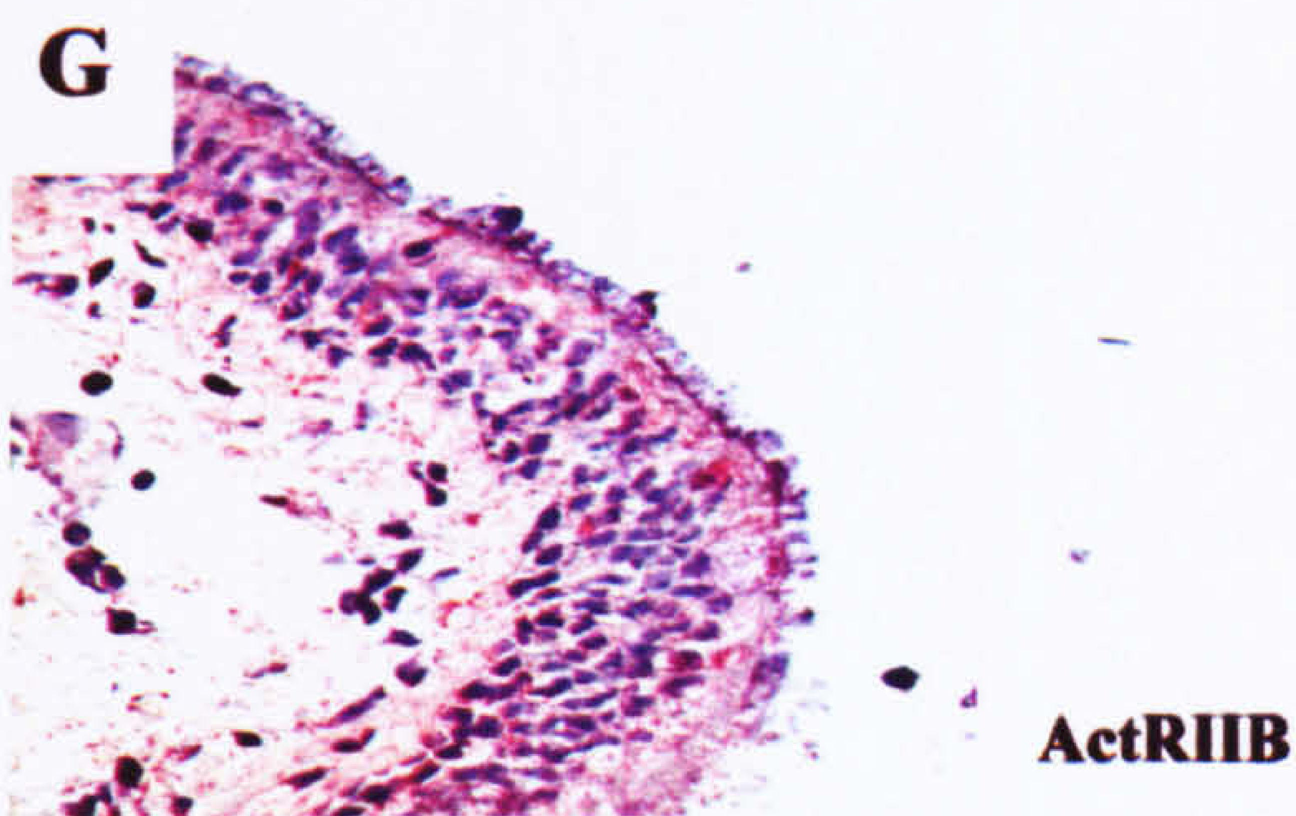












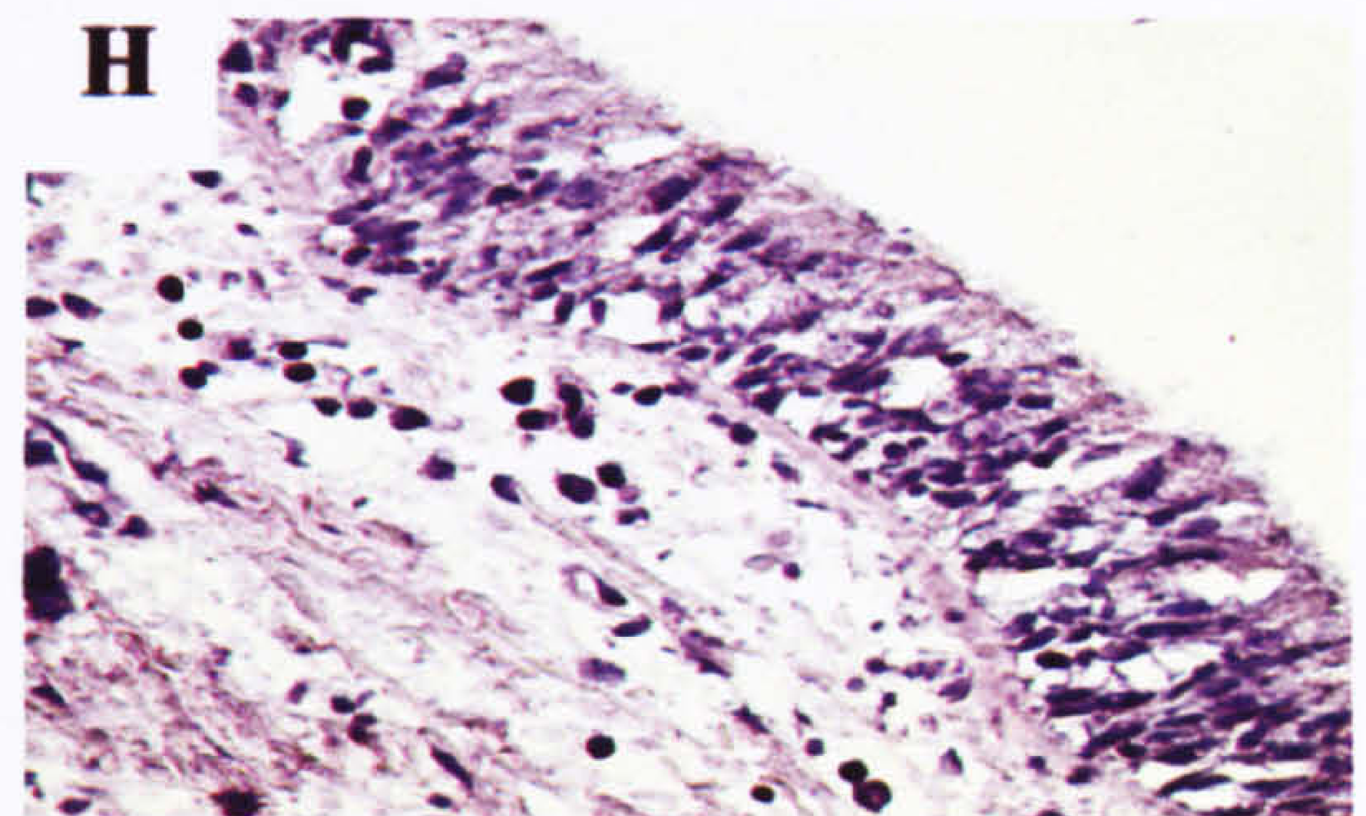


Figure 5.16: Representative photomicrographs of Type II receptor expression

Normal airway expression is presented in the left panel and asthmatic airway expression on the right. The expression of the TGF-β_{1-3} Type II receptor TβRII in the normal airway (A) was of similar intensity and distribution to that in asthma. In contrast the expression of the BMP Type II receptor BMPRII in the normal airway (C) was markedly increased compared to that in the asthmatic airway (D).The expression of ActRIIA and ActRIIB (Type II receptors for activins but also BMPs) in the normal airway (E and G respectively) is strongly immunoreactive compared to the asthmatic airway (F and H respectively).

Post-allergen challenge in asthma

On allergen challenge ActRIIA expression was rapidly up-regulated at both the 24 hours and 7 days ($p=0.02$ and $p=0.002$ respectively). There was no modulation of TβRII, ActRIIB or BMPRII expression in response to allergen at either time point in the airway epithelium (Figure 5.17). However, ActRIIA and TβRII expression was modulated in submucosal inflammatory-like cells at 7 days and 24 hours respectively ($p=0.04$ and $p= 0.004$ respectively) (Figure 5.18A-B).

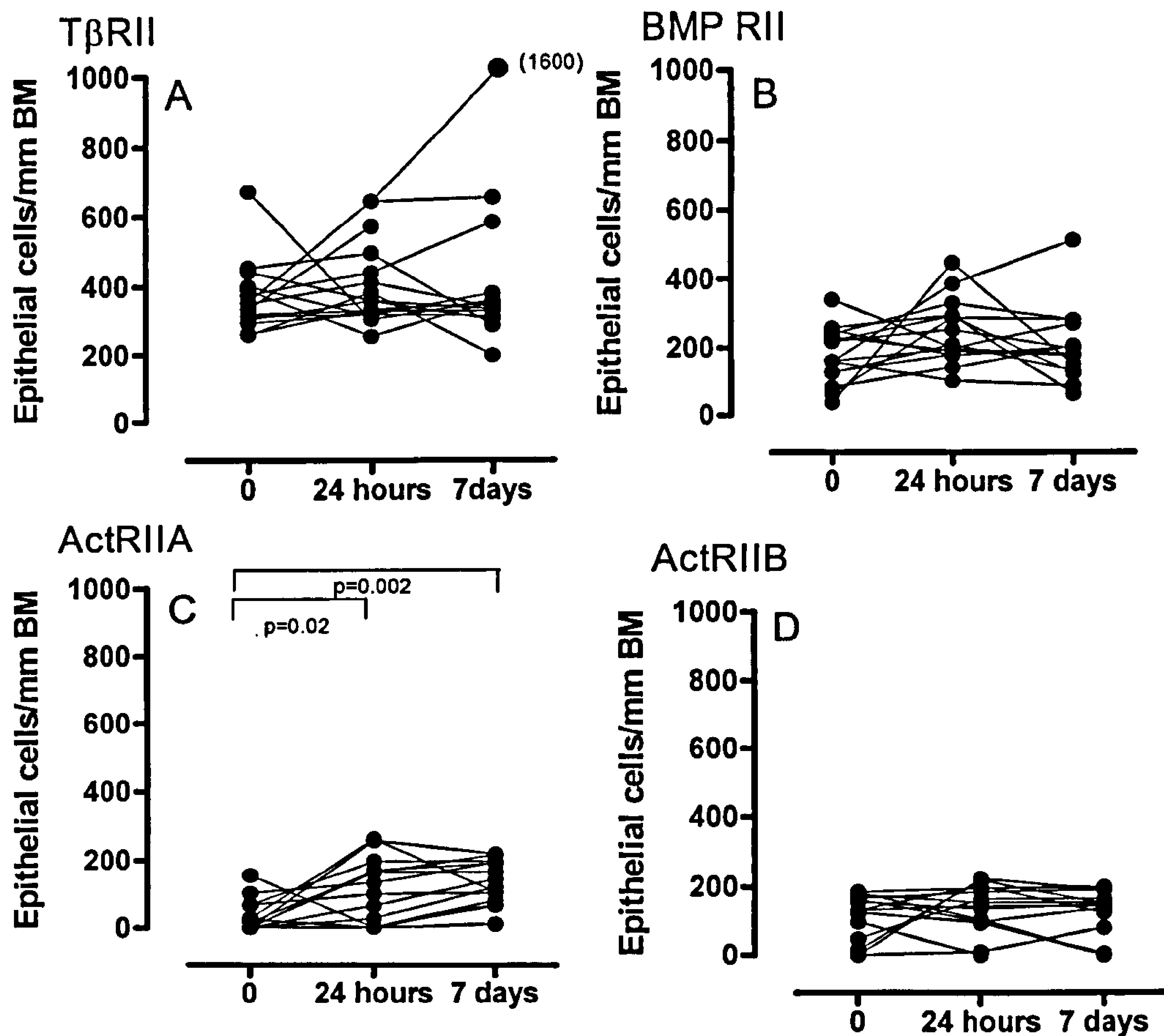


Figure 5.17: The expression kinetics of TGF Superfamily Type II receptors in the asthmatic airway 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/mm BM). Wilcoxon signed rank test was used to compare the change from baseline at the 2 subsequent time points. Predominant expression was confined to the airway epithelium. Significant expression of Act RIIA expression was seen at both 24 hours (p=0.02) and 7 days (p=0.002) after allergen challenge. No modulation of TβRII, BMPRII or ActRIIB was seen, consistent with concept that the Type II receptors may be constitutively expressed in tissues.

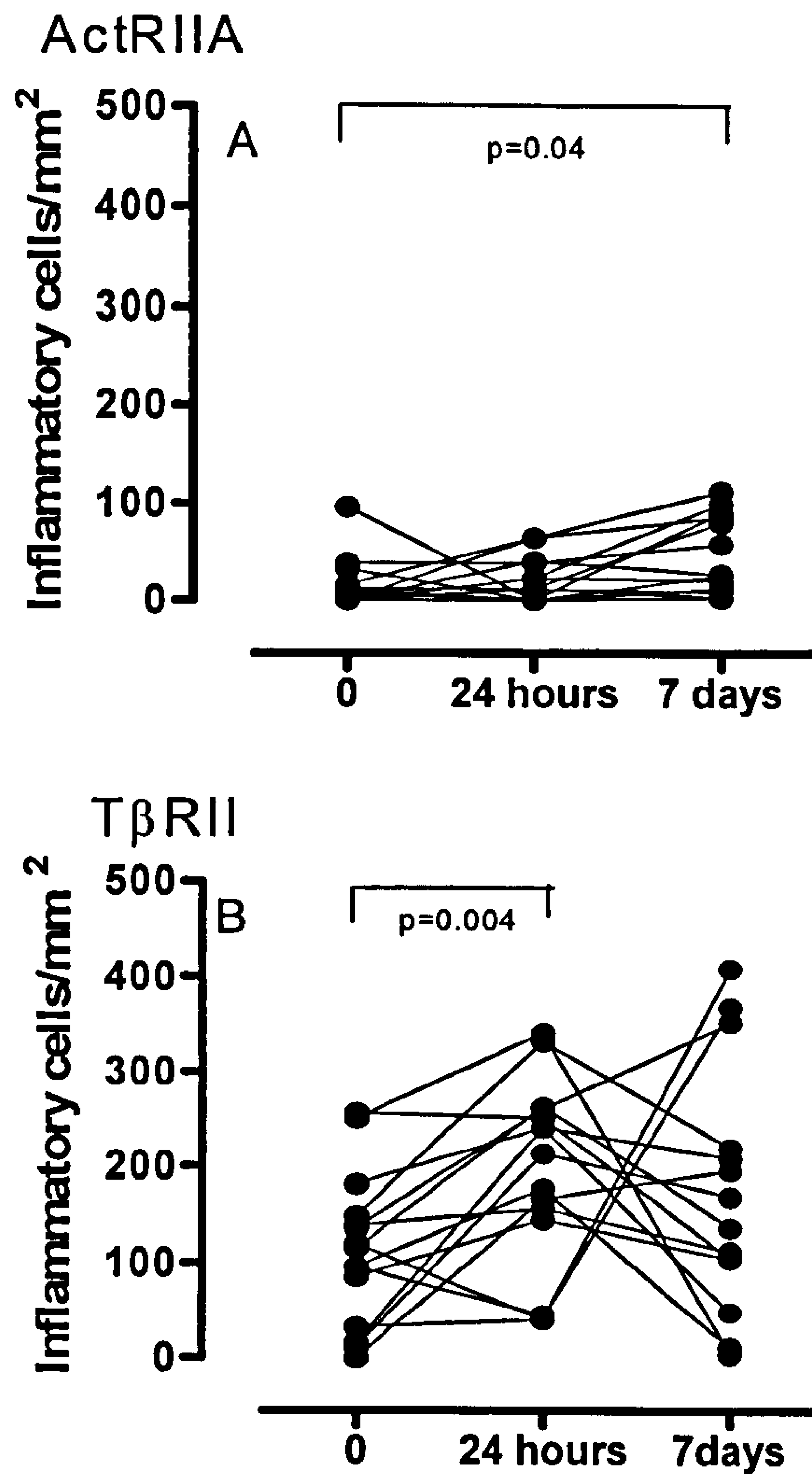


Figure 5.18: The expression kinetics of the Type II receptors ActRIIA and TβRIIA on submucosal inflammatory-like cells in asthma

Positive inflammatory-like cells present are expressed as cells/mm^2. The Wilcoxon signed rank test was used to compare the change from baseline at the 2 subsequent time points. $p<0.05$ was taken as significant.

5.3.3 Discussion

TβRII is expressed at a similar level and intensity in both the normal and asthmatic airway epithelium and this is consistent with the homeostatic role of TGF-β_{1-3} in airway biology. The ubiquitous expression of TβRII on epithelium, inflammatory cells, fibroblasts, airway and vascular smooth muscle in both the normal airway and asthma is keeping with the view that TβRII is constitutively expressed. Expression was not modulated with disease activation. These findings are consistent with other studies (Rosendahl *et al.* 2001; Khalil *et al.* 2002)(Balzar *et al.* 2005). In contrast the expression of BMPRII levels did not reach the level of expression seen in normal volunteers at any stage and this may have functional consequences. In diseases such as primary pulmonary hypertension, where there is defective or absent BMPRII mediated BMP signalling in endothelium, excessive smooth muscle cell proliferation is found as a result of the dominance of other non-TGF-β cellular signalling pathways. BMP signalling can also occur through the Type II activin receptors ActRIIA or ActRIIB. This may provide a partial explanation for the observation of significant up-regulation of ActRIIA in that BMP signalling may be through this receptor in asthma. There was almost no expression of ActRIIA at baseline but rapid up-regulation at 24 hours that was sustained a week later. ActRIIA was the only Type II receptor to be modulated with disease activation. The functional consequences of such altered signalling remains unknown but will be important to determine.

5.3.4 Summary of Type II receptor expression

Epithelial TβRII expression is similar in both the normal and asthmatic airway. This suggests that TβRII is a constitutive receptor, the expression of which is not changed in the context of an active diease setting. Expression of epithelial BMPRII, ActRIIA and ActRIIB is significantly less in the asthmatic airway. Allergen challenge leads to significant up-regulation of only ActRIIA at both the 24 hour and 7 day time point.

Allergen challenge was associated with significantly increased numbers of submucosal inflammatory–like cells expressing TβRII (24 hours) and ActRIIA (7 days).

5.4 Section C: Type I receptor expression

5.4.1 Introduction

TGF-β Superfamilly Type I receptor expression in the normal and baseline asthmatic airway is evaluated. Modulation of receptor expression in response to allergen-challenge in the asthmatic airway is then presented. The data is presented in the order of ALK-5 (TGF-β_{1-3} signalling), ALK-4 (activin-A signalling), ALK-1 (which binds TGF-β_1 but leads to activation of BMP regulated genes) and finally the BMP activated Type I receptors (ALK-2, ALK-3 and ALK-6).

5.4.2 Results

Normal versus Asthmatic airway

ALK-5 expression in the asthmatic airway was significantly decreased compared to normal volunteers (p=0.004) (Figure 5.19A) with predominant expression confined to the epithelium (Figure 5.23A-B). Scattered infiltrating inflammatory cells staining positive for ALK-5 were identified in low numbers only and not in all volunteers. Expression was not demonstrated in either fibroblast-like cells or airway smooth muscle.

Expression of ALK-4 was present in similar distribution in the airway epithelium with no difference between normal and asthmatics volunteers (Figure 5.19B). Staining intensity was marked in both the normal and asthmatic airway (Figure 5.24A-B). Significantly increased numbers of inflammatory cells expressing ALK-4 were present in the normal airway compared to the asthmatic group (p=0.001) (Figure 5.20A) suggesting that in inflammatory cells alone ALK-4 is down-regulated in asthma. Both groups demonstrated low numbers of fibroblast-like cells expressing ALK-4 (median 5.35 cells/mm^2 (interquartile range 0-88) in normals and 3.2 cells/mm^2 (0-25.6) in the asthmatic group).

ALK-1 is activated by TGF-β_1 but leads to activation of BMP regulated genes. ALK-1 expression was significantly down-regulated in the asthmatic airway compared to the normal volunteers in both epithelium (p=0.007) and infiltrating inflammatory cells (p=0.003) (Figure 5.19C and Figure 5.20B). Expression was also evident in the scattered fibroblast-like cells in the submucosa, vascular endothelium and smooth muscle (Figure 5.25A and 5.25C).

Compared to normal volunteers the expression of ALK-2 was markedly reduced in asthmatic airway epithelium (p=0.001) (Figure 5.19D and Figure 5.26A and 5.26B). ALK-2 expression was predominantly localised to the airway epithelium with only a few submucosal inflammatory-like cells staining positive in selected volunteers (Figure 5.26A).

ALK-3 expression was predominantly in the epithelium in both normal and asthmatic airways but was also observed in inflammatory cells, fibroblast-like cells and also airway smooth muscle (Figure 5.19E and Figure 5.27A and 5.27B). However, in the asthmatic airway the number of inflammatory cells expressing ALK-3 was significantly higher (p=0.03) (Figure 5.20C).

ALK-6 expression was confined to the airway epithelium. Expression was markedly decreased in asthma when compared to the normal airway (p=0.0009) (Figure 5.19F and Figure 5.28A and 5.28B)

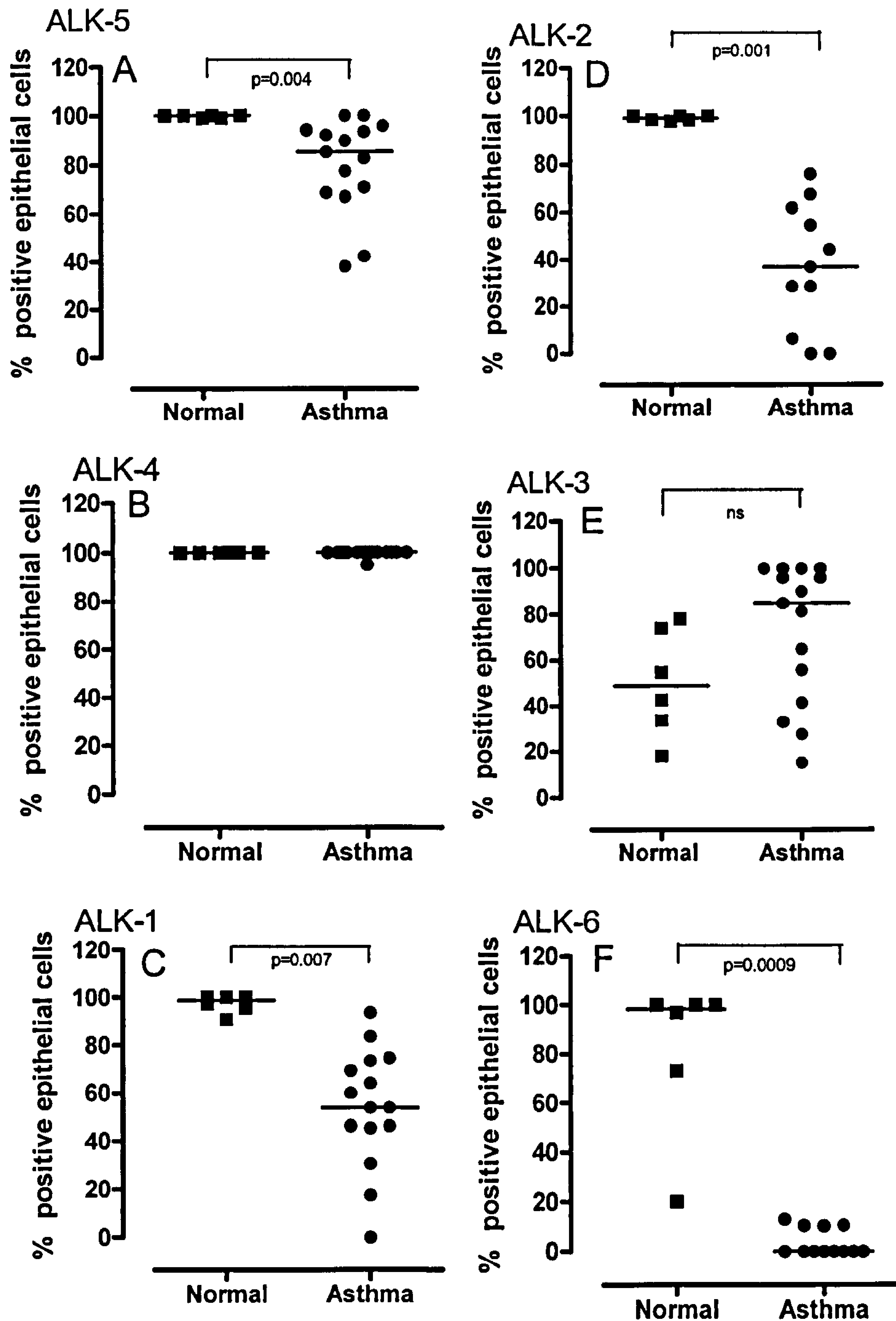


Figure 5.19: The expression patterns of Type I Receptors in the normal airway epithelium compared to asthma

The number of cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

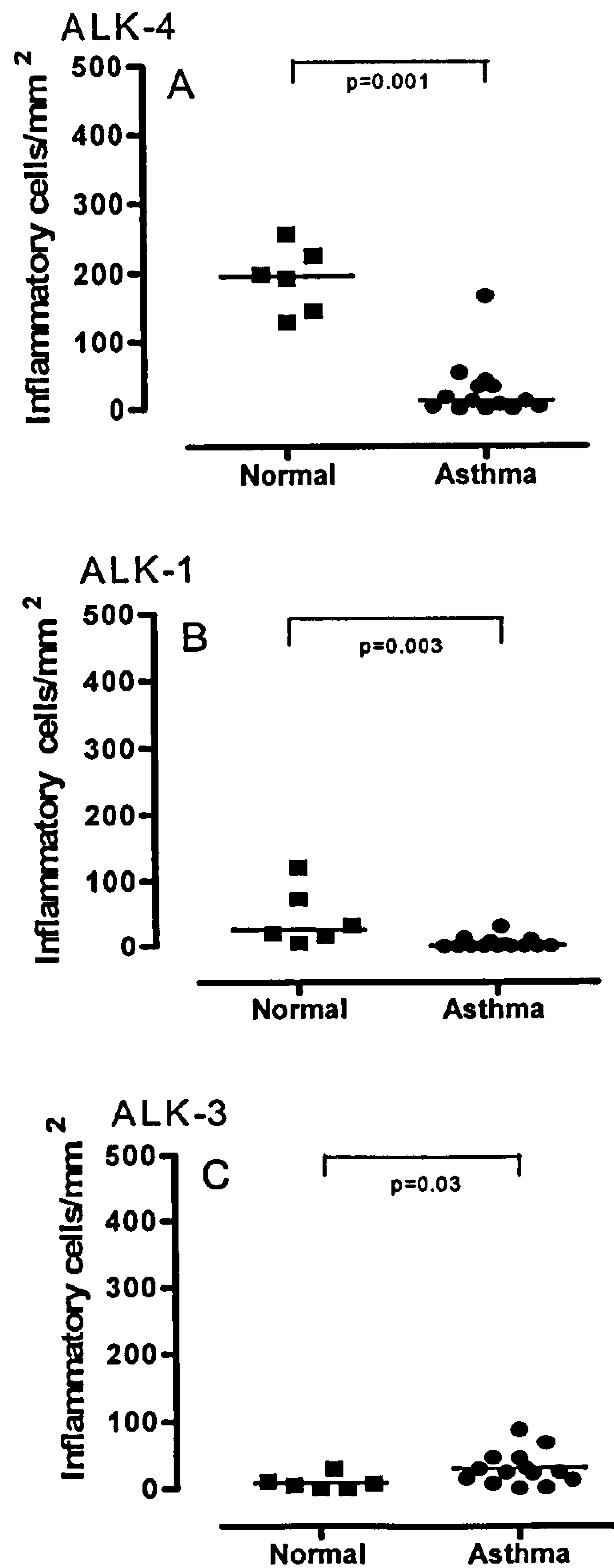


Figure 5.20: The expression patterns of Type I Receptors on inflammatory-like cells in the normal airway mucosa compared to asthma

The results are expressed as the number of positive cells/per mm^2. Significant differences between time points were analysed by the Mann Whitney U test. $p<0.05$ was taken as significant.

Post-allergen challenge in asthma

On allergen challenge there was marked down-regulation of ALK-5 epithelial expression at the 24-hour time point after allergen in the asthmatic volunteers (p=0.02) (Figure 5.21A and Figure 5.23C), returning to baseline levels at 7 days.

ALK-4 epithelial expression remained elevated in response to allergen challenge with no modulation of expression (Figure 5.21B and Figure 5.24) There was a small but noticeable upward trend in the number of the fibroblast-like cells staining for ALK-4 in response to allergen challenge with 3.2 (0-25.6) cells/mm^2 at baseline, 11.40 (4-88) cells/mm^2 at 24 hours and 19.5 (4-61.35) cells/mm^2 at 7 days (Figure 5.24C). Confirmation that ALK-4 expression was in fibroblasts transformed into the myofibroblast phenotype was evidenced by double immunofluorescence staining (Figure 5.24D-F).

With ALK-1, allergen challenge was associated with a trend towards increased expression at 24 hours with (p=0.23) with further significant increases in expression at 7 days with (p=0.04) (Figure 5.21C and Figure 5.25).

In terms of BMP signalling, allergen challenge was associated with marked and sustained increases in the expression of ALK-2 at both 24 hours (p=0.0006) and 7 days (p=0.001) (Figure 5.21D and Figure 5.26). Similarly the expression of ALK-6 was markedly up-regulated in response to allergen at 24 hours (p=0.004) with further increases at the 7 day time point (p=0.001) (Figure 5.21F and Figure 5.28). In contrast the level of ALK-3 expression was not modulated in response to allergen (Figure 5.21E and Figure 5.27). Inflammtory-like cells expressing ALK-4, ALK-3 and ALK-1 only were found to be significantly increased post allergen (Figure 5.22).

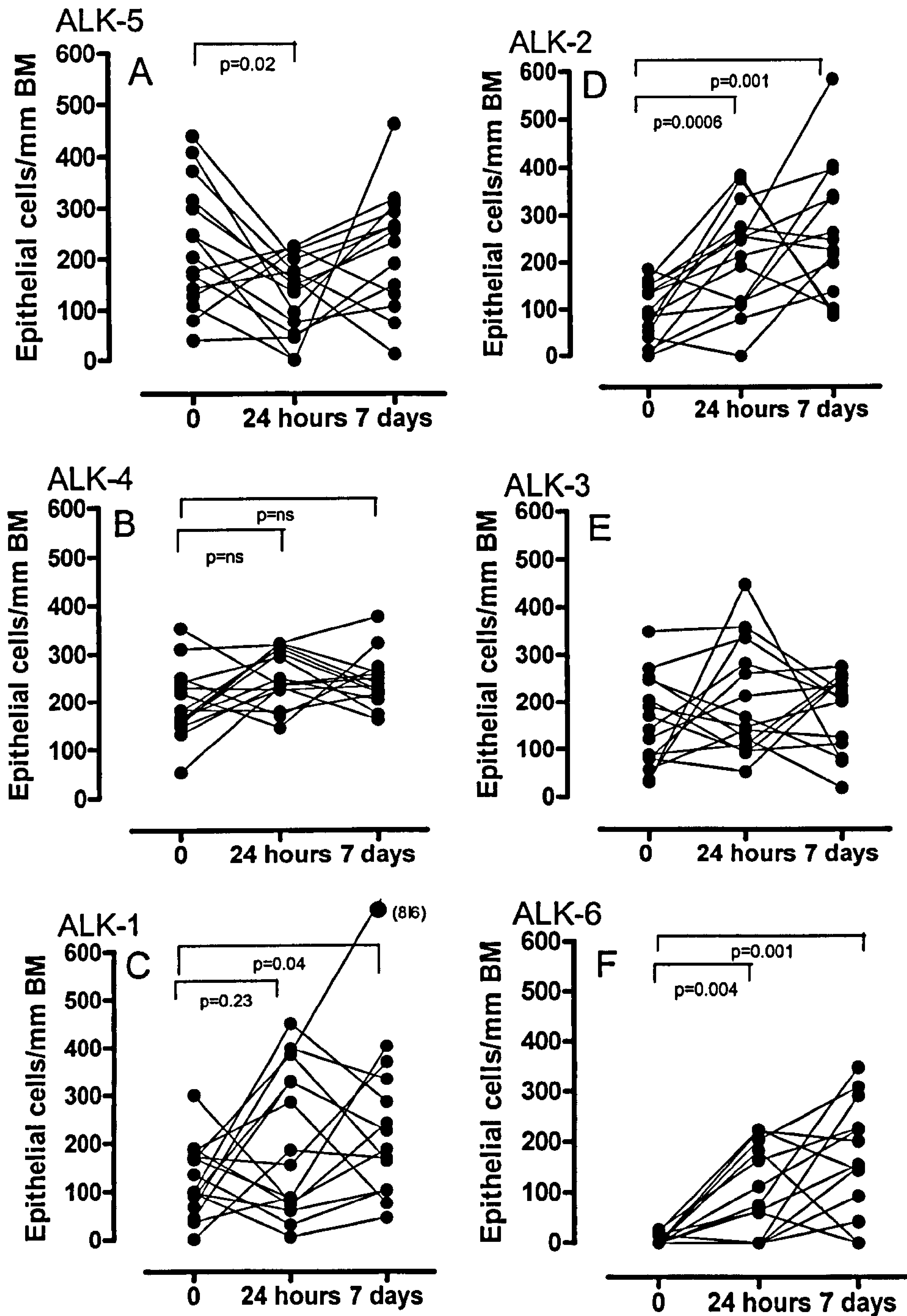


Figure 5.21: The expression kinetics of the TGF-β Superfamily Type I receptors in the asthmatic airway 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/ mm BM). Wilcoxon signed rank test was used to compare the change from baseline at the 2 subsequent time points. No modulation of either ALK-4 or ALK-3 expression was seen in response to allergen challenge. In contrast there was rapid down-regulation of ALK-5 expression whilst the Type I receptors that activate Smad1, Smad5 and Smad8 (ALK-1, ALK-2 and ALK-6) were rapidly up-regulated with sustained expression still at 7 days post allergen.

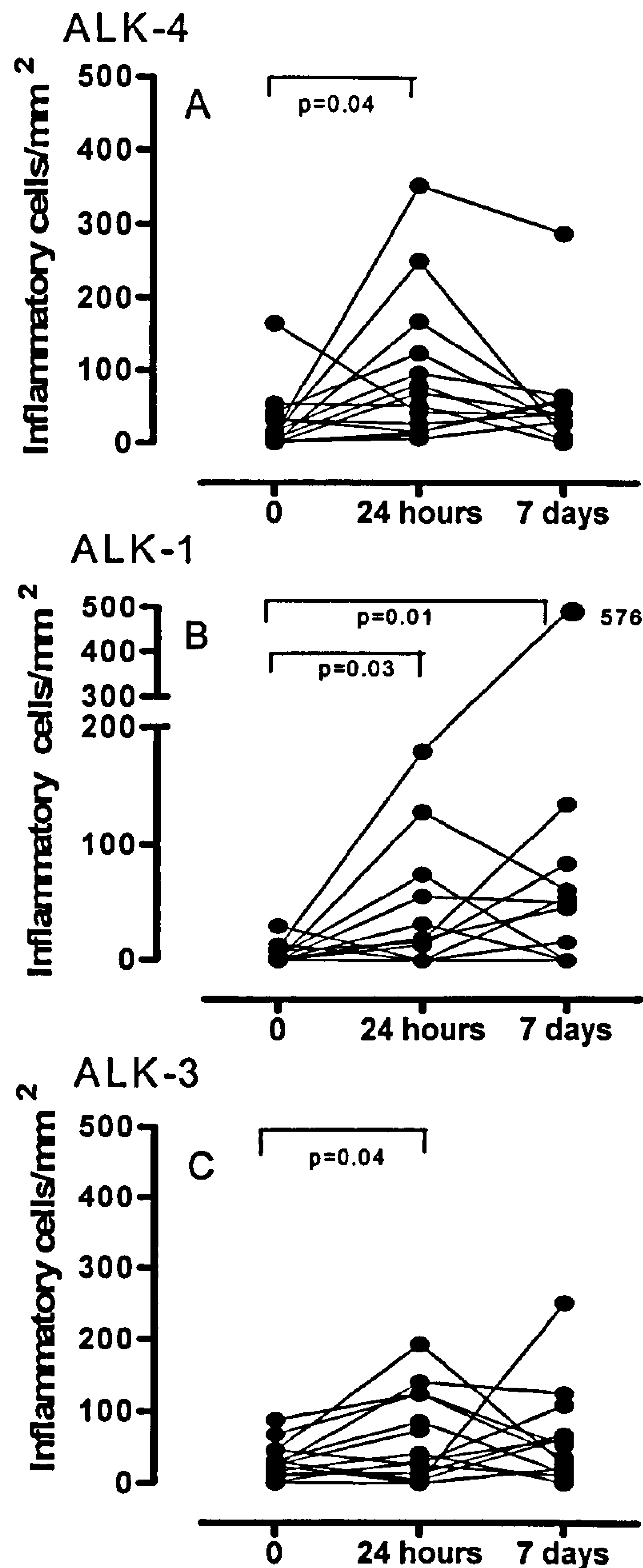


Figure 5.22: The expression kinetics of Type I receptors on inflammatory-like cells 24 hours and 7 days post allergen challenge

Positive inflammatory-like cells present are expressed as cells/mm^2. $p<0.05$ was taken as significant. The data is consistent with activin, TGF-β_{1-3} and BMP ligand signalling in inflammatory cells. No ALK-5 staining (the predominant Type I receptor for TGF-β_{1-3}) was evident which may be a result of down–regulation of ALK-5 on inflammatory cells as seen with the airway epithelium.

ALK-5

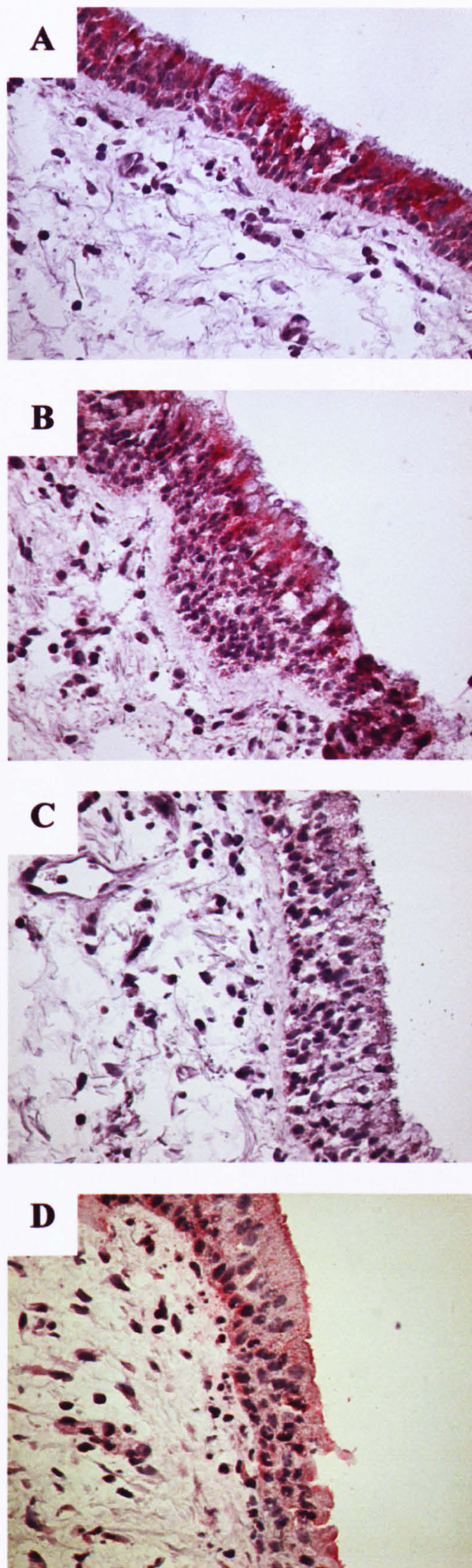


Figure 5.23: Representative photomicrographs of ALK-5 immunoreactivity

Normal volunteers demonstrate marked expression in epithelium and cells below the basement membrane (A). Asthmatics demonstrate down-regulation of ALK-5 expression at baseline (B) with further down-regulation 24 hours post-allergen (C). Expression levels return to baseline levels 7 days post-allergen (D).

ALK-4

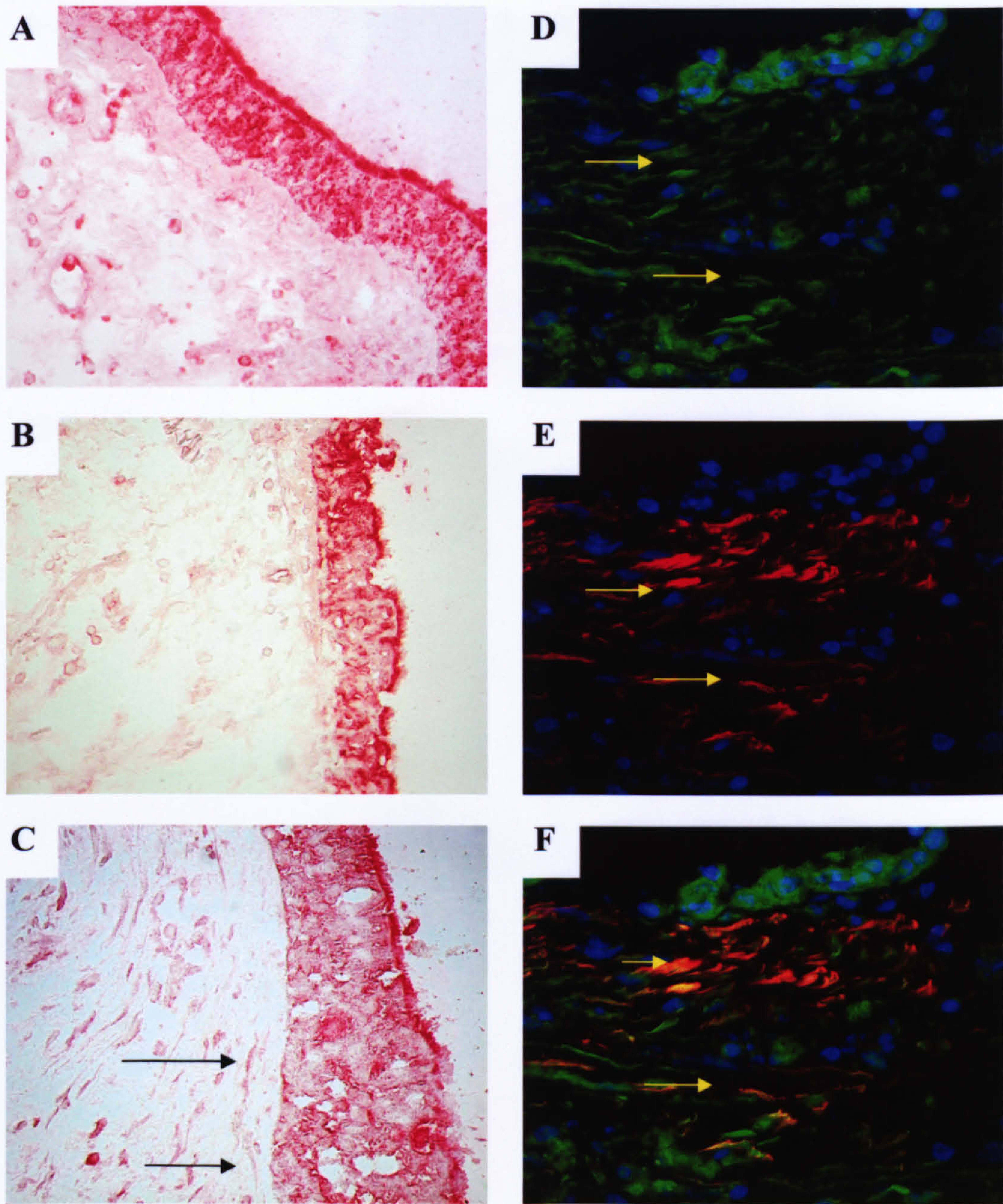


Figure 5.24: Representative photomicrographs of ALK-4 immunoreactivity

The expression of ALK-4 was similar in intensity and distribution both in the normal (A) and asthmatic (B) airway. Although no significant modulation of ALK-4 expression in epithelium was seen post-allergen, increased ALK-4 immunoreactivity in fibroblast-like cells was evident at Day 7 post-allergen (C) (arrowed). Confirmation of myofibroblast phenotype was confirmed using confocal microcrocopy. ALK-4 expression (D) is shown as green using a FITC-labelled secondary antibody and α-smooth muscle actin (SMA) identified as red using an anti-α-SMA-Cy3 primary antibody. The merged confocal image demonstrates fibroblasts staining yellow-orange indicating double positive cells (F).

ALK-1

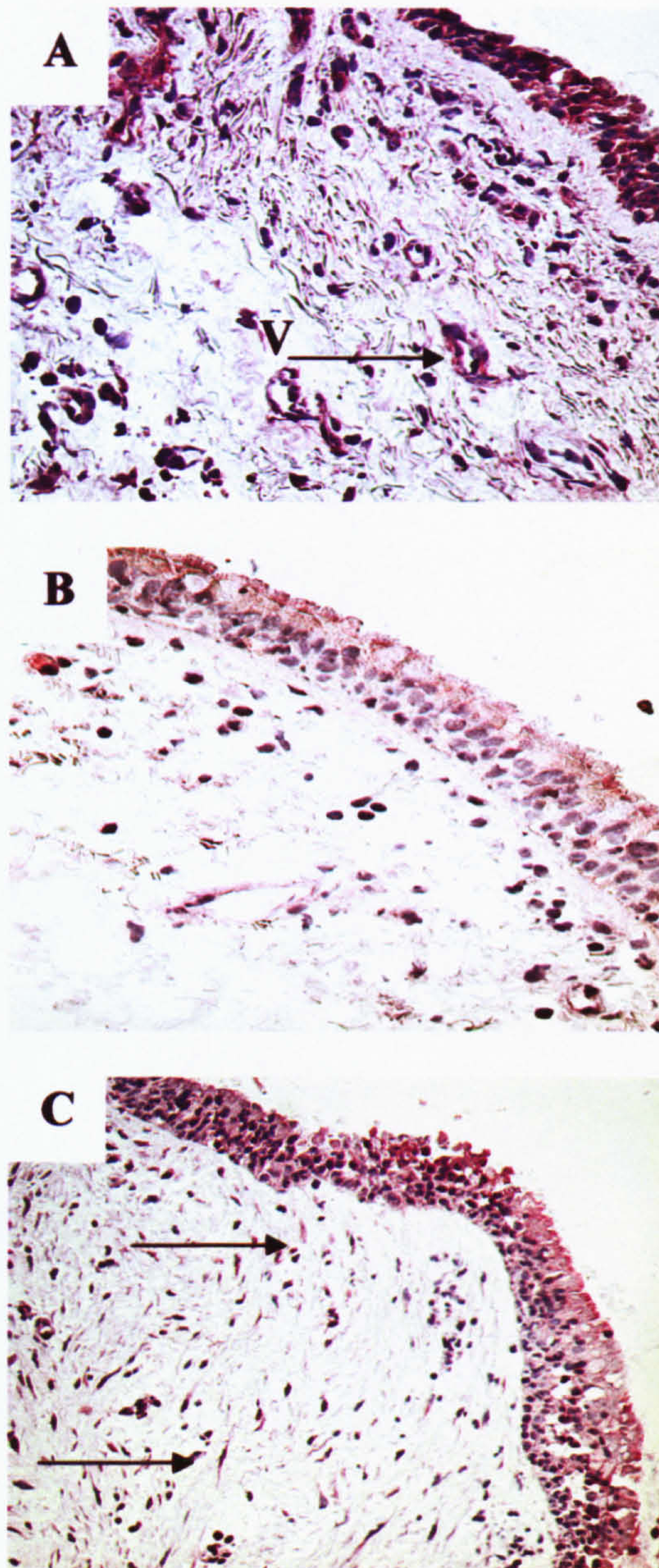


Figure 5.25: Representative photomicrographs of ALK-1 immunoreactivity

Normal volunteers demonstrate strong ALK-1 expression in epithelium and vascular structures (arrowed as **V**) (A). Asthmatics demonstrate weaker expression at baseline (B) but increased expression is demonstrated 7 days post-allergen (C) in epithelium as well as submucosal inflammatory-like and fibroblast-like cells (arrowed).

ALK-2

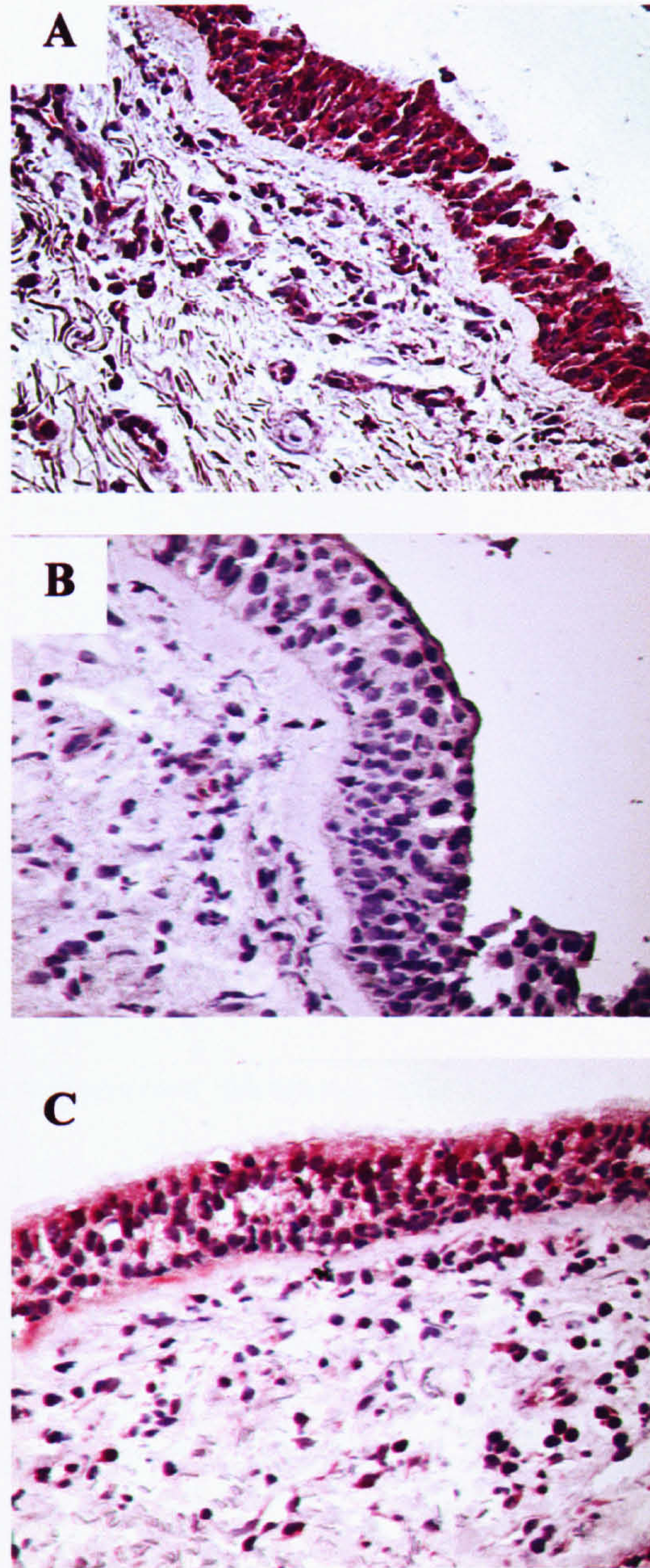


Figure 5.26: Representative photomicrographs of ALK-2 immunoreactivity

Normal volunteers demonstrate marked ALK-2 expression in epithelium, as well in some inflammatory-like cells below the basement membrane (A). Asthmatics demonstrate minimal ALK-2 expression at baseline (B) but marked up-regulation of expression is in the epithelium and infiltrating inflammatory-like as demonstrated 7 days post-allergen (C).

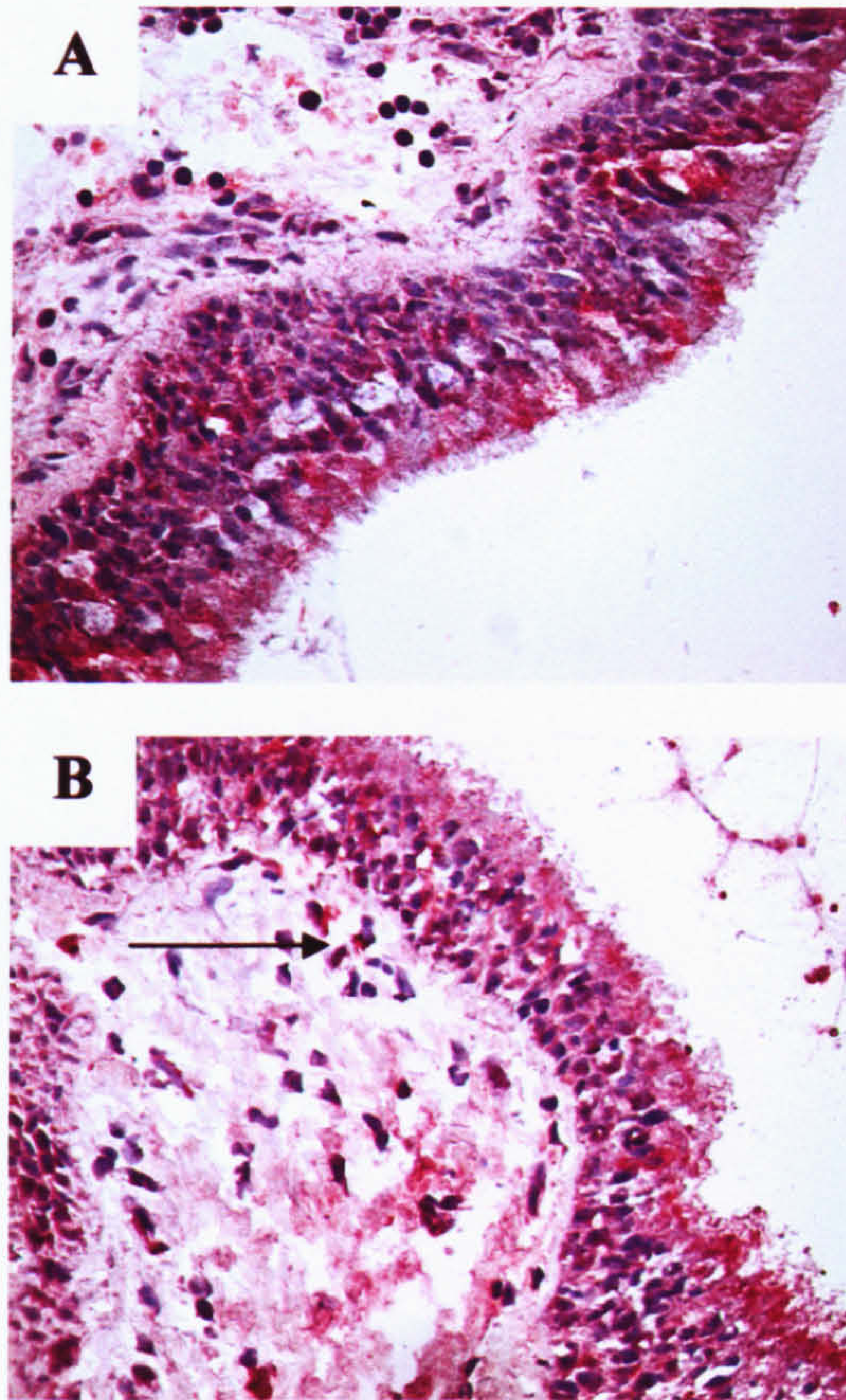


Figure 5.27: Representative photomicrographs of ALK-3 immunoreactivity in the airway

No difference in ALK-3 expression was detected between the normal (A) and asthmatic airway (B), with strong expression in both the airway epithelium and vascular structures demonstrated. Inflammatory-like cells demonstrated ALK-3 expression (arrowed) in the asthmatic airway consistent with BMP signalling in these cells.

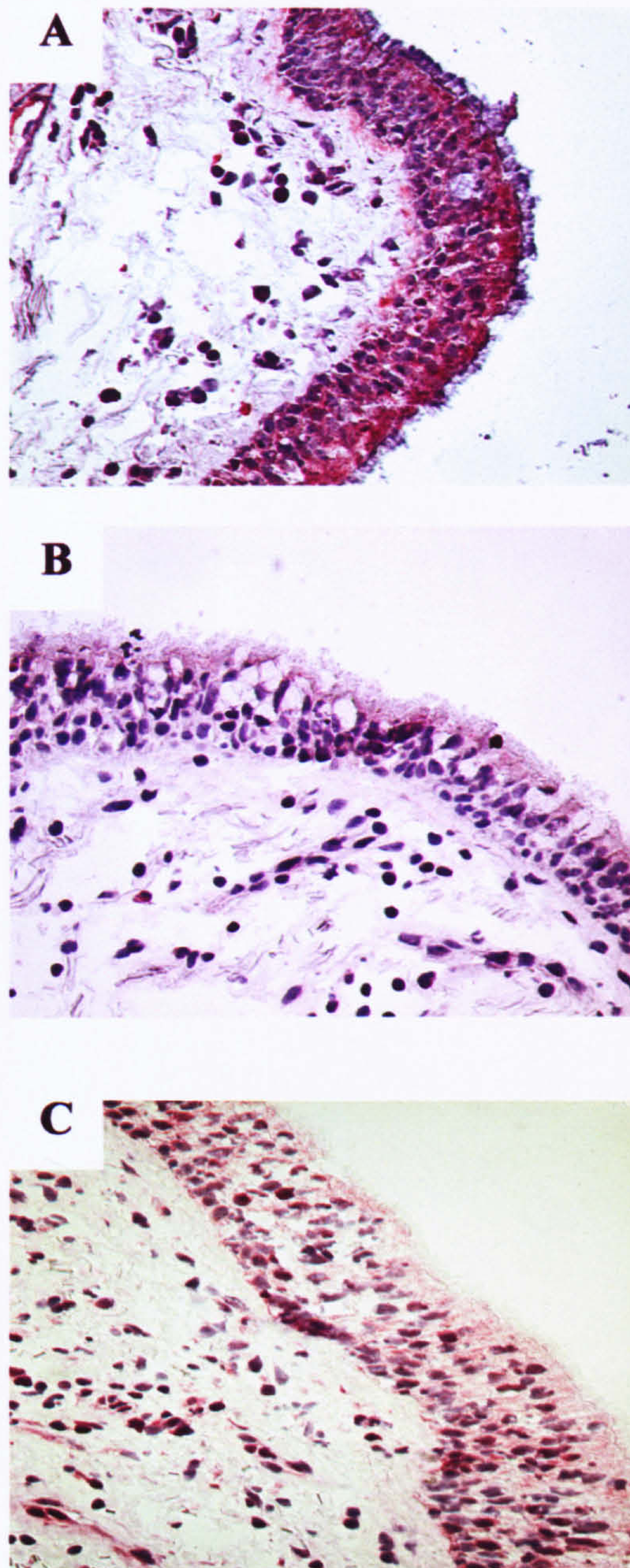


Figure 5.28: Representative photomicrographs of ALK-6 immunoreactivity

Normal volunteers demonstrate ALK-6 expression in epithelium and inflammatory cells (A). Asthmatics demonstrate no visible ALK-6 expression at baseline (B). Allergen challenge is associated with increased expression as demonstrated at the 7 day time point although expression intensity still remains less than that in normal volunteers.

5.4.3 Discussion

Modulation of receptor expression is an important mechanism in the regulation of the TGF-β Superfamily signalling pathway and changes in expression of receptors in a rapid fashion in response to activation of signalling would be expected. By confirming the modulation of Type I and Type II receptors in response to allergen challenge, it is possible to begin to provide a mechanistic explanation for our visualisation of active TGF signalling. Our data confirms that TGF-β Superfamily receptor distribution on the cell is very flexible and dynamic in response to activation of signalling.

TGF-β receptors are complex structures that are regulated at a number of different levels. It is the Type I receptor that directly activates the canonical Smad signalling pathway and it is therefore expected that Type I receptor modulation will serve as a point of pathway regulation. One of the interesting findings in this study is the marked difference in the levels of receptor expression in normal and asthmatic volunteers. Other than for ALK-3 and ALK-4, overall there is a marked down-regulation of Type I receptor expression in the asthmatic airway compared to normal airways. The down-regulation of the TGF-β Type I receptor ALK-5 in asthma compared to the normal airway has been previously detected (Balzar *et al.* 2005) but this is the first time it has been shown for ALK-1, ALK-2 and ALK-6 in airway epithelium alongside ALK-1 and ALK-4 in inflammatory-like cells. This suggests that there is a fundamental difference in the regulation of TGF-β signalling pathways in the asthmatic airway.

Several mechanisms may explain these observations. It is possible such TGF-β Superfamily signalling imbalance is a fundamental property of the asthmatic airway and reflects an intrinsic defect in the way an asthmatic airway can respond to injury. Asthma is the most common co-morbidity associated with inflammatory bowel disease (IBD) (Bernstein *et al.* 2005). The prevalence of asthma is in the range of 7.1-7.8 % in the IBD cohort of over 8000 patients, a prevalence that is higher than any of the other classic extraintestinal manifestations of IBD (Bernstein *et al.* 2005). Given the common embryological origins of the bronchial tree and gut, and the demonstration of dysregulated TGF-β signalling in IBD also, it is tempting to speculate that aberrant TGF-β signalling may be an intrinsic defect of the tissue itself.

It may also be that such receptor modulation is a reflection of ligand-induced down-regulation that occurs as a receptor regulatory response, as has been observed in other

signalling systems such as the IL-5 receptor on eosinophils (Gregory *et al.* 2003). Signal potency and duration is dependent on the rate of receptor internalisation. If the receptor-ligand dissociation is decelerated then there is enhanced and prolonged signalling. Receptor internalisation and degradation is a process that serves to reduce the number of functional receptors at the plasma membrane and attenuate the strength of the signal generated. Clathrin-dependent internalisation into early endosomes is important for signal propagation whilst entry into the caveolin positive lipid-rafts leads to receptor degradation and is associated with reduced Smad activation (Di Guglielmo *et al.* 2003). Of course it may be possible that the mechanisms of receptor processing are different in the asthmatic airway.

This study did not detect any obvious differences in the levels of ligand expression between the asthmatic and normal airways other than for activin-A and TGF-β_3. Despite the excess in activin-A expression in asthma ALK-4 and pSmad2 epithelial expression was not different between the two groups, indicating ubiquitous activin-A signalling in the airway and may suggest a homeostatic role for the activins in the airway. Consistent levels of ALK-4 expression, particularly so in myofibroblasts, indicates that the activin signalling pathway may remain active and suggests a dominant role for activins in airway inflammation and the repair response in asthma.

The rapid and consistent down-regulation in the expression pattern of ALK-5 at 24 hours after allergen exposure suggests that there may be a regulatory response in place to attenuate the cellular response to TGF-$\beta_{1\text{-}3}$ ligands. Such down-regulation was not observed for other Type I receptors post-allergen. Whilst this study is the first to demonstrate this modulation in response to allergen-induced injury in asthma, this observation has however been shown in a rat model of bleomycin-induced lung fibrosis (Khalil *et al.* 2002). Using both IHC and ISH it was demonstrated that the expression of ALK-5 on Type I alveolar epithelial cells was reduced whereas there was no change in the expression of the Type II receptor TβRII. This observation has also been made in our collaborator's allergen–induced mouse model of airway injury (Rosendahl *et al.* 2001). It may be that ALK-5 down-regulation is a response specific to tissue injury rather than a disease specific phenomenon. Interestingly, ALK-5 expression levels returned to baseline levels 7 days in this study, coinciding with return of pSmad2 signalling to baseline levels. The mechanism of ALK-5 expression down-regulation may be explained by the observations by Ebisawa et al (Ebisawa *et*

al. 2001). Smad7 is able to associate with Smurf 1 in the nucleus. Whilst Smurf 1 allows Smad7 to be exported out into the cytoplasm it through this interaction with Smad7 that Smurf 1 can be recruited to the site of activated ALK-5. Smurf 1 now induces the degradation of ALK-5 together with Smad7. The sensitivity of the cell to the effects of TGF-β_{1-3} is thus altered.

Given that the *in-vitro* effect of TGF-β_1 on epithelium is the inhibition of cellular proliferation, the specific down-expression of ALK-5 in epithelial cells in response to allergen may be an attempt by the airway to regenerate and repair itself by the loss of the TGF-β antiproliferative signal sensitivity. In asthma there is a lack of epithelial repair despite proliferation signals by factors such as EGFR ligands leading to a chronic wound scenario. Interestingly in the bleomycin injury model ALK-5 down expression coincided with a time point known to be associated with the peak of epithelial cell proliferation (Khalil *et al.* 1994) whilst the time point at which a return in expression of ALK-5 to baseline levels was seen coincided with cessation of epithelial proliferation (Khalil *et al.* 1994).

It was not possible to demonstrate a consistent expression of ALK-5 on inflammatory cells which is surprising as these cells are considered to be TGF-β_1 responsive. This observation has, however, been documented in the bleomycin model of lung injury discussed above where the inflammatory cells recruited in response to injury did not immunostain for ALK-5 despite the demonstration via ISH of ALK-5 expression in these cells. Immunostaining for ALK-5 was weak or absent also for fibroblast-like cells in the current study. These findings may be an indication that the expression of ALK-5 is even more down-regulated on inflammatory and mesenchymal cells that the immunohistochemical protocol used was not sensitive enough for the detection of ALK-5 in these cell types.

The functional consequences of TGF-β_1 on immune responses is partly dependent on the state of cellular differentiation and this in turn is related to the degree of cell TGF-β receptor expression, which in large part can determine the functional outcome in response to TGF-β ligands. For example, inactive monocytes express a relatively high proportion of ALK-5 but cell activation was associated with down-regulation of ALK-5 with concomitant loss of functional responses to TGF-β ligand (Brandes *et al.* 1991). Such receptor down-regulation did not occur in neutrophils (Brandes *et al.*

1991). In this *in-vitro* system there was however induction of TNF-α, an important observation given that in more severe asthma TNF-α may be implicated in pathogenesis (Howarth *et al.* 2005).

There was obvious staining for ALK-1 in our fibroblast populations. This identical observation in a mouse model of allergen induced airway injury with weak or absent ALK-5 immunostaining and an increase in ALK-1 is again consistent with findings here (Rosendahl *et al.* 2001). It would be important to understand the functional consequences of such ALK-1 expression on fibroblasts.

BMP Type I receptor ALK-2 and ALK-6 epithelial expression was rapidly increased following allergen challenge, and this increased expression was sustained at the 7 day time point when tissue repair was still evident. Epithelial ALK-3 expression was not modulated in response to allergen provocation. In the mouse model of allergen challenge a 2-fold increase in ALK-2 and a 6-fold increase in ALK-6 protein expression as detected using Western-blotting of whole tissue has been documented with no change in the level of ALK-3 expression. RT-PCR confirmed 5-fold induction of ALK-2 however but the ALK-3 mRNA level remained unchanged (Rosendahl *et al.* 2002). Further evidence that BMP signalling is down-regulated in asthma is suggested by the decreased expression of BMPRII, ActRIIA and ActRIIB at baseline compared to the normal airway.

5.4.4 Summary of Type I receptor expression

Epithelial ALK-5, ALK-1, ALK-2 and ALK-6 expression is significantly less in the asthmatic airway. ALK-5 expression decreases further 24 hours post-allergen. There is significantly increased expression of epithelial ALK-1 (7 days), ALK-2 (24 hours and 7 days) and ALK-6 (24 and 7 days) in response to allergen-induced activation of disease.

The asthmatic airway submucosal inflammatory-like cells express significantly less ALK-4 and ALK-1 compared to normal volunteers. Expression of ALK-3 is significantly increased in submucosal inflammatory-like cells in asthma. Allergen challenge leads to significant increased numbers of inflammatory-like cells expressing ALK-4 (24 hours), ALK-1 (24 hours and 7 days) and ALK-3 (24 hours).

5.5 Section D: Expression of TGF-β Superfamily signalling Smads

5.5.1 Introduction

TGF-β Superfamily ligands are regulated by synthesis but are also bound to ECM proteins as inactive forms which must be activated before functional signalling can occur. Thus signalling analysis is required to detect activity of these factors. In this section the expression of pSmad2, pSmad1/5, Smad6 and Smad7 in the baseline asthmatic airway, at 24 hours and 7 days post-allergen are evaluated. Expression is compared to that of the normal airway. The expression of Co-Smad4, Smad1 and Smad2 is also analysed.

5.5.2 Results

The overall expression of pSmad2 in the airway epithelium in the asthmatic airway did not differ from that in normal airway (Figure 5.29A and Figure 5.33A and B). pSmad2 expression was also evident, although weaker, in cells of the submucosal compartment in the asthmatic airway at the baseline time point. Given the weaker staining of pSmad2 in these cells (although some cells were easily identifiable as inflammatory cells or fibroblast-like cells), it was difficult to classify all cells precisely. There were almost no identifiable inflammatory or fibroblast-like cells staining for pSmad2 in the normal airway.

There was no significant difference in the level of pSmad1/5 in the baseline asthmatic airway compared to normal volunteers (Figure 5.29B), although there was a trend towards down-regulation of pSmad1/5 expression in asthma (Figure 5.34A and 5.34B).

The distribution and intensity of expression of inhibitory Smad7 was significantly less in the baseline asthmatic group compared to normal volunteers ($p=0.01$) (Figure 5.30A and Figure 3.35A and 5.35B). Whilst predominant staining was in the epithelium there were also scattered inflammatory-like cells, fibroblasts and smooth muscle cells expressing Smad7. The level of Smad6 expression did not differ between the normal and asthmatic airway (Figure 5.30B).

There was no difference in the level of Smad1, Smad2 or Smad4 expression between normal and asthmatic volunteers (Figure 5.31 A, B and C respectively).

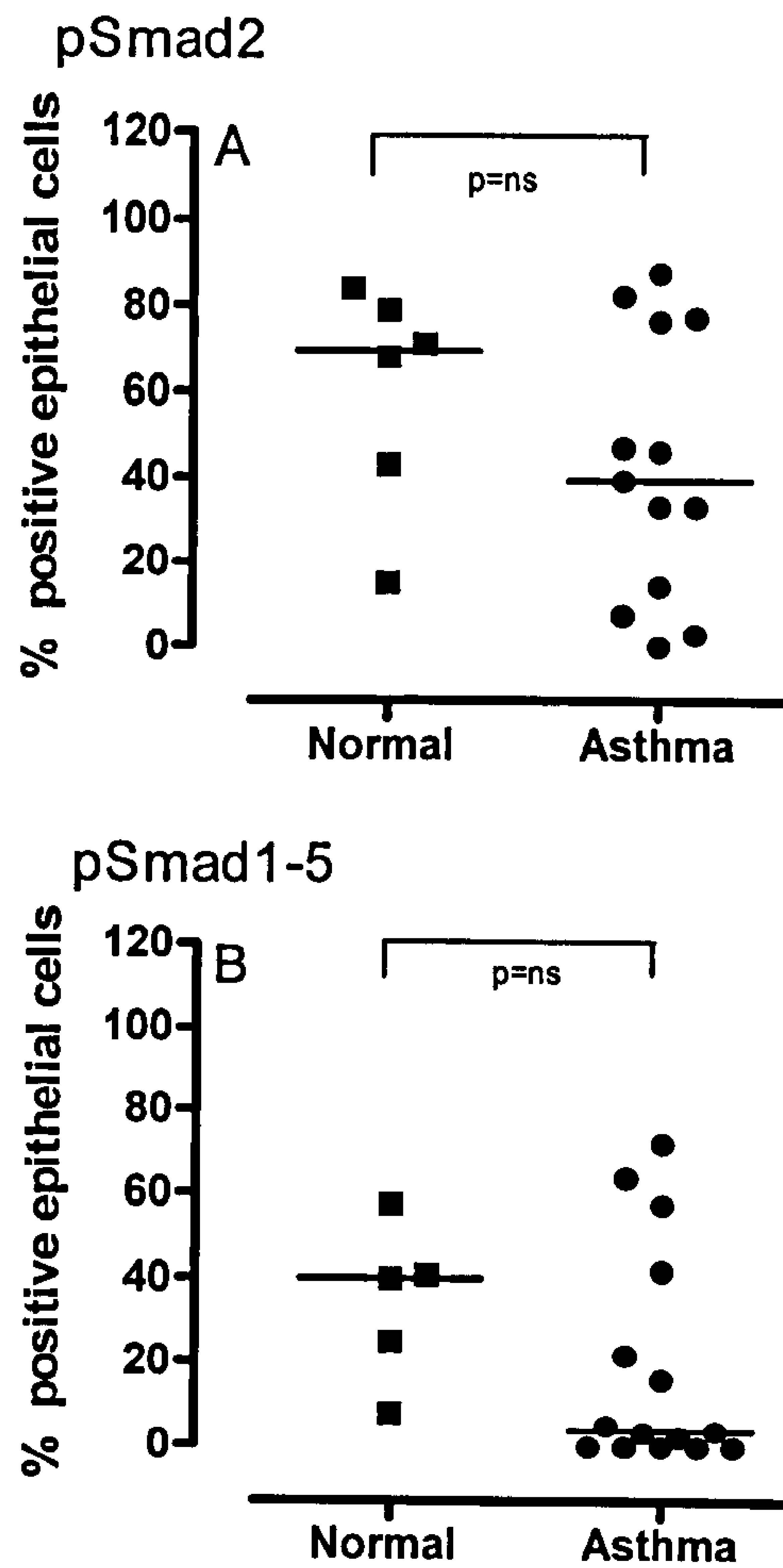


Figure 5.29: The expression of pSmad2 and pSmadl/5 in the normal airway epithelium compared to asthma

The number of cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

Figure 5.30: The expression patterns of inhibitory Smad7 and Smad6 in the normal airway epithelium compared to asthma

The number of cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

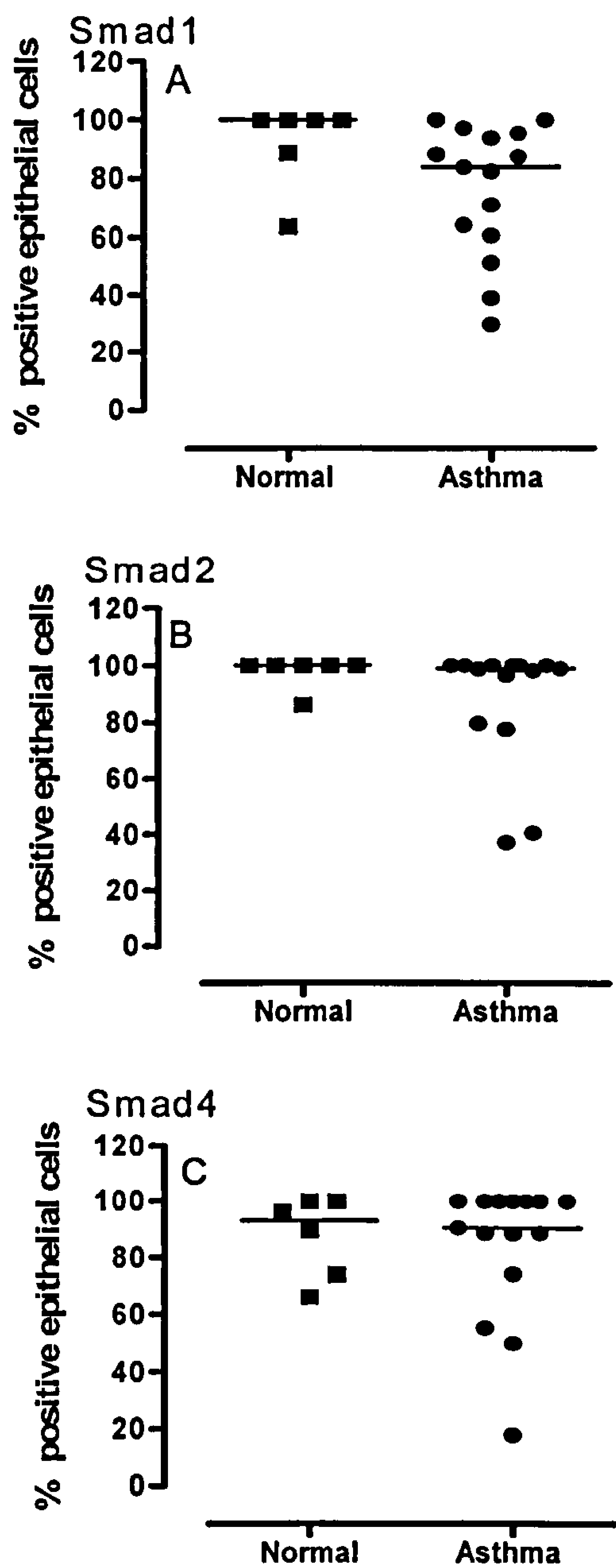


Figure 5.31: The expression patterns of Smadl, Smad2 and Smad4 in the normal airway epithelium compared to asthma

The number of cells expressing each isoform is expressed as a percentage (%) of the total number of epithelial cells present. Significant differences between the groups were analysed using the Mann-Whitney Test. $p<0.05$ was taken as significant.

Post allergen challenge in asthma

Evidence for activation of TGF-β $_{1-3}$ and activin downstream signalling was obtained by counting the number of pSmad2 positive cells. Allergen challenge was associated with significant increases in the total number of positive pSmad2 epithelial cells at 24 hours (p=0.03) with expression levels returning to baseline by 7 days (Figure 5.32A and Figure 5.33C). There were only scattered inflammatory-like cells and fibroblast-like cells staining for pSmad2. There was a pattern towards increased numbers of cells staining for pSmad2 below the basement membrane consistent with activation of TGF-β_{1-3} and activin signalling in inflammatory-like cells and fibroblast-like cells (median 2 (IQR 0-54) cells/mm^2 at baseline increasing to 17.60 (2.3-103) cells/mm^2 at 24 hours and 4.6 (0-128) cells/mm^2 7 days in fibroblast-like cells) following allergen challenge.

Activation of BMP signalling was evident with detection of significant increases in pSmad1/5 signalling in epithelial cells at 24 hours (p=0.04) and at 7 days (p=0.04) (Figure 5.32B and Figure 5.34C). Signalling was also evident in infiltrating inflammatory-like cells but no fibroblast-like cells could be convincingly identified.

There was increased expression of Smad7 in response to allergen challenge at 24 hours (p=0.003), with a trend towards sustained expression at 7 days (p=0.09) (Figure 5.32C and Figure 5.35C). There was no change in Smad6 expression in response to allergen challenge (Figure 5.32D).

There was no modulation of expression of Smad1, Smad2 or Smad4 in response to allergen challenge (Figure 5.36).

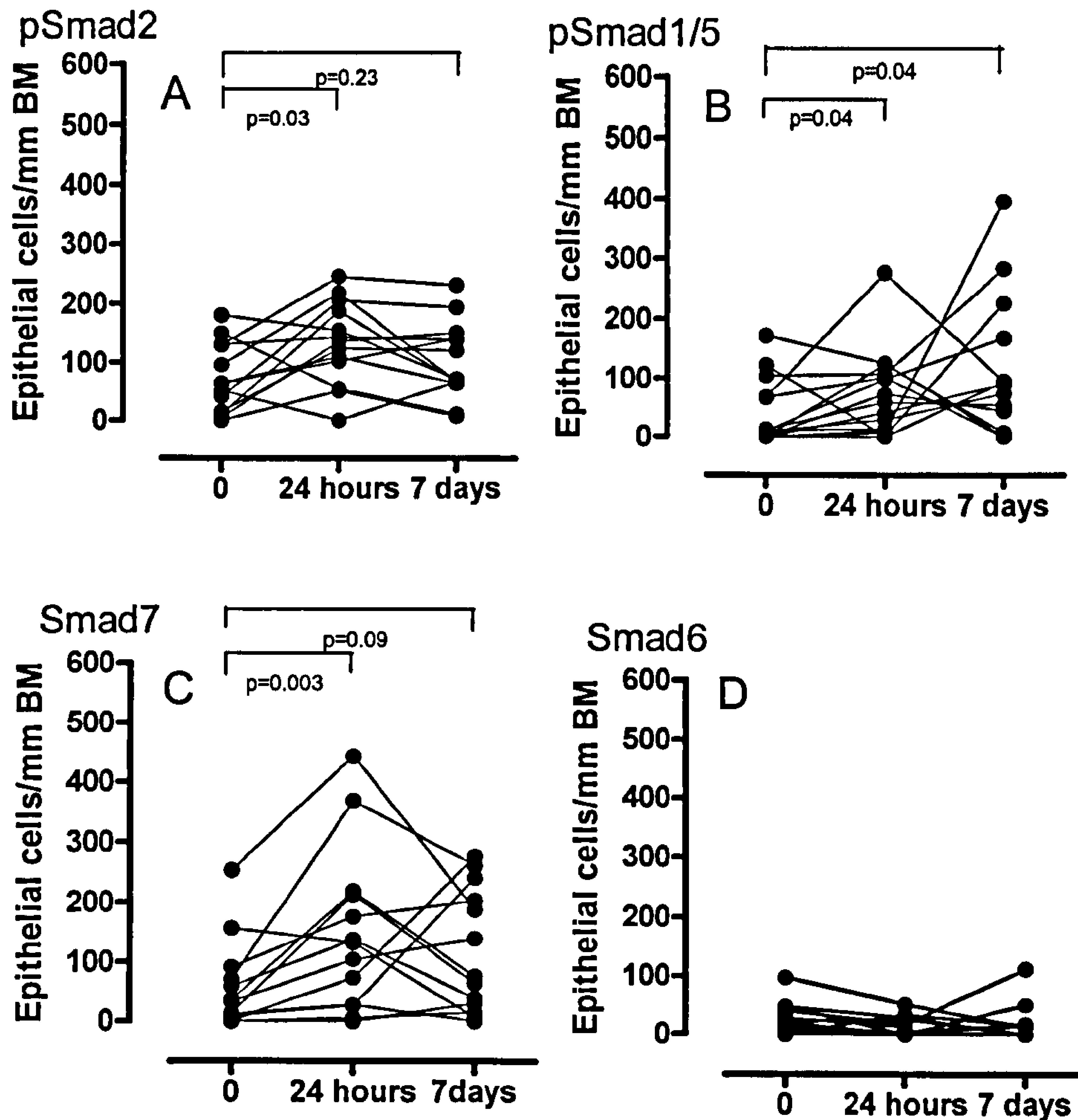


Figure 5.32: The expression kinetics of activated Smads in the asthmatic airway epithelium 24 hours and 7 days after allergen challenge

The number of epithelial cells expressing each isoform is expressed as the number per unit length of BM (cells/ mm BM). Wilcoxon signed rank test was used to compare the change from baseline at the 2 subsequent time points. $p<0.05$ was taken as significant.

Activation of both TGF-β isoform and activin (5.32A) and BMP (5.32B) signalling was seen predominantly in the airway epithelium, with active BMP signalling sustained even 7 days after allergen challenge(p=0.04). Increased numbers of cells staining positive for Smad7 post-allergen (p=0.003) is consistent with the view of pSmad2 and pSmad1 induced up-regulation of Smad7 as part of the negative feedback loop that is known to regulate TGF-β signalling pathways.

pSmad2

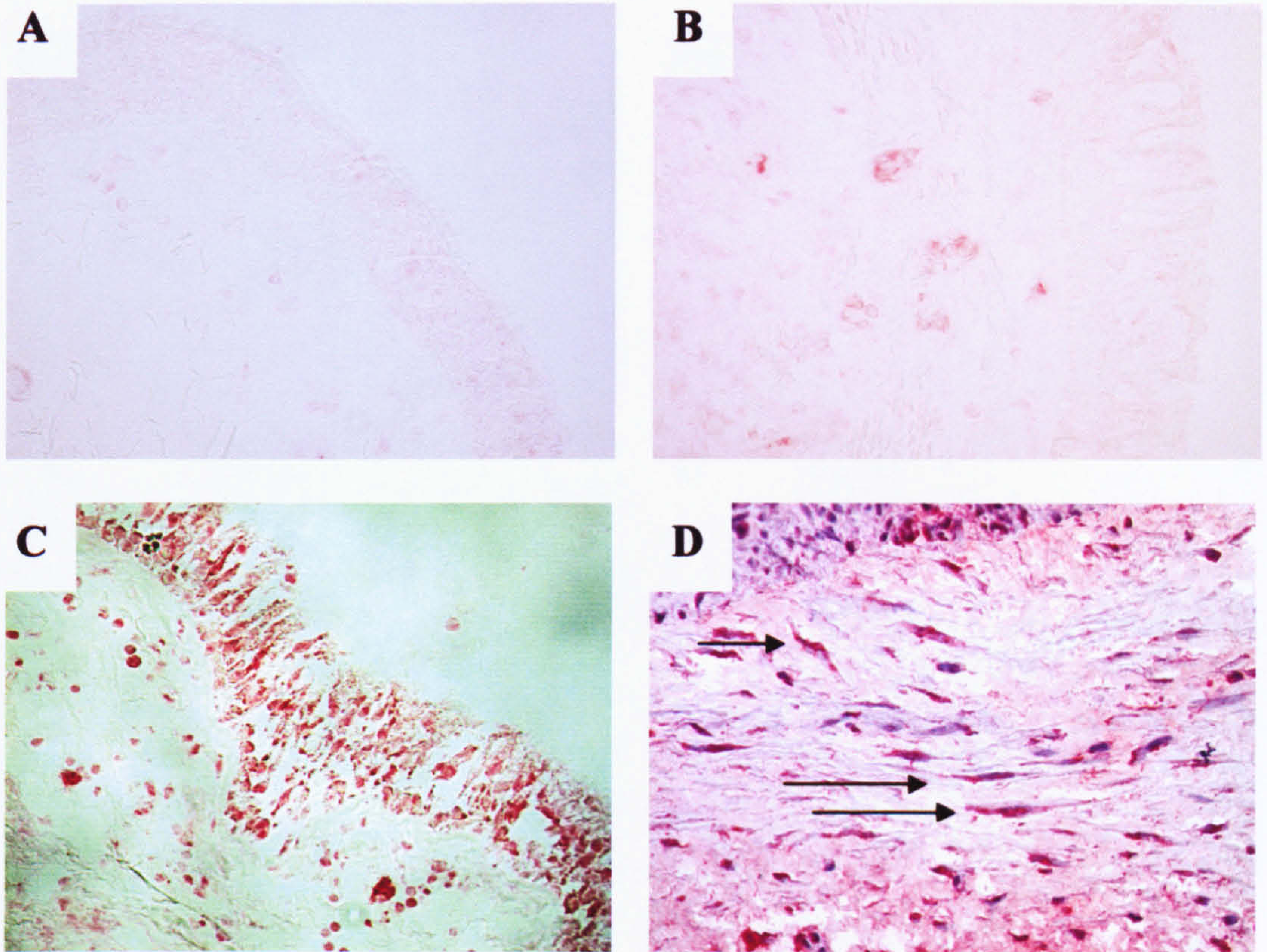


Figure 5.33: Photomicrograph of pSmad2 expression

The expression of pSmad2 at in a normal airway (A) alongside a baseline asthmatic airway (B) and 24 hours post-allergen (C) is presented. Weak or absent expression is seen at baseline. Allergen provocation is associated with increased intensity and numbers of epithelial cells and submucosal inflammatory-like cells staining positive for pSmad2. Immunohistochemistry did not detect many fibroblast-like cells staining for pSmad2. H+E background staining was omitted given the low level of staining intensity of pSmad2 at baseline. In contrast to pSmad2 staining, fibroblast-like cells staining positive for Smad2 (arrowed) were readily detectable (D).

pSmad1/5

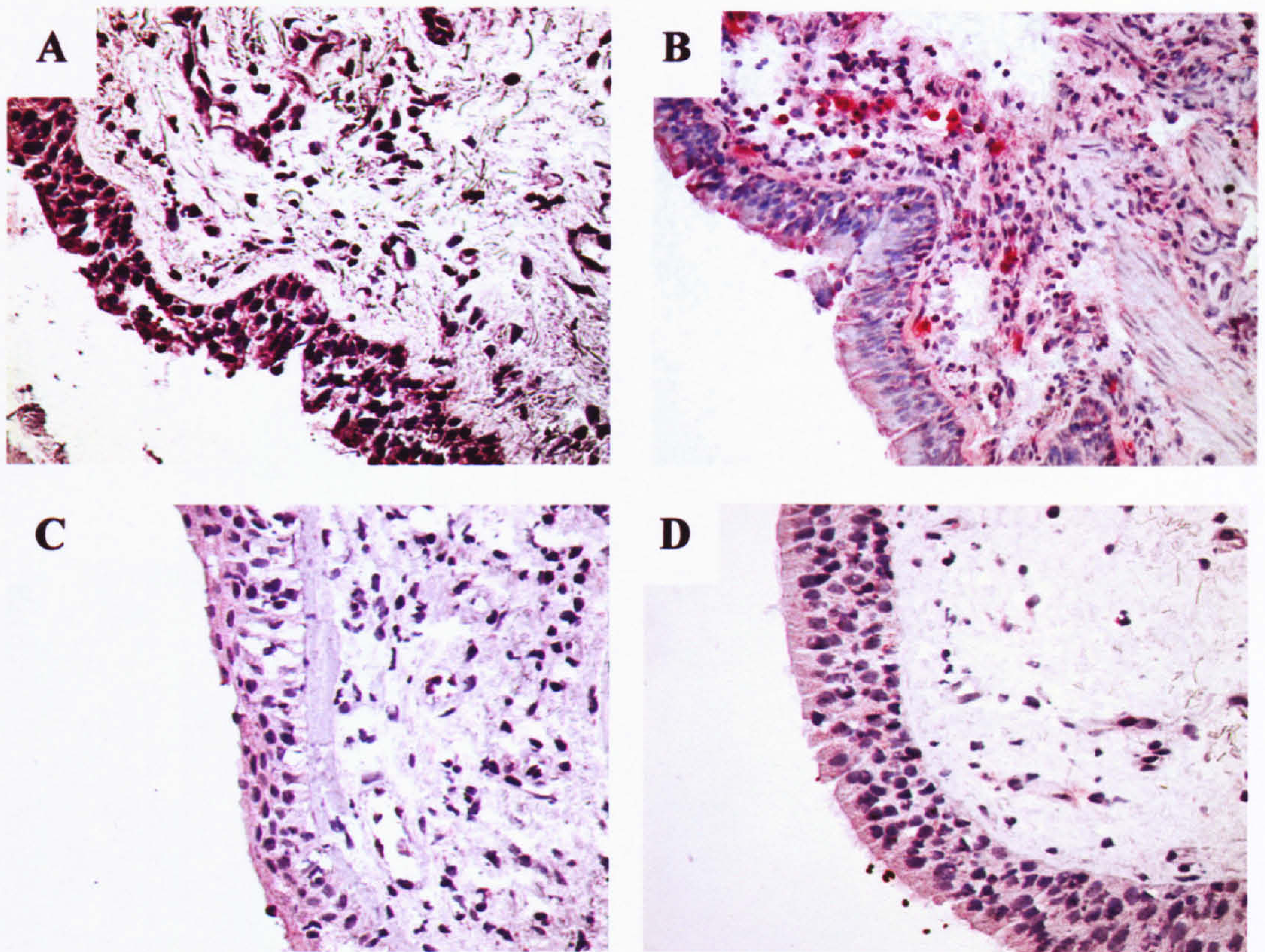


Figure 5.34: Representative photomicrographs of pSmad 1/5 immunoreactivity

Normal volunteers demonstrate expression of pSmad1/5 in epithelium and inflammatory cells (A). Asthmatics demonstrate less epithelial expression of pSmad1/5 (B) suggesting active BMP signalling maybe down-regulated in the asthmatic airway. However submucosal inflammatory cells of the normal airway still express pSmad1/5. Allergen challenge is associated with increased expression at 24 hours (C) and this was sustained at the 7 day time point (D) although the intensity of staining was still less than that in seen in the healthy adult volunteers.

Smad7

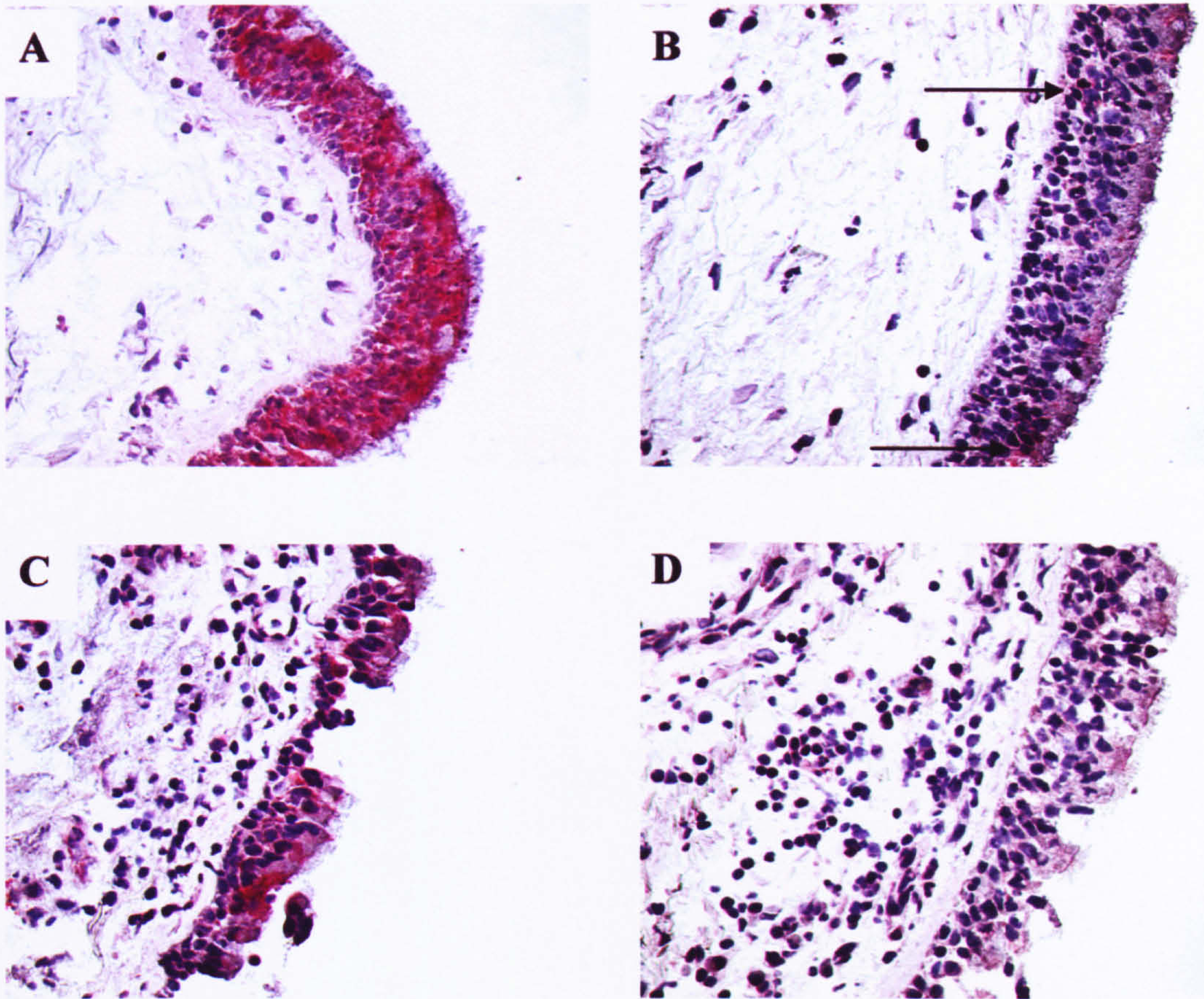


Figure 5.35: Representative photomicrographs of Smad7 immunoreactivity

Normal volunteers demonstrate marked expression of Smad7 in nearly all of the epithelium and also some inflammatory-like cells (A). Asthmatics demonstrate weak (arrowed) or absent expression of Smad7 at baseline (B) suggesting dyregulated TGF-β signalling in the asthmatic airway. Allergen challenge is associated with increased expression of Smad7 at 24 hours (C) with expression levels returning to baseline levels at the 7 day time point (D) when airway remodelling marker expression was maximal.

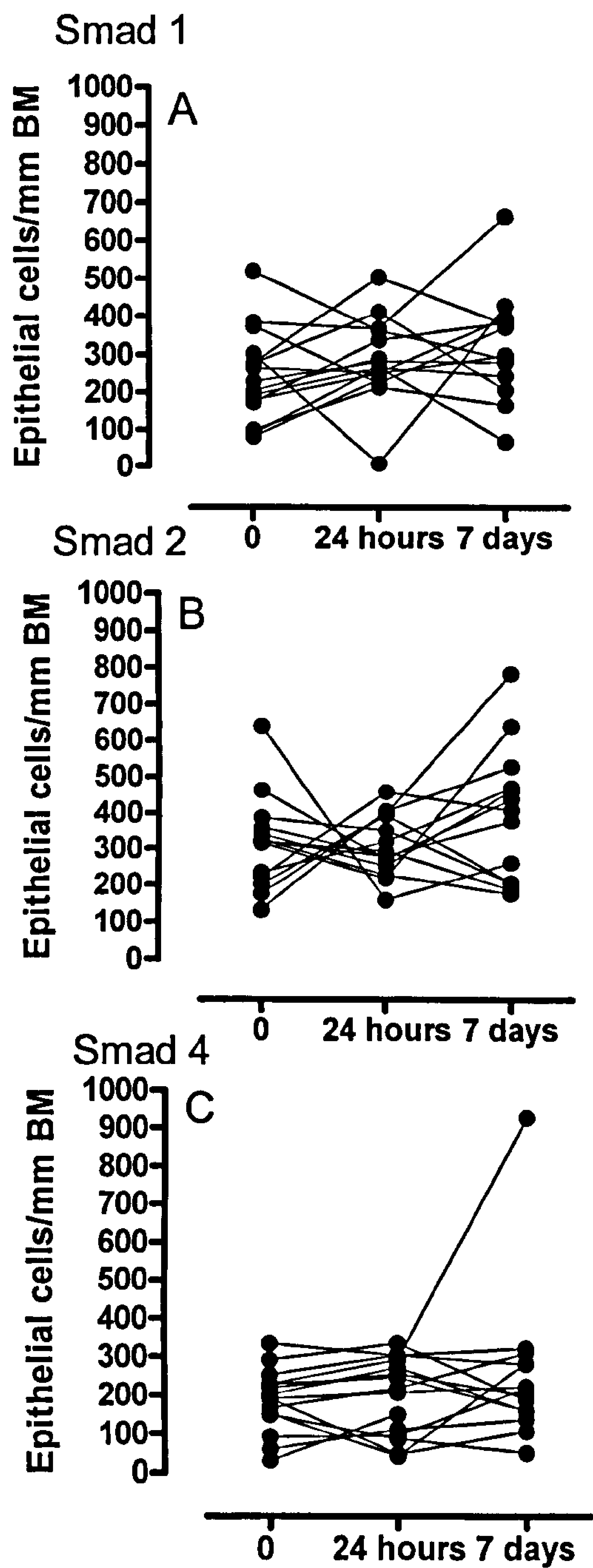


Figure 5.36: The expression of Smad1, Smad2 and Smad4 in the asthmatic airway

Immunohistochemical detection of Smad1, Smad2 and Smad4 expression did not reveal any modulation. The regulation of Smad levels in the cell is complex and the findings of similar levels of Smad protein expression probably reflect the fine balance between Smad synthesis, nucleus-cytoplasm shuttling and Smad degradation.

5.5.3 Discussion

Overall there was no significant difference between the levels of pSmad2 or pSmad1/5 epithelial cell expression between normal and asthmatic individuals, consistent with the view that TGF-β Superfamily ligand co-ordinated signalling is constitutively active in the maintenance of tissue homeostasis. There was however a tendency towards down-regulation of active BMP signalling in the asthmatics. Having confirmed the presence of TGF-β Superfamily ligands in the airway the study then evaluated whether allergen-induced airway injury would lead to increases in the level of activation of ligand signalling. Active TGF-β_{1-3} and activin signalling was evident by the detection of significantly increased immunostaining of pSmad2. Activation was rapid with predominant localisation to the airway epithelium consistent with the increasingly recognised role of epithelium in the airway injury response. Whilst there was evidence of signalling in infiltrating inflammatory-like cells, there were only few cells with fibroblast-like morphology that immunostained for pSmad2 suggesting that Smad2 may not be the predominant signalling Smad in fibroblasts. This observation is in line with studies that suggest it is rather Smad3 that is the predominant signalling Smad in fibroblasts (Roberts *et al.* 2001). α-SMA and Type I collagen expression in dermal fibroblasts is only Smad3 induced (Verrecchia *et al.* 2001). Attenuation of fibrosis is seen in the Smad3 KO mouse despite adenoviral over-expression of TGF-β_1 in fibroblasts (Flanders 2004) and is seen in the bleomycin-induced lung fibrosis model again using the Smad3 KO mouse (Zhao *et al.* 2002). No commercial or collaborator-produced pSmad3 antibody was available at the time of this study to define pSmad3 expression in the tissue sections.

Rapid and sustained activation of BMP signalling was evidenced by the up-regulation of pSmad1/5 expression. This finding is not surprising given the essential role of BMPs in tissue repair and function. This confirms that TGF-β Superfamily signalling in response to airway injury is a complex and co-ordinated response. Activation of BMP signalling was again predominantly confined to the airway epithelium. There were however inflammatory and fibroblast-like cells immunostaining for pSmad1/5 suggesting that BMPs may have a role in the functional modulation of these cells.

Whilst both Smad7 and Smad6 can inhibit of BMP signalling, Smad6 with greater potency, Smad7 is considerably more potent than Smad6 inhibiting TGF-β_{1-3} mediated signalling responses (Hanyu *et al.* 2001). Expression of such inhibitory

Smads (I-Smads) is a major determinant of the TGF-β Superfamily signalling response. Consistent with previous observations (Nakao *et al.* 2002) the study was able to demonstrate that in asthma the expression of Smad7, as determined by immunostaining, is dramatically less in baseline asthmatics compared to normal volunteers. Dysregulated TGF-β signalling as a result of aberrant Smad7 expression has been demonstrated in the fibrotic disease systemic sclerosis (Dong *et al.* 2002), inflammatory bowel disease (Monteleone *et al.* 2001) and in models of pulmonary fibrosis (Venkatesan *et al.* 2004).

With allergen challenge a rapid induction of Smad7 at 24 hours was evident. This observation is in keeping with *in-vitro* studies that have demonstrated rapid induction of Smad7 and Smad6 gene transcription by TGF-β_1, activin-A and BMP-7 in several cell types (Afrakhte *et al.* 1998; Nakao *et al.* 1997). What may be important in terms of therapeutic implications is our observation that in asthma, despite Smad7 up-regulation, the degree of expression was still only half of that seen in the normal bronchial tissue indicating that in asthma there is a failure to adequately regulate TGF-β Superfamily signalling and hence the repair response. This may again indicate an intrinsic abnormality of TGF-β regulation in asthma or a reflection of the effect of other inflammatory signalling pathways operational in asthma that can effect Smad7 gene transcription. The finding that Smad7 over-expression in a transgenic model leads to enhanced inflammation and AHR may suggest the down-regualation of Smad7 in asthma is an attempt also to regulate inflammation.

The mechanism by which the Smad7 promoter is down-regulated is currently unknown. However, marked induction of Smad7 transcription occurs in response to TGF-B_1 (Nakao *et al.* 1997) and the Smad7 promoter is also responsive to MAPK signalling such as JNK1, signal transducers such as IFN-γ (via JAK/STAT1 signalling) and activators of transcription such as NFκB in response to stimulation by TNF-α (Ulloa *et al.* 1999; Bitzer *et al.* 2000). Binding of Smads and other transcription factors to unmethylated CpG islands of the Smad7 promoter stimulate basal promoter activity and induction of gene transcription. TGF-β signalling is the most important regulator of Smad7 expression as shown by point mutations in the palindromic GTCTAGAC sequence of the Smad3-4 binding element (SBE) completely abolishing TGF-β induction whilst deletion of binding sites for AP-1 or SP-1 only attenuates the basal transcriptional activity of the promoter (Brodin *et al.*

2000). TGF-β mediated Smad7 induction is also attenuated in this instance (Brodin *et al.* 2000). By the ability of Smad7 transcription to respond to multiple factors, Smad7 not only serves as a TGF-β antagonist but also fine tunes the cellular response to TGF-β ligands by the integration of these different signalling pathways. Restoration of Smad7 expression in asthma may offer a therapeutic target whereby a specific component of the signalling pathway is manipulated with the aim of restoring regulation to TGF-β signalling responses.

Regulation of the TGF-β signalling is also dependent on the continuous nucleocytoplasmic shuttling of the Smad proteins (Xu *et al.* 2002)(Inman *et al.* 2002). Ligand-receptor binding leads to receptor mediated phosphorylation of R-Smads leading to activation for a few hours and residence in the nucleus of R-Smads before dephosphorylation and shuttling out of the nucleus to go back and detect the activation status of the receptor. Nucleocytoplamic shuttling of Smad-4 is independent of TFG-β signlling (Watanabe *et al.* 2000). Smads eventually undergo degradation via the ubiquitin-proteasome pathway (Lo & Massague 1999). In this study immunohistochemical detection of R-Smad1, R-Smad2 and Co-Smad4 expression did not reveal any overall modulation. It may be that exact nuclear versus cytoplasmic Smad localisation is required. It was not possible to be certain of such distribution using just basic IHC. The regulation of Smad levels in the cell is complex and our findings of similar levels of Smad protein expression probably reflect the fine balance between Smad synthesis, nucleocytoplasmic shuttling and Smad degradation.

5.5.4 Summary of Smad signalling

Allergen challenge in asthma is associated with activation of TGF-Superfamily ligand signalling with significant increases in epithelial pSmad2 and pSmad1/5 expression. The baseline asthmatic airway demonstrates significantly less Smad7 expression but there is rapid up-regulation of expression following allergen provocation.

5.6 General discussion of chapter

TGF-β Superfamily ligand expression and the functional activation of signalling pathways in response to allergen-provocation specific to asthma are defined in this chapter. It is demonstrated that the airways of mild to moderate atopic asthmatics have markedly altered expression of TGF-β Superfamily signalling pathway components compared to the normal airway. Allergen-induced airway injury is associated with the swift functional activation of TGF-β ligand signalling together with rapid modulation of selected Type I and Type II receptor expression that is sustained for at least 7 days after airway injury. It is confirmed therefore that there is an imbalance of TGF-β Superfamily signalling in asthma.

The baseline expression of TGF$\beta_{1\text{-}3}$ mRNA isoforms in asthmatic airway epithelium did not differ from baseline expression in airway epithelium from normal volunteers. Increased numbers of submucosal inflammatory-like cells staining positive for TGF-β_1 and TFG-β_3 mRNA in the asthmatic airway at baseline compared to normal volunteers was found, with statistical significance demonstrated for only TFG-β_3. If it is presumed that the majority of these cells are inflammatory cells, then this finding supports the concept that inflammatory cells may be important sources of growth factors. Allergen challenge was not associated with modulation of any TGF-β isoform mRNA expression in either the epithelium or submucosal cell population. In contrast, significantly increased amounts of activin–A protein expression, with both epithelial and inflammatory-like cells identified as significant sources, were found in the asthmatic airway at baseline. Whilst the levels of BMP-2, BMP-4 and BMP-7 expression were similar between the normal airway and asthmatic airway, there was significant up-regulation of BMP-7 in inflammatory-like cells after allergen challenge at 24 hours and sustained to 7 days. Eosinophils were the predominant inflammatory cell source of BMP-7. There were marked differences in the degree of Type I and Type II receptor expression in the asthmatic airway compared to normal volunteers. Interestingly there was strong and widespread ALK-4 staining throughout the airway in both the normal airways and that of asthmatics, indicating that activin signalling may have an important homeostatic role in the airway. Overall there was no difference in the level of pSmad2 or pSmad1/5 expression in asthma compared to normals although there was a trend towards down-regulation of BMP signalling in asthma. There was decreased expression of Smad7 in the asthmatic airway but not Smad6.

Allergen challenge was associated with activation of TGF-β_{1-3}, activin and BMP signalling pathways identified as rapid modulation of selected Type I receptors and pSmad2 and pSmad1/5 expression, together with the induction also of regualatory Smad7 signalling, confirming that these pathways act in a fast but co-ordinated manner.

Growth factor imbalance and inappropriate signalling is implicated in several pathological fibrotic processes including asthma, although it remains to be determined whether such imbalance is a cause or a consequence of the disease. Such imbalance will lead to a situation in which the activity of a growth factor predominates over another often opposing factor with functional consequences. An excellent example of such imbalance is the over-expression of EGF family signalling in the asthmatic airway (Kretzschmar *et al.* 1997). Despite such over-expression there is absent epithelial proliferation in response to EGFR signalling (Puddicombe *et al.* 2003). In the context of asthma it appears that TGF-β_1 can override the proliferative effects of EGF. The functional outcome is therefore is a determinant of summation of such opposing signals in the context of a specific disease environment.

The diversity of TGF-β ligands that utilise these receptors is extensive, the corollary to which is that other regulatory receptor mechanisms operate. In fact, given the remarkable diversity of responses that can be mediated by what is a limited number of receptor-ligand combinations, it is not surprising that the TGF-β Superfamily ligands can interact with several alternative cell-signalling pathways. Several non-TGF-β signalling pathways can also converge on the Smad pathway (Derynck & Zhang 2003) (Kretzschmar *et al.* 1997). The mitogen-activated protein (MAP) kinases are an enzyme cascade system comprised of at least 3 different enzymes. Activation (by phosphorylation) of the first enzyme leads to the sequential activation of the other enzymes in the system. One end result is the activation of the enzyme extracellular receptor-activated kinase (Erk), the prototypic member of the MAP kinase family. Activated ERK can now phosphorylate the protein Elk that in turn leads to the transcription of Fos component of the transcription factor activator protein-1 (AP-1). Erk MAPK can effect R-Smad signalling by phosphorylation of the Smad2 MH1 domain and Smad1, Smad2 and Smad3 linker domains (Kretzschmar *et al.* 1997) (Funaba *et al.* 2002). JNK phosphorylation of Smad3 enhaces TGF-β responsiveness

via increased nuclear translocation and transcription (Engel *et al.* 1999). Smad6 and Smad7 can also be regulated by phosphorylation independent TGF-β ligand induced stimulation. It is only Smad4 that is not regulated in this manner.

The demonstration of TGF-β signalling with either Smad4 knock out cells or cells with dominant negative Smad transfection has confirmed that TGF-β ligands can signal independent of the Smad system. Although the exact mechanism by which the JNK, ERK and p38 MAPK activated signalling cascades can interact with TGF-β pathways are still unclear what is apparent is the cellular convergence of these alternative signalling pathways can lead to not only cooperation but also counteraction, leading to cell specific responses to TGF-β signalling, which can be further defined in response to the alteration of these signalling processes in disease states.

It is likely therefore that other complex signalling cascades operative in asthma will have influenced the marked modulation of TGF-β Superfamily signalling observed in this study. In asthmatic epithelium it is shown that there is extensive up-regulation of EGFR expression leading to continuous activation of ERK, JNK and p38 MAPK signalling (Puddicombe *et al.* 2000; Duan & Wong 2006). Such pathways can interact with the TGF-β signalling components. For example ERK signalling activation *in-vitro* experiments leads to phosphorylation of Smad1 at the linker region that leads to inhibition of the BMP signalling pathway (Kretzschmar *et al.* 1997). It is interesting to speculate that our observed down regulation of pSmad1/5, the Type I receptors ALK-2 and ALK-6 as well as the Type II receptor BMPRII is a reflection of other cellular pathways down-regulating BMP signalling in the asthmatic airway. Imbalance of growth factor signalling is a reoccurring theme in asthma.

The modulation of Type I receptors suggest that the regulation of signalling at the receptor level occurs by modulation of ALK-5 for TGF-β_{1-3} and through predominantly ALK-2 and ALK-6 for BMPs.

It is therefore concluded that the airways of mild-moderate atopic asthmatics have markedly altered expression of TGF-β Superfamily signalling pathway components compared to the normal airway. There is an imbalance in TGF-β Superfamily signalling in asthma. Allergen-induced airway injury is associated with the swift

activation of TGF-β Superfamily signalling. This activation is associated with rapid modulation of receptor expression and induction of inhibitory Smad7 signalling pathways. Co-ordinated activation of several TGF-β Superfamily signalling pathways occur in response to allergen predicting that therapeutic targeting of just a single pathway ligand or receptor is unlikely to effective. It therefore remains to be identified if the process of airway remodelling can be antagonised for therapeutic benefit in asthma by modulating the TGF-β signalling system. The elucidation of such novel targets in TGF-β Superfamily signalling will hopefully lead to valuable therapeutic intervention in airway remodelling.

5.7 Overall summary of chapter

The aim of this chapter was to determine TGF-β_{1-3}, activin-A, BMP-2, BMP-4 and BMP-7 ligand expression together with the respective Type II receptors, Type I receptors and activated Smads in the normal and asthmatic airway. In addition the modulation of expression was evaluated at 24 hours and 7 days after allergen challenge.

It was shown that TGF-β_3 (inflammatory-like cells) and activin–A (epithelium and inflammatory-like cells) expression were increased in the asthmatic airway with no further increase after allergen challenge. BMP-2, BMP-4 and BMP-7 were predominantly expressed in airway epithelium with no significant difference between normals and asthma at baseline. Allergen challenge was associated with marked and sustained up-regulation of only BMP-7 in infiltrating inflammatory-like cells at 24 hours and at 7 days and the epithelium at 7 days. Eosinophils, $CD4^+$ T cells, mast cells and macrophages were sources of BMP-7

Epithelial ActRIIA, ActRIIB and BMPRII expression was significantly less in asthma. Only ActRIIA up-regulation was significant at 24 hours and 7 days post-allergen challenge.

Epithelial ALK-5 and ALK-1 expression was significantly less in asthma with further down-regulation of ALK-5 at 24 hours. There was significant down-regulation of ALK-2 and ALK-6 in asthma compared to the normal airway. Receptor up-regulation was seen for ALK-2 and ALK-6 at 24 hours and at 7 days post-allergen.

Overall there was no difference in the level of epithelial pSmad2 or pSmad1/5 expression in asthma compared to the normal airway although there was a trend towards down-regulation of BMP signalling in asthma. There was decreased expression of only Smad7 in asthma. pSmad2 and Smad7 expression increased at 24 hours after allergen and Smad7 expression was still raised at 7 days. pSmad1/5 expression increased at 24 hours and 7 days suggesting active BMP signalling. Thus allergen challenge is associated with activation of TGF-β_{1-3}, activin and BMP signalling as evidenced by increased pSmad2, pSmad1/5, and Smad7 expression.

Chapter 6

Safety and tolerability of three consecutive bronchoscopies in asthma

6.1 Introduction

In this chapter the safety and tolerability of the model involving three consecutive bronchoscopies with broncho-alveolar lavage (BAL) and bronchial biopsy (BB) before and following inhaled allergen challenge is presented.

Fibreoptic bronchoscopy (FOB) with BAL in human volunteers was first reported in 1972 (Reynolds & Newball 1974) and the first study of volunteers with asthma involving BB was in 1977 (Molina *et al.* 1977). Concerns over the potential for BAL to exacerbate airway obstruction prevented the development of studies with BAL in asthma until 1982 (Godard *et al.* 1982). Currently there are over 200 reports of research utilising FOB and has lead to the publication of several reports in safety and volunteer tolerability (Djukanovic *et al.* 1991; Humbert *et al.* 1996; Van Vyve *et al.* 1992) leading to the development of consensus guidelines (Busse *et al.* 2005). The continued collection and collation of safety and tolerability data in a standardised form in defined volunteer groups is essential to develop protocols that ensure volunteer safety.

6.2 Results

6.2.1 Bronchoscopy volunteer characteristics

Data is presented on the fifteen individuals with mild to moderate asthma who each underwent three bronchoscopies (except one volunteer who only undertook two bronchoscopies with no BAL on the second occasion), a total of 44 bronchoscopies. The volunteers (9 male and 6 female) were of median age 25 (range 19-46) years, FEV_1 % predicted of 97 (range 75.41-125.7) % with a baseline methacholine PC_{20} of 2.1 (1.2-3.6) mg/ml (geometric mean ± 95% CI) at study entry.

None of the volunteers developed any significant complication from bronchoscopy. The only discomfort reported was of a mild sore throat which had resolved the next day. There was only one incidence of haemoptysis post-procedure but was mild and clinically insignificant. One volunteer declined the final procedure because she found bronchoscopy intolerable despite sedation

6.2.2 Baseline bronchoscopy

At baseline bronchoscopy, the median FEV_1 was 93.97 (range 80-120.1) % predicted before bronchoscopy and 92.81 (73.59-119.0) % predicted at discharge (p=0.08). The median prebronchoscopy oxygen saturation on room air was 99 (range 95-100) % with no significant change at discharge at 97 (96-100) % (p=0.25). During the course of bronchoscopy the median maximum oxygen saturation recorded was 99 (range 98-100) % whilst the minimum was 96 (92-100) % (p=0.005). End procedure median saturation was 98 (range 91-100) % on supplemental oxygen. Oxygen saturation on room air 10 minutes post procedure was maintained at median 98 (range 95-99) %.

6.2.3 Bronchoscopy 24 hours after inhaled allergen challenge

On attendance for the second bronchoscopy 24 hours after allergen challenge the median FEV_1 was 94.94 (range 75.1-111.1) % predicted before bronchoscopy (no difference from FOB1) but dropped to 85.48 (62.40-119) % (p=0.05) at discharge. The median oxygen saturation prebronchoscopy on room air was 99 (range 96-100) % and 97 (94-100) % on discharge (p=0.02). During the course of the procedure the peak median oxygen saturation recorded was 99 (range 98-100) % and the lowest level recorded was 96 (94-100) % (p=0.0002). End of procedure median saturation was 97 (93-100) % on 2-4 litres of entrained oxygen whilst the saturation on room air 10 minutes post-procedure was 98 (94-100) %.

6.2.4 Bronchoscopy 7 days post-allergen

Although the median FEV_1 % predicted had fully recovered with a pre-bronchoscopy value at 100.1 (range 70.56-119) % predicted there was still a significantly marked reduction in the FEV_1 at discharge after bronchoscopy at 90.15 (66.13-119) % (p=0.009). The prebronchoscopy oxygen saturation was 98.5 (96-100) % and 97.5 (96-100) % on discharge (p=0.05). The difference between the highest recorded median oxygen saturation at 99.5 (range 98.5-100) % and lowest at 96 (92-98) % was again statistically significant at p=0.0002. There was, however, no clinical consequence as a result of desaturation seen during the course of bronchoscopy.

The % BAL volume recovered did not significantly differ between the groups at each bronchscopy. The median volume recovered as the percentage of that instilled for Group A was 61.9 (range 50-72.22) % at FOB1 vs 61.11(52.38-74) % at FOB2 vs 62.50 (57.17-77.78) % at FOB3. The median volume percentage recovered for Group B was 56.95(range 37.03-72) % at FOB1 vs 57.14% (30.55-83.33) at FOB2 vs 58.3 (41.67-72) % at FOB3. There was however a significant correlation between the % BAL volume recovered and change in FEV_1 on discharge (r=0.31, p=0.04). Overall, there was no significant correlation between the change in oxygen saturation and the percentage volume of BAL recovered.

6.2.5 Bronchoscopy and asthma control

The effects of FOB on asthma control the day after bronchoscopy are summarised in Table 6.1. FOB was associated with increased symptoms on all occasions. Significant fall in FEV_1 was only seen following FOB that was preceded by allergen challenge (p=0.002) and was associated with the most significant increases in symptoms (p=0.001) and corresponding medication usage (p=0.004). None of these changes required treatment other than inhaled short-acting β_2 agonists and all had resolved by the second day after bronchoscopy.

FOB1	Day-Before FOB	Day-After FOB	Significance
FEV1%	84.80 (68.06-120)	87.98 (63.71-113)	p=ns
Symptom Score	0 (0-4)	4.5 (0-10)	p=0.002
Medication	0 (0-2)	1 (0-8)	p=0.02
FOB2			
FEV1%	90.17 (67.10-120.4)	82.18 (56.25-111.9)	p=0.002
Symptom Score	0 (0-4)	3 (0-9)	p=0.001
Medication	0 (0-4)	2 (0-16)	p=0.004
FOB3			
FEV1%	86.78 (69.06-125.0)	86.43 (68.71-120)	p=ns
Symptom Score	0 (0-10)	2 (0-7)	p=0.05
Medication	0 (0-10)	1 (0-7)	p=ns

Table 6.1: Summary the effect of bronchoscopy on asthma control in terms of FEV_1% (median ± range), symptom scores and frequency of reliever medication recorded the day before and day after FOB

Data was analysed further according to volunteer groups with no increased AHR following allergen challenge (n=5, termed Group A) and with increased AHR (n=10, termed Group B). The methacholine PC_{20} (geometric mean) was 3.1 mg/ml at baseline with no significant change at either 24 hours (4.2 mg/ml) or 7 days (2.22 mg/ml) in Group A. Group B demonstrated a baseline PC_{20} of 1.56 mg/ml with significantly increased AHR at both 24 hours (0.39 mg/ml) and 7 days (0.44 mg/ml) (p=0.002 and p=0.0078 respectively).

Although both groups demonstrated reduction in FEV_1 post bronchoscopy, it was Group B with marked AHR at the second bronchoscopy that demonstrated the most significant decrease in FEV_1 at discharge. The data is summarised in Table 6.2. None of the volunteers required any medication other than short acting β_2 agonists for asthma symptoms.

Table 6.3 summarises the FEV_1 % data from the day before and the day after bronchoscopy. Reduction in FEV_1 was seen in both Group A (no increased AHR following allergen challenge) and Group B (increased AHR after allergen challenge) only after the bronchoscopy that followed allergen challenge (FOB2), although only in Group B was this statistically significant (p=0.01). Only Group B reported significant increases in reliever medication usage (p=0.02) in response to increased symptoms (p=0.03) (Table 6.4 and 6.5 respectively). The diary data is illustrated in Figure 6.1.

	Group A			Group B		
Bronchoscopy	Pre-FOB FEV_1%	Discharge FEV_1%	p	Pre-FOB FEV_1%	Discharge FEV_1%	p value
1	104.7 (80.65-118.5)	98.15 (73.59-119)	ns	92.5 (80.65-120.1)	91.97 (75.39-114.9)	ns
2	104.4 (74.60-111.1)	92.92 (81.65-119)	ns	94.59 (79.03-104.4)	83.80 (62.9-107)	0.02
3	100.3 (70.56-119)	92.68 (81.65-119)	ns	100.1 (77.42-113.6)	88.17 (66.13-108.4)	0.004

Table 6.2 FEV_1 % immediately before bronchoscopy and prior to discharge

Summary of the FEV_1% (median ±interquartile range) of volunteers before bronchoscopy and after (just prior to discharge) in terms of no increased AHR (Group A) and increased AHR (Group B) status. Each measurement was taken 30 minutes after nebulised salbutamol. Both groups demonstrate reduction in FEV_1 following bronchoscopy but it only Group B with significantly increased AHR following allergen challenge that demonstrated a significant fall in FEV_1.

	Group A			Group B		
Bronchoscopy	Day -Before FOB FEV_1%	Day-After FOB FEV_1%	p	Day -Before FOB FEV_1%	Day-After FOB FEV_1%	p
FOB 1	84.22 (74.60-130)	84.46 (63.71-113)	ns	84.40 (68.06-98.10)	87.98 (73.78-98.06)	ns
FOB2	91.21 (70.96-130.4)	83.17 (56.25-111.9)	ns	88.85 (67.1-126.4)	78.55 (56.77-84.91)	0.01
FOB 3	92.54 (72-125)	92 (81-120)	ns	83.09 (69.03-105.6)	84.3 (68.68-98.69)	ns

Figure 6.3 Summary of the effect of FOB on asthma control in terms of FEV_1 measured the day before and the day after

Values are expressed as the median ± interquartile range.

	Group A			Group B		
Bronchoscopy	Day Before FOB	Day-After FOB	p	Baseline	Day-After FOB	p
FOB 1	0 (0-4)	4 (0-10)	ns	0 (0-4)	5 (1-9)	0.02
FOB2 2	0 (0-3)	3 (0-4)	ns	0 (0-4)	4 (0-9)	0.02
FOB 3	0 (0-2)	3 (0-9)	ns	0 (0-10)	3 (1.5-7)	ns

Table 6.4: Summary of the effect of FOB on asthma control in terms of volunteer inhaler usage frequency

Only the group with increased AHR (group B) demonstrate significantly increased inhaler usage post bronchoscopy. Values are expressed as then median (± interquartile range).

	Group A			Group B		
Bronchoscopy	Day Before FOB	Day-After FOB	p value	Baseline	Day-After FOB	p
FOB 1	0	0 (0-1)	ns	0 (0-2)	3 (0-8)	ns
FOB 2	0	2 (0-2)	ns	0 (0-4)	3 (0-15)	0.03
FOB 3	0	0.5 (0-2)	ns	1 (0-10)	2 (0-7)	ns

Table 6.5: Summary of the effect of FOB on asthma control in terms of volunteer symptom scores the day before and the day after FOB

Only Group B demonstrates significantly increased symptoms in relation to FOB. Values are expressed as the median (± interquartile range).

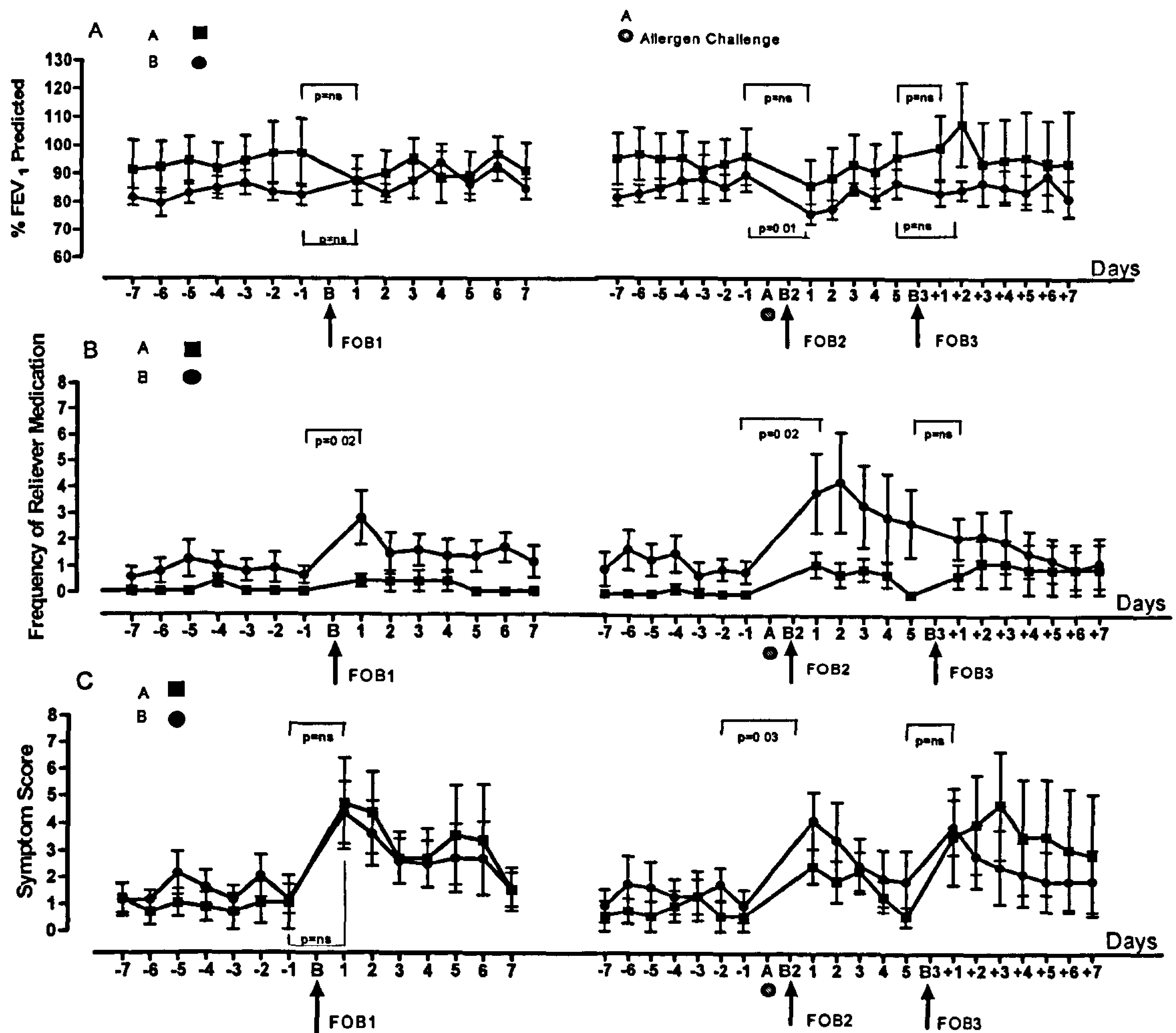


Figure 6.1 Summary of volunteer diary data a week before and after each bronchoscopy

Following the screening visit, volunteers recorded FEV_1 (A), salbutamol inhaler usage (B) and symptoms (C) in a run in period of two weeks before a baseline bronchoscopy with BAL and BB (FOB 1) and throughout the study period. A hand held Piko Device FEV_1 recorder (Ferraris Respiratory Europe Ltd, Hertford, UK) was used with the volunteers fully trained in its use. Volunteers were asked to take measurements in the morning before any reliever medication.

A ■ Group A (no increased AHR)

B ● Group B (increased AHR)

6.2.6 Volunteer follow-up spirometric data

Out of the 15 volunteers that entered the study 12 returned for follow up 2-6 weeks after the end of the study. The median FEV_1% predicted was 99.88 (range 79-109) % at follow up compared to 95.18 (75.41-114.8)% measured at the study entry screening visit. All 12 of these volunteers were still maintained on short-acting β_2 agonists only and none reported clinical deterioration of asthma control in the weeks following the study.

6.3 Discussion

In this study we have shown that three consecutive bronchoscopies of volunteers with mild and moderate asthma were not associated with adverse events and that falls in FEV_1 post-procedure were associated with allergen-induced increased AHR. In addition there were no long term adverse effects. The model is validated by the demonstration of reproducibility of increased cellular recruitment into the airway at the 24 hour time point (Bentley *et al.* 1993)(Robinson *et al.* 1993) in association with AHR which is sustained 7 days later (Cockcroft *et al.* 1977). We have successfully set up a longitudinal model of human asthma where we can sample the airways in a time course manner which can be used to look at the induction and resolution of inflammatory and remodelling events in relation to symptoms and changes in airway physiology.

The limited evidence suggests that FOB with bronchial biopsy (BB) does not exacerbate AHR and no deterioration in asthma control occurs measured in terms of the peak expiratory flow (PEF)(Humbert *et al.* 1996). In both normals as well as asthmatics there is, however, an increased risk of airway obstruction when bronchial biopsy is combined with BAL (Djukanovic *et al.* 1991). The falls in FEV_1 were greater in the asthmatics rather than normals however suggesting that the risk of bronchospasm may also be related to AHR. The data in the study supports this finding in that falls in FEV_1 following bronchoscopy were greater in the presence of allergen induced increases in AHR. In terms of PEF, the degree of airway obstruction is less when BAL is omitted and only bronchial biopsy was performed (Humbert *et al.* 1996). In this study the fall in FEV_1 was significantly correlated with the BAL volume recovered in this study, adding to the concept that BAL can adversely effect airway obstruction. This has important implications for BAL with more severe asthma.

Theoretically BAL can also lead to an increased alveolar-arterial (A-a) gradient with resultant hypoxia but only minor falls in end procedure saturation were seen in volunteers, reaching statistical significance only after FOB2 and FOB3 when increases in AHR had been induced by allergen challenge. There was no significant correlation between the percentage BAL volume recovered and the change in oxygen saturation.

A major concern for clinicians performing FOB in asthmatics is whether the procedure will have an adverse effect on disease control. There is no available data on the degree of airway obstruction measured in terms of FEV_1 measurement at present. Diary data confirmed increased airway symptoms necessitating reliever after each FOB, but the FEV_1 did not deteriorate following baseline bronchoscopy or indeed after the final bronchoscopy. Significant falls in FEV_1 was seen only the day after bronchoscopy related to allergen challenge. This suggests that the increased AHR after allergen increased the impact of bronchoscopy on symptoms and FEV_1, or that these changes were related to allergen challenge alone as previously reported in allergen studies without bronchoscopy (Cockcroft *et al.* 1977). There were no asthma exacerbations requiring additional treatment. Overall FOB was well tolerated. Only one volunteer found repeat bronchoscopy unacceptable despite sedation and declined to complete the final study FOB.

6.4 Summary

Three consecutive bronchoscopies combined with BAL and bronchial biopsy was well tolerated by these mild to moderate asthmatic volunteers. By careful volunteer selection, close monitoring and dedicated aftercare it is possible to develop longitudinal studies in human asthma.

Chapter 7
General Discussion

Developing a human model of asthma whereby the dynamic events of airway remodelling can be examined in relation to changes in cellular inflammation and airway function was the primary aim of this study. The airway in stable disease was sampled with repeat sampling immediately after disease activation following allergen challenge as previously reported (Phipps *et al.* 2004), and again at a time when the disease should either have been in resolution or in a state of further propagation. The development of such a study as this is particularly important in asthma as it is a chronic complex heterogenous condition and no animal model can ever mimic the human disease completely. Animal models provide valuable insights into the potential roles of complex molecular pathways, but ultimately studies of human asthma are required (Kariyawasam & Robinson 2005; Wenzel & Holgate 2006).

The study presented here confirms that longitudinal studies, whereby disease activity defined by airway physiological changes in relation to inflammation and remodelling events, are possible and as such should encourage research groups to undertake similar studies in the future. Careful volunteer selection and close monitoring is essential in any further studies involving bronchoscopy. This study reports on the safety and tolerability of bronchoscopy with allergen challenge in 15 volunteers and although generalisation from such numbers is not possible the data does suggest that, provided that an established bronchoscopy protocol is followed and the procedure is performed by an experienced group of operators with dedicated after-care, three consecutive bronchoscopies can be carried out in volunteers with asthma. A repeat study might consider airway biopsy at a fourth time point with a reduction in the number of biopsies taken at each bronchoscopy. Such an extended study would likely provide insights into what aspects of remodelling and TGF-β Superfamily signalling may be yet sustained when AHR returns to baseline levels.

An important point of current discussion is what relationship airway inflammation has to airway remodelling? There are only three possibilities; firstly that inflammation leads to airway remodelling; secondly that the asthmatic airway has an intrinsic propensity to remodelling in response to obvious environmental insults such as viral infection, pollution or allergen and that inflammation is both promoted and sustained in response to such an abnormal airway architecture; or thirdly both inflammation and remodelling are independent separate events in the airway each contributing to different aspects of the asthma phenotype.

The findings examined in this thesis confirm that allergen provocation is associated with the rapid and simultaneous activation of cellular inflammation and airway remodelling but dissociation of selected aspects of remodelling from inflammation occur at a later time point. An explanation for this observation may be that the epithelial–mesenchymal trophic unit (EMTU) initially responds and interacts with the steroid responsive Th2 mediated aspects cellular of inflammation and mediators, but that the EMTU may itself take on a more active and sustained role in driving remodelling in the absence of further inflammatory cell recruitment. In support of this concept is the finding that airway myofibroblasts (α-SMA) and collagen are partially responsive to corticosteroids (Bergeron *et al.* 2005), presumably by attenuation of the inflammatory contribution to remodelling. IL-4 and IL-13 are established key cytokines in inflammatory propagation. Recent studies have identified airway structural cells as additional targets for these cytokines. IL-13 knockout mice display decreased subepithelial fibrosis, epithelial changes and goblet cell hyperplasia (Kumar *et al.* 2002) as well eosinophils and other inflammatory cell recruitment. Eosinophils are implicated in both inflammation and remodelling by the release of cytokines and fibrogenic factors (Phipps *et al.* 2002). Such a mechanism of EMTU mediated remodelling may explain the more complex and severe disease phenotypes where airway obstruction and AHR becomes less sensitive to anti-inflammatory therapy (i.e steroid refractory) and less dependent on factors such as environmental allergens which are considered more important in the milder disease phenotypes. These findings also provide an explanation for why some aspects of remodelling may be steroid responsive whilst others are not, and why disease progression can occur despite anti-inflammatory therapy. Why some asthmatics develop such complex and steroid refractory phenotypes is not known.

Whilst it is likely that inflammatory events may initiate remodelling through epithelial-mesenchymal activation it is possible that such activation may occur independently of inflammation, for example through allergen-induced injury of epithelium directly or mechanical stretch leading to epithelial activation, both events leading to underlying mesenchymal signalling and activation. Epithelial-fibroblast *in-vitro* co-culture experiments support such a mechanism. The development of animal models with an inducible vulnerability for airway epithelial injury and abnormal repair is eagerly awaited as such models may help in the evaluation of what aspects of

remodelling events can proceed in the absence of inflammation as a result of aberrant epithelial-mesenchymal signalling alone .

Further studies into understanding the mechanisms of how inflammation and remodelling occur in asthma and how these contribute to altered airway function, in particular AHR, are required. An important follow on study would be to look at the inflammatory and remodelling changes allergic of rhinitic patients in a time course manner with allergen provocation as defined in this thesis. Allergic rhinitic (AR) patients show some features of airway remodelling (Chakir *et al.* 1996). If the asthmatic airway is predisposed to dysregulated and exaggerated repair, then it can be expected that in the AR group inflammation and remodelling will be similar to the asthmatic group in the acute phase at 24 hours with resolution of remodelling markers that were otherwise sustained in the asthmatic group at the 7 day time point. Another important study would comprise a group of asthmatics on high-dose inhaled steroids to evaluate the remodelling response when cellular inflammation is more attenuated.

In this thesis the expression of TGF-β Superfamily ligands and signalling pathway components in the asthmatic airway compared to the normal airway have been defined and the activation and modulation of these signalling pathways in response to disease activation in response to allergen challenge evaluated. These are the initial steps towards an understanding of the complex signalling pathways that are operative in the asthmatic airway. The data confirms that TGF-β Superfamily signalling pathways are markedly abnormal in the asthmatic airway. Rapid activation and modulation of signalling components is seen with allergen-induced disease provocation. However, despite modulation of signalling components, the expression levels of selected receptors and inhibitory Smads, at least in immunohistochemical terms, remains markedly abnormal. This suggests that TGF-β Superfamily signalling in the asthmatic airway is occurring through alternative receptor combinations without strict regulation. Combinatorial interactions of different Type I and Type II receptors provide a versatile system by which TGF-β ligands can achieve multiple outcomes in a cell. At present comprehension into signalling by this complex pathway is at a very basic stage but it can be predicted that such aberrant signalling leads to functional outcomes that contribute to disease pathology in asthma. The challenge is to understand the exact functional consequences of such altered signalling has for the airway. In terms of therapeutic intervention, it is obvious even at this stage that

targeting a single TGF-β ligand by itself is unlikely to be effective given the complexity of receptor modulation that is present and the ability for other signalling cascades to interact and regulate TGF-β signalling pathway components. Only by understanding the initiation and integration of these complex pathways can TGF-β signalling become a therapeutic target.

The inherent weakness of the data in this study is that it is predominantly derived from immunohistochemical work which is a semi-quantative method. In addition there is no functional data to support the concepts of TGF-β Superfamily signalling highlighted by this work. Nevertheless it is possible to begin to propose an overall mechanism of TGF-β Superfamily signalling in asthma (Figure 7.1). In immunohistochemical terms, similar levels of TGF-β_{1-2} and BMP ligand expression is present in both the normal and asthmatic airway, such expression being consistent with the known regulatory role in airway inflammatory and repair processes. The asthmatic group demonstrated increased numbers of cells expressing TGF-β_3 and activin-A. It appears that activin-A may be a more a disease specific growth factor, as has been shown for other inflammatory and fibrotic disease such as Crohn's disease. Increased staining of activin-A in baseline asthma with identification of inflammatory cells as significant sources suggests that the asthmatic airway has a tendency for a pro-fibrotic response following injury. The level of Smad7 expression is a determinant of overall TGF-Superfamily responsiveness. A pro-fibrotic tendency is also suggested by the significantly low levels of inhibitory Smad7 in asthma, indicating that TGF-β signalling pathways are inherently dysregulated compared to the normal airway. Allergen-induced airway injury is associated with the rapid activation of stored ligand rather than rapid synthesis, although there was evidence for increased epithelial and inflammatory cell sources of BMP-7. The down-regulation of the TGF-β_{1-3} Type I receptor ALK-5 but not the down-regulation of the predominant activin receptor ALK-4 may suggest that the rapid increase in pSmad2 signalling is a predominant response to activin signalling. The activin Type II receptor ActRIIA is down-regulated in asthma compared to normal volunteers. Thus activin signalling may be predominantly through the other Type II Receptor ActRIIB, a combinatorial interaction that leads to an alternate functional outcome in the cell. The rapid increase in ActRIIA at the 24 hour post allergen time point may be an attempt by the airway to regulate the activin signalling response. The versatility of the signalling system is such that the increased expression of the ActRIIA is perhaps a response to local

activation of BMP ligands. Baseline expression of BMPRII in the asthmatics group was less compared to the normal airway with no further modulation despite rapid BMP signalling as evidenced by increases in pSmad1/5 signalling. BMP can also signal through ActRIIA and ActRIIB. Therefore it is possible that up-regulation of ActIIA is also related to BMP mediated signalling. BMP-ActRIIA signalling will lead to a separate functional outcome compared to signalling via BMPRII. Rapid induction of Smad7 suggests that some regulation of the signalling process occurs but overall the intensity and degree of Smad7 expression fails to reach that of the normal airway. Thus altered and dysregulated TGF-β signalling occurs in asthma.

It is also possible to begin to propose how the allergen induced injury-repair process may proceed in atopic asthma. Allergen challenge is associated with inflammatory cell influx, an important source of cytokines such as IL-4 and IL-13 but also TGF-β Superfamily ligands. There is also local activation of stored TGF-β Superfamily ligands. Activation of structural cells can now occur. Epithelium in particular is an important source of TGF-β Superfamily growth factors. Given the dysregulated TGF-β signalling pathways present in asthma at baseline, ligand activation may lead not only to an exaggerated and dysregulated response but also to an altered functional cellular outcome. Such aberrant signalling may thus contribute to a sustained and excessive repair response that remains associated with AHR in the absence of further inflammatory cell influx.

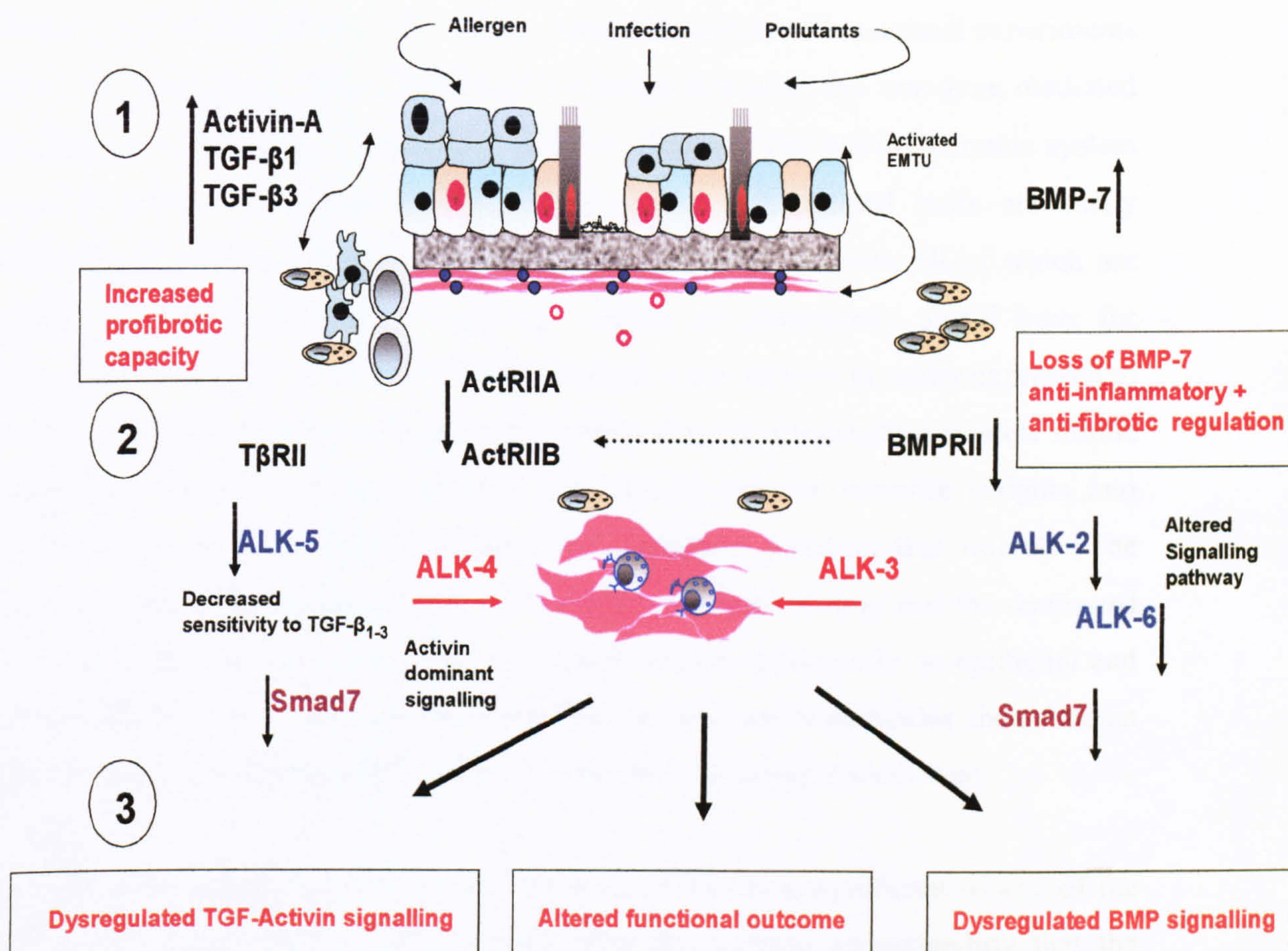


Figure 7.1 Illustration of a proposed pathway of TGF-β Superfamily signalling in asthma.

(1). The excess of TGF-β_3 and activin-A alongside the low levels of the inhibitory Smad7 in the asthmatic airway leads to an increased fibrotic tendency in response to environmental insults. (2) The TGF-Superfamily response is dysregulated Altered expression of Type II and Type I receptors leads to alternative ligand-receptor combinatorial interactions. Down-regulated ALK-5 expression suggests attenuation of cellular sensitivity to TGF-β_1-$_3$; epithelial ALK-4 expression remains unaltered and is further up-regulated in inflammatory cells and myofibroblasts, suggesting activin mediated signalling may be the dominant ligand leading to active signalling via pSmad2. BMP-7 may be less efficient in its anti-inflammatory and anti-fibrotic role as its signalling receptors are markedly altered. (3) Activation of TGF-β Superfamily signalling in asthma may therefore leads to an altered functional outcome for the cell.

The functional significance of the modulation of signalling pathways cannot be effectively studied in humans and it is in these circumstances that animal experiments are valuable. Already many hypotheses involving molecular and one-gene mediated diseases have been effectively studied in whole animals. The murine immune system in particular is well characterised, in-bred strains with defined traits are easily available and there is the ability to easily manipulate the genome, all of which are distinct biological advantages. Trangenic models are particularly useful here, for example where a specific Type I or Type II receptor can be over or under expressed to evaluate tissue responses to injury. Not withstanding that transgenic models mimic isolated aspects of a complex process, they clearly provide valuable insights into dissecting the complex TGF-β Superfamily signalling cascades that operate. The increased amounts of activin-A expression in the asthmatic airway and the continued high levels of expression of its Type I receptor ALK-4, particularly on epithelial and mesenchymal cells, would indicate this particular receptor in particular should be an area of focus in such a transgenic model combined with allergen challenge.

An important finding in this study is that eosinophils are a significant source of the anti-fibrotic ligand BMP-7. This concurs with the growing understanding that the eosinophil may be a repair cell. Given that eosinophils are also the predominant inflammatory cell source of TGF-β_1 (Minshall *et al.* 1997) it may be that BMP-7 is co-secreted alongside profibrotic TGF-β_1 in order to regulate the repair response. Although BMPRII is the predominant Type II receptor for BMP ligand signalling, BMP-7 can also bind either ActIIA or ActRIIB and can subsequently recruit ALK-2, ALK-3 or ALK-6. Given the findings in this study of abnormal BMPRII expression both in baseline asthmatic airway and after allergen provocation, it may be that BMP-7 signalling leads to a functional outcome in the airway that is not necessarily regulation of repair processes. Such hypotheses will be important to investigate, examining also factors that induce BMP-7 expression in eosinophils.

By characterising the expression pattern and the functional activation of TGF-β system components in human asthmatic airways in a time-course manner, it is possible to begin to identify which aspects of TGF-β signalling may be relevant to human asthma. Although fibrosis is very relevant to other organ systems such as the kidney, liver and heart it is impossible to provoke disease and obtain tissue in a time

course manner to follow the dynamic events of TGF-β signalling in relation to remodelling events in these organs. This thesis has validated that this is possible in the asthmatic airway. It therefore presents a very novel and important model of human asthma.

An important future step would be to quantitate mRNA of the TGF-β signalling pathway components at the cellular level. This is important as not only will it provide quantitative measures of expression but will also determine whether the difference in expression in asthma is a result of down-regulation at the level of gene transcription and translation. Tissue has been processed and preserved to be available for future laser capture micro-dissection (LCM) which can be used to isolate specific cell types within the biopsy after delineation by immunostaining. This technique is becoming increasingly established in research centres. So far, direct analysis of tissues from mammals has not been straightforward due to the cellular complexity of the material. The affected cellular component often represents a small fraction of the total cellular mass and the composition of the tissue is commonly altered in consequence of the disease. Therefore changes in protein expression can be masked or can be due to comparison of heterogeneous samples, thus not reflecting specific changes in their regulation. The development of the laser capture microdissection technology has provided a powerful way of overcoming these problems. This technology allows the isolation of tissue sub-compartments i.e. epithelial, endothelial or inflammatory cells from either diseased or healthy tissue, restricting the analysis/comparison to similar tissue sub-compartments or even single cells. LCM can be used to isolate epithelial cells, fibroblasts, eosinophils and smooth muscle cells for subsequent quantitative real-time PCR analysis of mRNA expression for TGF-β Superfamily ligands and receptors. Such data obtained will be essential for the design of blocking interventional studies in both animal models and *in-vitro* cell culture systems. Experiments using dominant-negative Smads and receptors will provide insight into which components of the signalling pathway have most relevance to the signalling process.

In summary this thesis presents a model of airway remodelling and TGF-β Superfamily signalling in asthma. Encouraging safety and tolerability data for three consecutive bronchoscopies is presented. It is shown for the first time that sustained increases in cellular inflammation is not required for the maintenance of AHR and

that inflammation can be dissociated from AHR and airway remodelling. These findings allow further hypotheses to be proposed and support the view that remodelling may contribute some component to AHR. The study has also confirmed that the TGF-β Superfamily signalling pathways are markedly abnormal in the asthmatic airway. There is rapid activation and sustained modulation of signalling pathways in response to allergen provocation suggesting TGF-β Superfamily signalling has functional consequences for the asthmatic airway. This study is a first step towards identifying remodelling and TGF-β Superfamily signalling events for planning future mechanistic and interventional studies in the future. Conclusions from such studies may lead to novel and target-specific therapeutic intervention in asthma.

Chapter 8
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