

# **Exploring the expression and function of CRTh2 in asthma**

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## ABSTRACT

### Exploring the expression and function of CRTh2 in asthma

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CRTh2 (DP2) is implicated in the pathogenesis of asthma; however, currently there is a lack of data describing the protein expression of CRTh2 in bronchial biopsies in asthma. This has limited the cell types that CRTh2 function has been explored within. A thorough understanding of CRTh2 expression within the airways and whether changes in receptor expression correlates with disease severity, may aid in the design of future CRTh2 antagonist clinical studies.

This study aimed to investigate the expression of CRTh2 expression in bronchial biopsies of subjects with asthma and healthy controls. The novel finding that CRTh2 was expressed on bronchial epithelial cells in asthma prompted further investigation into the expression and activation of CRTh2 on bronchial epithelial cells *in vitro*, using the selective CRTh2 agonist 13, 14-dihydro-15-keto prostaglandin D<sub>2</sub> (DK-PGD<sub>2</sub>) and the CRTh2 selective antagonist AZD6430.

This study is the first to describe differential CRTh2 expression within bronchial tissue in asthma compared to healthy controls. The number of sub-mucosal CRTh2<sup>+</sup> cells was found to be increased in asthma compared to healthy controls. CRTh2 was found to be expressed on the bronchial epithelium and its expression was decreased in asthma compared to healthy controls with similar differences observed *in vitro* by primary epithelial cells. Squamous metaplasia of the bronchial epithelium was increased in asthma and related to decreased CRTh2 expression. DK-PGD<sub>2</sub> promoted epithelial cell migration, and in air-liquid interface cultures increased the number of MUC5AC<sup>+</sup> and involucrin<sup>+</sup> cells, which were blocked with the CRTh2 antagonist, AZD6430.

This study describes the novel findings that CRTh2 is expressed by the bronchial epithelium in both health and asthma, and its activation drives epithelial differentiation. These data suggests that CRTh2 could contribute to airway remodelling in asthma and this information may contribute to the understanding of the effects of CRTh2 antagonists in asthmatic patients.

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## **Publications and presentations arising from this thesis**

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## Abbreviations

AHR	Airway hyper-responsiveness
ALI	Airway liquid interface
ANOVA	Analysis of variance
AP	Activator protein
ASM	Airway smooth muscle
AZ	AstraZeneca
BDR	Bronchodilator reversibility
C5a	Complement receptor 5a
CCR	C-C Chemokine receptor
CD	Cluster of differentiation
CK	Cytokeratin
CLC	Calcium activated chloride channel
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRTh2	Chemoattractant receptor homologous molecule expressed on Th2 cells
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAPI	Diamidino-2-phenylindole dihydrochloride
DK-PGD2	13, 14-Dihydro-15-keto prostaglandin D2
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	D-prostanoid
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial mesenchyme transition
EMTU	Epithelial mesenchymal trophic unit
EPO	Eosinophil peroxidase
FEV <sub>1</sub>	Forced expired volume (in 1 second)
FGF	Fibroblast growth factor
FMLP	Formyl methionyl leucyl phenylalanine
FP	Fluticasone propionate
FVC	Forced Vital Capacity
GCS	Glucocorticosteroids
GINA	Global Initiative for Asthma
GMA	Glycol Methacrylate
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G-Protein Coupled Receptor
GR	Glucocorticoid Receptor
H&E	Haematoxylin & Eosin
HDM	House Dust Mite
HEK	Human Embryonic Kidney
HETE	Hydroeicosate traenoic acid
HLMC	Human Lung Mast Cell
HMC-1	Human mast cell line-1
H-PGDS	Hematopoietic Prostaglandin D Synthase

ICS	Inhaled Corticosteroids
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate Lymphoid Cell
LABA	Long Acting $\beta$ 2 Agonists
L-PGDS	Lipocalin Prostaglandin D Synthase
MBP	Major Basic Protein
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix Metalloprotease
MTT	3-4,5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide
NBF	Neutral Buffered Formalin
PBS	Phosphate Buffered Saline
PC <sub>20</sub>	Provocative Concentration (of methacholine required to cause 20% fall in FEV <sub>1</sub> )
PCR	Polymerase Chain Reaction
PG	Prostaglandin
RNA	Ribonucleotide acid
SMA	Smooth Muscle Actin
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TER	Trans-Epithelial Resistance
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TP	Thromboxane A2 receptor
VEGF	Vascular endothelial growth factor



---

# **Chapter 1**

## **Introduction**

---



### ***1.1. The burden of asthma***

The prevalence of asthma ranges from 1-18% of the population across different countries, affecting as many as 300 million people worldwide and is associated with a large socio-economic burden (1). This disease remains incurable with no significant new treatments introduced in the last 30 years despite intense investigation. This highlights the need for both further understanding of the disease and the need for new asthma therapies.

### ***1.2. Clinical description of asthma***

As defined by the Global Initiative for Asthma guidelines (GINA), "Asthma is a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation" (1). This description highlights that the exact pathogenesis of asthma is unknown and that there is heterogeneity in the symptoms of the disease. These aspects make the application of current asthma therapies for patients difficult, and the development of novel asthma therapies challenging.

### ***1.3. Clinical parameters***

A number of clinical parameters are used to assess if individuals have asthma and once diagnosed to assess the progression of the disease. Changes in forced expiratory volume in one second ( $FEV_1$ ), is used as one parameter to diagnose asthma.  $FEV_1$  decreases with bronchoconstriction, and a significant increase in  $FEV_1$  after bronchodilator indicates reversible airflow obstruction and supports the diagnosis of asthma (2,3). However, an absent responsiveness to bronchodilator does not exclude asthma (4). A reduced ratio of  $FEV_1$  to Forced Vital Capacity

(FVC) ( $FEV_1/FVC$ ) indicates airflow limitation. Population studies suggest that the  $FEV_1/FVC$  is normally greater than 0.75 to 0.80 (5), and any values less than this suggest airflow limitation. Airway hyper-responsiveness (AHR) is defined as an increased bronchoconstrictor response to a non-specific stimulus (6). The response can be measured using a non-selective stimulus that provokes bronchoconstriction and this is typically performed by inhalation of agents, such as methacholine. Methacholine  $PC_{20}$  is the provocative concentration of methacholine required to cause a 20% fall in  $FEV_1$ . A normal range for this parameter is  $>8\text{mg/ml}$  of methacholine and an increased AHR is suggested by a reduced  $PC_{20}$  (7-10). Presence of atopy increases the probability that a patient with respiratory symptoms has allergic asthma. Atopic status can be identified by skin prick testing or by measuring the level of allergen specific immunoglobulin E (sIgE) in serum (1). Symptoms scores of asthma as assessed by the patient can be measured using an asthma control questionnaire (ACQ) (1). Induced sputum differential counts can be used to assess if a patient has a predominance of a specific inflammatory cell phenotype such as eosinophils or neutrophils within the airways. Eosinophil inflammation can also be evaluated using exhaled nitric oxide concentration ( $FE_{NO}$ ). Higher sputum eosinophil counts are associated with more marked airways obstruction and reversibility, greater asthma severity and atopy (11). A normal sputum eosinophil count is  $<2\%$  (12,13). There is growing evidence that measures of eosinophilic airway inflammation are more closely linked to a positive response to corticosteroid treatment (14-16), demonstrating the importance of these measurements in the control of asthma, and the targeting of therapies.

#### ***1.4. Disease stratification***

Factors that determine the pattern of airflow obstruction, response to treatment, and natural history of asthma are highly variable, giving rise to the concept of disease heterogeneity. This has led to alternative classification methods such as clinical and inflammatory phenotypes, endotypes or trigger type being proposed with the aim of providing more targeted treatments (17-19). These classification methods are based on the grouping of several observable characteristics that tend to track together (20,21). Examples of phenotypes in asthma include hereditary, early onset allergic asthma, poorly reversible, very severe, neutrophilic asthma, late onset eosinophilic asthma and late onset, symptom dominant, obese minimal inflammation asthma (22-26).

#### ***1.5. Severe asthma***

While most asthma is well treated with current medications, approximately 10-20% of patients remain refractory to current gold standards of treatment (27,28). This remaining percentage represents a heterogeneous group of individuals, some of whom appear to have a more severe form of traditional allergic asthma, whereas many others do not (21). Severe asthma is defined as “asthma which requires treatment with high dose inhaled corticosteroids (ICS) plus a second controller to prevent it becoming ‘uncontrolled’ or which remains ‘uncontrolled’ despite this therapy” (29). There is a positive correlation between healthcare costs and asthma severity (30), with patients with severe disease and frequent exacerbations accounting for 50% of the costs of asthma (30). This group of asthmatics is therefore an important area of focus for research into the pathogenesis of asthma and for the development of new therapies.

Disease severity within the current study was categorised using the Global Initiative for Asthma (GINA) treatment guidelines (1). Using these guidelines, mild asthma incorporates patients that are classed as GINA I and II, moderate asthma as GINA III, and severe asthma as GINA IV and V.

### ***1.6. Current asthma therapies***

Currently, the most effective anti-inflammatory drug used for the treatment of persistent asthma are inhaled corticosteroids (ICS), with prolonged administration found to substantially improve symptomatic lung function (31). However, corticosteroids have shown limited, if any, benefit in reducing airway remodelling (32-36). Glucocorticoids (GCs) are effective in the control of mild to moderate asthma. They exert their effects by binding to the intracellular glucocorticoid receptor (GR) and the activated GR-GC complex rapidly translocates to the nucleus where it up-regulates anti-inflammatory proteins (an affect called transactivation) or down-regulates the expression of many inflammatory related genes (an affect called transrepression) (37,38).

$\beta_2$ -agonists have been shown to reduce airflow limitation by improving airway diameter through direct induction of airway smooth muscle relaxation via the  $\beta_2$ -receptors. They are broadly classified according to their duration of action; short-acting  $\beta_2$ -agonists (SABA) and long acting  $\beta_2$ -agonists (LABA) (39,40).

Some asthma therapies currently used are administered to a specific group of asthmatics, such as patients with severe asthma. Long-acting anticholinergic agents (LAMAs) such as tiotropium bromide have been shown to control disease in patients who have severe asthma (41-43). Methylxanthine therapeutics such as theophylline

are considered to have bronchodilator, anti-inflammatory and anti-tussive properties (44) and can be used for the treatment of severe asthma (45).

Asthma treatments have also been developed to interfere with the production of pro-inflammatory mediators such as the leukotrienes (LT), including cysteinyl LT (CysLT) and LTB<sub>4</sub>. These lipid mediators, together with their respective receptors CysLT and BLT are considered to be important in the pathogenesis of asthma (46,47). To date, no conclusive studies have demonstrated efficacy for leukotriene receptor antagonists (LTRAs) in severe asthma. However, the CysLT<sub>1</sub> receptor antagonist, montelukast has been shown to improve FEV<sub>1</sub> and the number of exacerbations for asthmatic patients with particular genetic variants of genes involved within the leukotriene pathway (48).

Monoclonal antibody treatments have also been developed for the treatment of asthma. Omalizumab, an anti-IgE therapy which prevents the link of IgE to high-affinity IgE receptors (FcεRI) (49) on a number of inflammatory cells thus preventing subsequent triggered inflammatory cascades (50,51) is currently used to treat patients with severe uncontrolled allergic asthmatics with raised IgE levels and a positive skin prick test to a perennial allergen (52).

These currently used asthma therapies reduce, but do not eliminate, the impact of day-to-day symptoms and the frequency of exacerbations for asthmatic patients. In addition, due to the heterogeneity of asthma and the genetic variation of asthmatic patients, there is still a requirement for the development of new asthma therapeutics.

### ***1.7. Pathogenesis of asthma***

The direct cause of asthma is not known. A number of gene association studies have implicated genes encoding for inflammatory cytokines and receptors including those involved in Th2 type responses such as the IL-4 gene cluster (53), IL-1 (54) and IL-33 (55). In asthma, the airway wall is known to be infiltrated with mononuclear cells, which are mostly CD4+ T-cells, and with eosinophils. In addition, mast cells, macrophages, B-cells, and neutrophils have been reported as being variably increased in the airways of asthmatics compared with those of controls (56). Another area of research into the pathogenesis of asthma that has attracted significant interest is the structural changes of the airway wall, collectively referred to as airway remodelling. These are thought to be a result either of the interaction of inflammatory mediators with stromal cells or of abnormal tissue injury repair mechanisms (57).

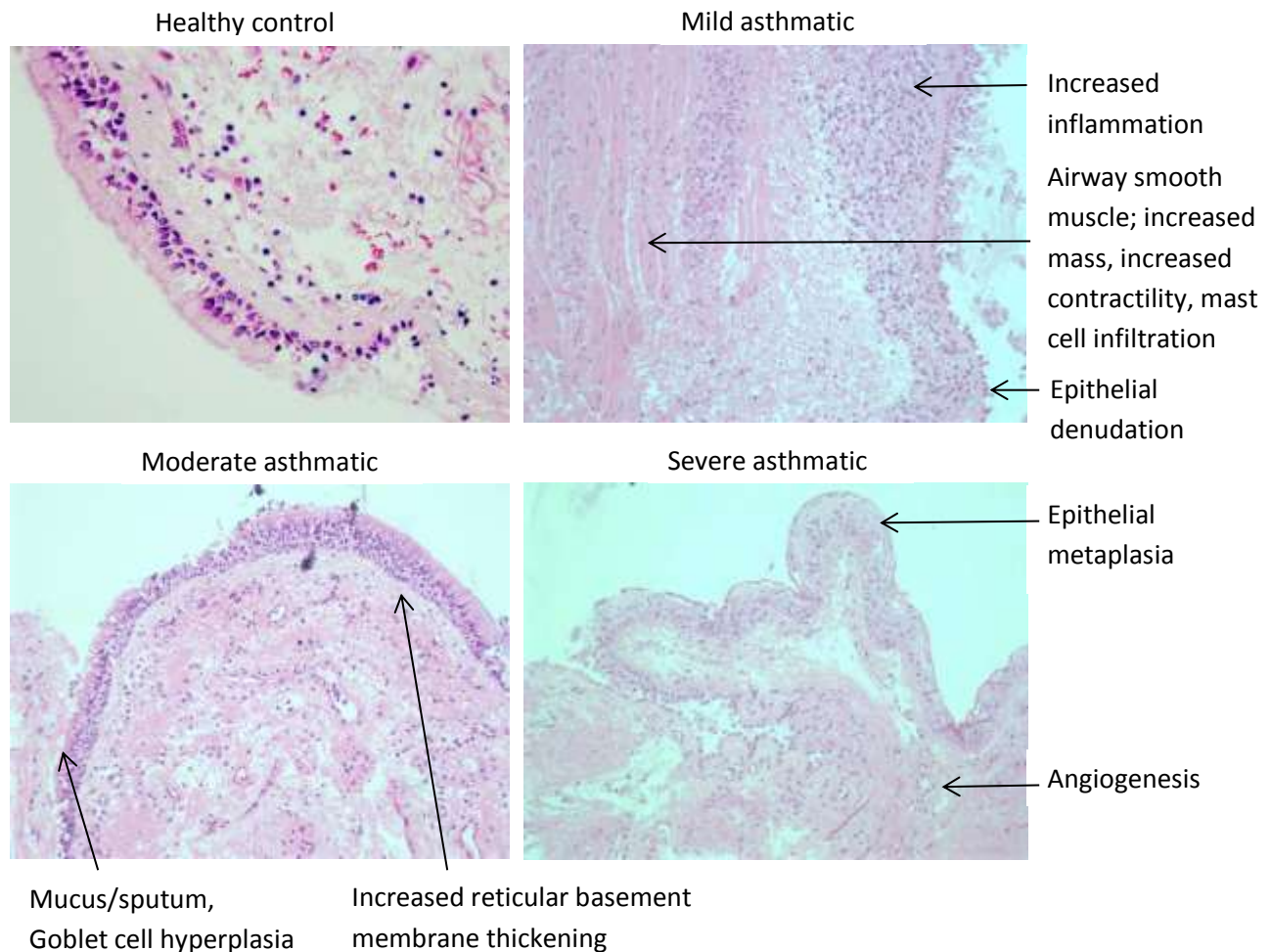
### ***1.8. Airway remodelling in asthma***

Airway remodelling in asthma is the term used to describe the structural changes in the airway walls thought to be caused by repeated cycles of injury and repair (58). Although structural changes in the airway are strongly correlated with inflammation, aspects of airway remodelling may occur independently of airway inflammation, such as mechanical stresses resulting from bronchoconstriction (59,60). The main components of airway remodelling are considered to be (61);

- 1) Surface epithelial metaplasia, goblet cell hyperplasia, increased mucus secretion

- 2) Fibrosis with deposition of abnormal extracellular matrix (ECM) components in the basement membrane layer beneath the epithelium (collagen, tenascin, matrix proteins)
- 3) Increased thickness of smooth muscle due to muscle cell and myofibroblast hyperplasia
- 4) Angiogenesis

**Figure 1.1** highlights the remodelling changes that have been identified for asthmatic subjects. The airway epithelial cells, fibroblasts and smooth muscle cells undergo significant phenotypic differentiation during the remodelling process and result in thickening of the airway wall, which makes the airways stiffer and less distensible, as well as narrowing of the airway lumen. Biopsy specimens from asthmatics have been found to have thickened airway epithelium and reticular basement membrane, goblet cell hyperplasia and mucus hyper-secretion (62-64). In severe asthmatic samples a thickened epithelial layer has been found with epithelial cells that express activation and repair markers (64-66). Chronic exposure to pro-inflammatory stimuli has been reported to lead to excessive activation such as the up-regulation of NF $\kappa$ B, with damage and structural changes to the epithelium, for example, Th2 cytokine induced goblet cell hyperplasia, which is frequently seen in the airways of asthmatic patients (67). It is also not uncommon to observe epithelial cell metaplasia as part of the remodelling response, especially in severe asthmatic biopsies (68). Metaplasia is defined as the transformation of differentiated tissue into differentiated tissue of another type (69). The most frequent metaplasia within epithelial tissue is squamous metaplasia (69).



**Figure 1.1.** Haematoxylin and eosin (H&E) images describing the airway remodelling changes in asthmatic subjects (x10 magnification).

### ***1.9. Why is airway remodelling important in asthma?***

Structural changes have been observed in airway biopsies of children years before the symptoms of asthma can be measured (70). Airway biopsy studies in children suggest that pathologic changes such as epithelial cell loss, basement membrane thickening, and angiogenesis occur early on in the development of asthma (71-76). Biopsies from children with asthma contain evidence that the epithelial cells are stressed, such as expression of heat shock proteins, leading to further remodelling changes such as collagen deposition within sub-mucosal tissue, even in the absence of inflammation (77). These studies demonstrate the impact of



airway remodelling changes in influencing the structural components of the airways even without significant inflammatory cells being present. In addition, clinical consequences of airway remodelling and inflammation that have been described include fixed airway obstruction and AHR leading to asthma symptoms such as breathlessness and wheezing (78). Reduced airway diameter, an increase in smooth muscle contractility, the degree of epithelial injury, dysfunctional neuronal regulation, an increase in microvascular permeability, and many inflammatory mediators, have also been associated with AHR (79). In addition, an increase in smooth muscle mass has been found to correlate with lower lung function in patients with asthma (80). Further still, changes to bronchial epithelial cells, such as loss of tight junction proteins and increase in expression levels of 15-HETE have been linked to clinical status and AHR (81,82). Elevated mucus levels in asthma are thought to be important as acute degranulation of hyperplastic goblet cells are thought to be a possible mechanism for asthma exacerbations in mild and moderate asthma (62). Also, MUC5AC rich mucus has been found in asthmatic patients (62), and mucus hyper-secretion has been found to strongly contribute to morbidity and mortality during exacerbations (83). Finally, further understanding of airway remodelling changes are required as to date the structural changes established in asthma are poorly responsive to current treatments such as corticosteroids (34,84,85).

#### ***1.10. Allergic asthma***

Clinically, most asthmatic patients exhibit acute immediate hypersensitivity responses to common inhaled allergens (allergic/atopic asthmatics), which leads to reversible airway obstruction through the triggering of mast cell degranulation in an

immunoglobulin E (IgE) dependent way. However, a proportion of patients with asthma can't be classified on the basis of immunological mechanism (non-allergic/non-atopic) (86). Allergens which may trigger asthma include components of house dust mite faeces (87,88), fungal spores (89,90), and pollen (91,92). Non-allergic triggers include respiratory viral infections (93), air pollution (94), occupational exposures (95), exercise (96), aspirin (97), and lifestyle factors (98,99).

In allergic asthma, Th2 cells and the cytokines they produce lead to eosinophilic inflammation, mucus hyper-secretion, AHR, and persistence of disease (57). Th2 cells are characterised by the secretion of IL-4, IL-5, IL-9, and IL-13. The transcription factor GATA-binding protein (GATA3) is crucial for the differentiation of uncommitted naïve T-cells into Th2 cells and the regulation of the secretion of Th2 cytokines (100). Mediators that enhance Th2 cell induction and activation, or augment allergen-driven asthma, are considered important targets to modulate asthma (57). Recent clinical trials of anti-cytokine antibodies that block IL-5 or IL-13 showed therapeutic efficacy in selected populations of patients with asthma with evidence of Th2 cytokine-driven disease (101). In addition a recent randomised controlled trial using an anti-IL-4 $\alpha$  receptor antibody called dupilumab, reduced the number of exacerbations and improved lung function for persistent, moderate-severe asthma with elevated eosinophil levels (102). These clinical studies demonstrate that Th2-targeted therapy combined with strict subject selection can effectively improve asthma symptoms. A molecule found to be involved in the Th2 pathway that recently has attracted significant interest for the development of antagonists for asthma is, CRTh2 (Chemoattractant Receptor-Homologous molecule expressed on Th2 cells) also called DP2 or GPR44.

### ***1.11. Description of CRTh2***

CRTh2 is a G-protein coupled receptor (GPCR) that has been found to be expressed on Th2 cells (103,104). Activation of Th2 cells through CRTh2 leads to the release of IL-4, IL-5 and IL-13 (105,106). Due to these findings, and other observations described in more detail below, the development of CRTh2 antagonists for asthma and allergic-related diseases has been a very active area in recent years (107-109).

The major endogenous ligand for CRTh2 is prostaglandin D<sub>2</sub> (PGD<sub>2</sub>). This ligand is released in large amounts by mast cells during allergic reactions and is found at high levels in the bronchoalveolar lavage fluid of asthmatics both constitutively (110-112) and following acute antigen challenge (113-115).

### ***1.12. Clinical and pre-clinical evidence for a role for CRTh2 in allergy and asthma***

An increase in the number of CRTh2 positive inflammatory cells in allergic disease (116) and genetic association of CRTh2 sequence variants with asthma and allergy phenotypes (117,118) have strengthened the linkage between CRTh2 and inflammatory related disorders. Activity directed toward the development of CRTh2 antagonists has steadily increased with twenty antagonists having progressed to clinical development for diseases such as asthma and allergic rhinitis (107-109). To date, limited efficacy has been demonstrated for CRTh2 antagonists in asthma (119,120); however, it remains to be determined whether CRTh2 antagonism is more effective in a sub-set of patients. **Table 1.1** summarises the published CRTh2 antagonists used in clinical studies in recent years. This table demonstrates that a

number of clinical studies have been carried out in asthmatic patients. The mixed results achieved for CRTh2 antagonist studies in asthmatic patients is likely to be influenced by the patient population involved in the study. This is demonstrated by more significant findings being demonstrated for patients selected based on their sputum eosinophil count (121) or for allergic status (122). For a moderate persistent asthma group, where the patients were free of inhaled steroids a CRTh2 antagonist demonstrated significant beneficial effects with respect to FEV<sub>1</sub>, peak flow, circulating IgE levels, night time asthma symptoms and quality of life score (119). The same compound also reduced sputum eosinophil counts post allergen challenge in asthmatic subjects (123). However, in a study looking at patients with inadequately controlled moderate to severe asthma, with continued inhaled corticosteroid treatment, a dual antagonist of human D-prostanoid receptor (DP1) and CRTh2 was not effective in improving asthma symptoms or lung function (120). The lack of efficacy achieved within this study utilising a dual PGD<sub>2</sub> receptor antagonist, could be attributed to opposing functional roles of the DP1 receptor and CRTh2. Recent studies have demonstrated that in response to PGD<sub>2</sub>, DP1 may promote anti-inflammatory actions, whereas CRTh2 may promote pro-inflammatory actions in animal models of ulcerative colitis and skin inflammation (124,125). Similar actions may therefore be present within asthma and if this is the case, antagonism of CRTh2 pro-inflammatory actions only could be more effective in improving asthma clinical end-points.

The CRTh2 antagonist clinical studies highlight that a thorough understanding of CRTh2 expression within the airways and whether changes in receptor expression correlates with disease severity, may aid in identifying the most

responsive asthmatic group. In addition, determining the distribution of CRTh2 expressing cells may provide information for alternative target cells for CRTh2 antagonists, potentially suggesting additional clinical end-points for evaluation of antagonist efficacy.

**Table 1.1.** Summary of recently published CRTh2 antagonist clinical studies

<b>Reference</b>	<b>Antagonist</b>	<b>Study</b>	<b>Reported significant effects compared to placebo</b>	<b>Reported non-significant effects compared to placebo</b>
Gonem (2014)(121)	CRTh2, QAW039	Asthmatics with sputum eosinophil count >2%	Significant reduction in sputum eosinophilia, asthma symptoms. Significant improvement in post bronchodilator FEV <sub>1</sub>	
Diamant (2014)(122)	CRTh2, Setipirant	Allergic asthmatics	Significant reduction in allergen-induced late asthmatic response and AHR to methacholine.	No difference in early asthmatic response or in allergen-induced changes in eNO.
Pettipher (2014)(126)	CRTh2, OC000459	Asthmatics predicted FEV <sub>1</sub> 60-85%	Significant improvement FEV <sub>1</sub> Improvements in ACQ. Lower incidence of exacerbations and respiratory infections	
Krug (2014)(127)	CRTh2, BI 671800	Seasonal allergic rhinitis	Significant reduction nasal eosinophil numbers, nasal inflammatory cytokine levels	
Snell (2013)(128)	CRTh2, AZD1981	Moderate-severe COPD		No significant changes in lung function, COPD symptom score
Straumann (2013)(129)	CRTh2, OC000459	Eosinophilic esophagitis	Significant decrease in esophageal eosinophil load. Reduced extracellular deposits of eosinophil peroxidase and tenascin C	

Busse (2013)(120)	CRTh2, DP1, AMG 853	Moderate-severe asthma		No significant changes in ACQ, FEV <sub>1</sub> , symptom scores and exacerbations
Horak (2012)(130)	CRTh2, OC000459	Rhinoconjunctiv itis	Significant reduction in nasal and ocular symptoms following grass pollen challenge	
Singh (2013)(131)	CRTh2, OC000459	Steroid-naïve asthmatics	Significant reduction in late asthmatic response to bronchial allergen challenge	No significant changes in early asthmatic response to bronchial allergen challenge
Barnes (2012)(119)	CRTh2, OC00459	Moderate- persistent asthma, steroid- free	Significant improvement in FEV <sub>1</sub> for full-protocol group, improvement in quality of life score	Mean eosinophil sputum count reduced but not significant

### ***1.13. CRTh2 in animal models***

A number of CRTh2 antagonists have been tested in models of asthma and atopic dermatitis. For mouse models it is important to note that in mice CRTh2 expression is not biased towards Th2 like in humans (132,133), so there are some limitations in extrapolating data from animal models to humans. In allergen mouse models CRTh2 antagonists have been shown to reduce allergic pulmonary and large airway inflammation, Th2 associated cytokines levels and goblet cell metaplasia (134). In allergic guinea pig models CRTh2 antagonists have been shown to reduce eosinophilia (135,136) and in an aspergillus fumigatus, allergen induced rat model, a CRTh2 antagonist was found to reduce airway eosinophilia (137). In an OVA-sensitised skin model of atopic dermatitis a decrease in inflammatory infiltrate, levels of cytokines (IL-13, TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , IL-4, IL-9, eotaxin, TARC, KC, MIP-2) and levels of antigen specific antibodies were observed (138). There was

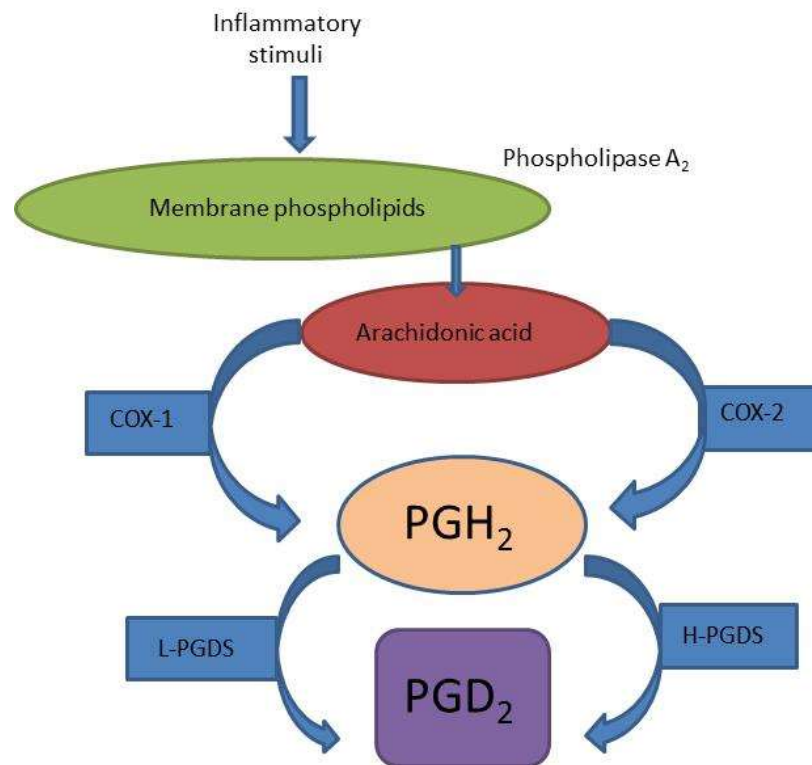
also a decrease in the gene expression of members of the calgranulin family, genes associated with epithelial differentiation in the skin (138).

For CRTh2 knock-out model studies how the antigen is delivered and the subsequent presentation of the antigen and the genetic background of the mice has been found to be important in determining effects (133). In mice bred on a C57BL/6 background, CRTh2 knock-out mice were found to have increased eosinophil recruitment into the lung following antigen challenge, which was related to an increase in the levels of IL-5 for the knock-out mice (139). In an allergic rhinitis model, CRTh2 knock-out mice bred on a BALB/c background (a strain which preferentially develops Th2 responses) had a reduction in antigen specific IgE levels, nasal eosinophilia and numbers of CD3+ cells (140). These studies all indicate that CRTh2 has a role in Th2-associated pathways and additional pathways that have been associated with the pathogenesis of allergic diseases and asthma. The reported effects of CRTh2 antagonists on eosinophils within these animal models links with some of the effects seen in human clinical studies, where a reduction in eosinophil numbers is also seen with CRTh2 antagonist treatment (see table 1.1).

#### ***1.14. Evidence for a role of Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) in allergy and asthma***

In humans, increased levels of PGD<sub>2</sub> in allergic patients have been demonstrated, following bronchial allergen challenge (114,141) and PGD<sub>2</sub> has been found to be released during early and late phase asthmatic responses following allergen challenge in asthma (142). However, other studies have found no increase in PGD<sub>2</sub> levels in asthmatic patients compared to normal (143). This negative finding could be due to the difficulty in measuring levels of PGD<sub>2</sub> due to its short half-life and the fact that it is quickly degraded (144-146).

In response to inflammatory stimuli,  $\text{PGD}_2$  is produced following a number of steps (**Figure 1.2**). Arachidonic acid is liberated from membrane phospholipids by phospholipase  $\text{A}_2$ . Arachidonic acid is then converted to prostaglandin  $\text{H}_2$  ( $\text{PGH}_2$ ) by cyclooxygenase enzymes COX-1 and COX-2. Lipocalin-type PGD synthase (L-PGDS) and hematopoietic PGD synthases (H-PGDS) then convert  $\text{PGH}_2$  to  $\text{PGD}_2$ .  $\text{PGD}_2$  is released predominantly from mast cells, but is also released from dendritic cells, Th2 cells and eosinophils (105,147).



**Figure 1.2:** Production of  $\text{PGD}_2$

### ***1.15. $\text{PGD}_2$ in animal models***

Animal models have demonstrated that the presence of excess  $\text{PGD}_2$  provokes an increase in Th2 associated cytokine release and the recruitment of eosinophils. A number of animal model experiments have demonstrated that  $\text{PGD}_2$



is an important mediator in allergic asthma. Fujitani et al (2002) demonstrated that mice overexpressing L-PGDS in an OVA model had elevated IL-4, IL-5, eotaxin and an increased eosinophilia in the BAL of the L-PGDS overexpressing mice compared to control mice (148). Honda et al (2003) demonstrated that PGD<sub>2</sub> nebulisation to mice before challenging with aerosol antigen challenge enhanced the Th2 inflammatory responses including eosinophilia (149). Mandal et al (2004) demonstrated that reducing PGD<sub>2</sub> levels by using uteroglobin (an anti-inflammatory protein), reduced allergic inflammation (150). Administration of a H-PGDS inhibitor was found to improve mouse airway inflammation (151).

#### ***1.16. Components of PGD<sub>2</sub> pathway***

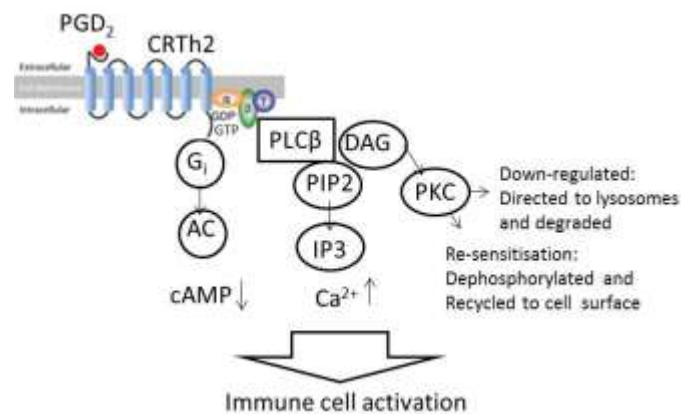
As well as PGD<sub>2</sub>, components within the PGD<sub>2</sub> production pathway have also been shown to be up-regulated in allergy and asthma. COX-2 has been found to be up-regulated in asthmatic airways (152,153). The distribution of prostaglandin synthases has been determined in a number of cell types with L-PGDS being found to be expressed in the central nervous system (154,155), heart (156), retina (157) and genital organs (158). H-PGDS has mainly been localised to immune cells such as mast cells and Th2 cells (159-161). H-PGDS levels have been found to be increased in the airway of asthmatics (116). Of the cells that have been reported to date to express CRTh2, some of them have also been found to express H-PGDS, and release PGD<sub>2</sub>. For these cells a potential autocrine function has therefore been suggested. A proportion of CRTh2 positive Th2 lymphocytes have been found to co-express H-PGDS and abundantly produce PGD<sub>2</sub> in response to antigenic stimuli (105,161). Eosinophils and basophils have also been found to express H-PGDS and release PGD<sub>2</sub> (147,162). Mast cells contain the predominant cellular domain of H-

PGDS among resident and recruited cells in allergic tissues (163) but to date the expression of CRTh2 on mast cells has not been explored. During injury processes fibroblasts have been shown to express H-PGDS and release PGD<sub>2</sub> (164). There have been some reported links of H-PGDS expression, PGD<sub>2</sub> levels and epithelial cells, with them both being found to be increased in the later stages of wound healing in skin models (165). This suggests a potential link between H-PGDS expression and the production of PGD<sub>2</sub> from epithelial cells. In addition, a study investigating the role of cyclooxygenase derived metabolites and epithelial cells in airflow-induced bronchospasms in dogs, showed a significant correlation between the epithelial cell number and PGD<sub>2</sub> recovered in the lavage fluid after dry air challenge (166). In a study looking at strips of human bronchioles, PGD<sub>2</sub> treatment on epithelial-denuded strips caused increased sensitivity to methacholine challenge (167). These findings suggest that as well as being important for inflammatory cell responses in asthma PGD<sub>2</sub> could play a role in driving abnormal airway sensitivity via airway remodelling processes.

### ***1.17. Other PGD<sub>2</sub> receptors***

Other receptors that have been demonstrated to respond to PGD<sub>2</sub> are the D prostanoid receptor (DP1) and thromboxane A<sub>2</sub> receptor (TP). **Table 1.2** summaries the similarities and differences between these receptors. There appears to be more overlap in expression and function for CRTh2 and DP1. Importantly there are selective agonists available for both CRTh2 and DP1, DK-PGD<sub>2</sub> is a CRTh2 selective agonist and BW 245C is a DP1 selective agonist, which allow for selective activation of each receptor (168-171). PGD<sub>2</sub> binds to CRTh2 and DP1 with similar affinity (172) but CRTh2 mediated signals predominate over DP1 when cells are

exposed to PGD<sub>2</sub> (173-175). CRTh2 is not structurally related to DP1 and CRTh2 signals through a different mechanism (see **Figure 1.3**). Unlike DP1, CRTh2 is a member of the chemoattractant receptor family and has higher sequence homology with FMLP and C5a receptors than DP1.



**Figure 1.3.** Summary of CRTh2 signalling pathway. PGD<sub>2</sub> binds to CRTh2 transmitting a signal into the cell resulting in activation of bound G-protein and second messenger's causing immune cell activation. AC adenylyl cyclase, PLCβ phospholipase C, PIP2 phophatidylinositol 4, 5 biphosphate, DAG diacyl glycerol, IP3 inositol triphosphate, PKC protein kinase C.

**Table 1.2.** Similarities and differences between the Prostaglandin D<sub>2</sub> receptors.

<b>Receptor</b>	<b>CRTh2</b>	<b>DP1</b>	<b>Thromboxane A2 receptor (TP)</b>
<b>Peripheral blood expression to date</b>	Peripheral blood Th2 cells, eosinophils, basophils	Platelets, dendritic cells, Th1 and Th2 cells.	Platelets
<b>Additional expression</b>		Bronchial smooth muscle, vascular smooth muscle	Bronchial smooth muscle, vascular smooth muscle
<b>Main effects investigated to date</b>	Activation of Th2 lymphocytes, eosinophils, basophils	Relaxation of vascular and airway smooth muscle leading to vasodilation and bronchodilatation  In platelets linked to anti-aggregatory function  Dendritic cell activation	Platelet aggregation, constriction of vascular and airway smooth muscle  The bronchoconstrictor effects of TP dominate over the bronchodilator effects of DP1 in the airways
<b>Signalling</b>	G <sub>i</sub> -dependent elevation in intracellular calcium levels and reduction in intracellular levels of cyclic AMP	G <sub>s</sub> - mediated elevation in cyclic AMP	G <sub>q</sub> -coupled
<b>Agonists</b>	PGD <sub>2</sub>  13,14-dihydro-15-keto-PGD <sub>2</sub> (DK-PGD <sub>2</sub> )  11, deoxy-11-methylene-15-keto-PGD <sub>2</sub> (MKPGD <sub>2</sub> )  Indomethacin	PGD <sub>2</sub>  BW245C 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3hydroxypropyl)-hydantoin J)	PGD <sub>2</sub>  I-BOP  Thromboxane A2  U44612

### ***1.18. CRTh2 structure and signalling***

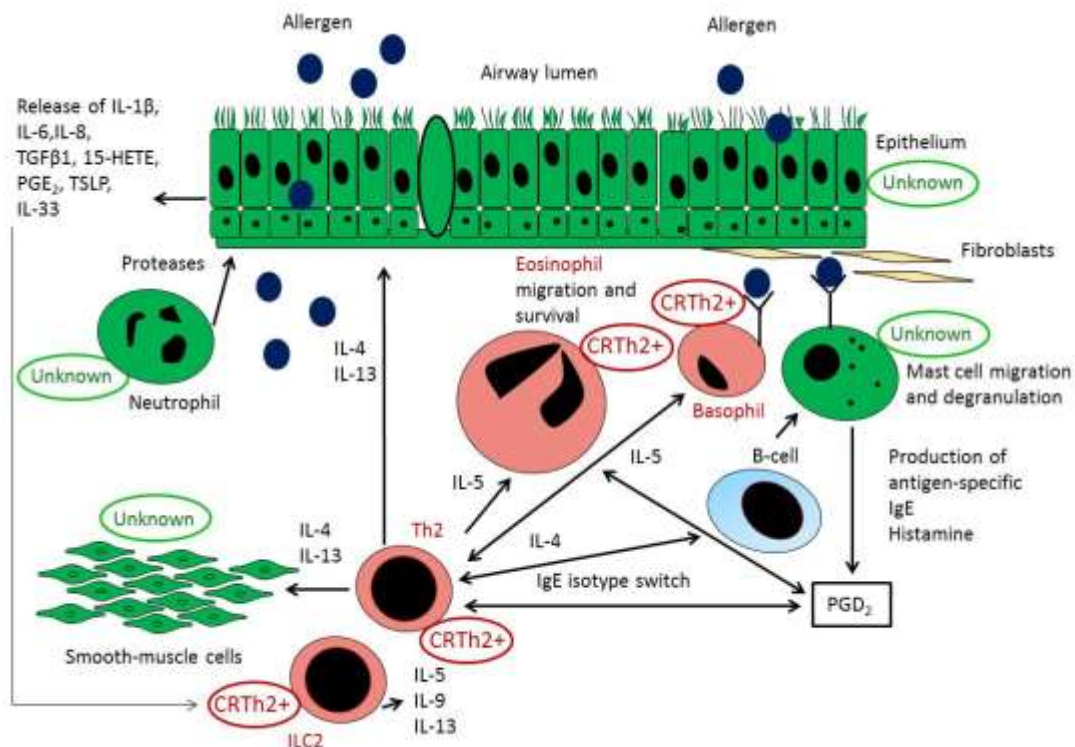
CRTh2 is a characteristic seven-hydrophobic-transmembrane GPCR belonging to the class A family (176). The CRTh2 gene is located on chromosome 11q13. To date, there have been two single nucleotide polymorphisms (SNPs) identified, G1544C and G1651A in the 3' untranslated region (177). The downstream signalling pathway of CRTh2 has been found to be different depending on the cell type and cellular conditions (178), as reported for other chemoattractant receptors (179). CRTh2 activation by PGD<sub>2</sub> does not lead to the stimulation of cAMP but does cause an increase in intracellular calcium (104). In T-lymphocytes CRTh2 activation has been found to be pertussis toxin sensitive, suggesting that CRTh2 couples to the G-protein G<sub>ai/0</sub> class (104). However, some of the probable CRTh2 mediated responses in primary eosinophils such as induction of cell morphological change by indomethacin has been found to be partly dependent on PTX resistance G proteins (G<sub>q</sub> class), phospholipase C, phosphatidylinositol 3 kinase and p38 mitogen activated protein kinase (**Figure 1.3**).

The C-terminus tail of CRTh2 is considered to be important for its G-protein signalling as it contains the arrestin-3 site and a recycling motif (176). Following internalisation, arrestin-3 dissociates from CRTh2 in the endocytic vesicles (176).

### ***1.19. CRTh2 expression and function on effector cells in asthma***

CRTh2 has been reported to be expressed on a number of the cell types that have been implicated in asthma pathobiology (**Figure 1.4**). However, for some of the cell types such as the structural cells of the airways; epithelial cells and smooth muscle cells, the expression of CRTh2 has not been explored. In **Figure 1.4**, the

cells on which CRTh2 expression has been confirmed in the literature are coloured in red; cells where expression stills need to be determined are highlighted in green.



**Figure 1.4.** Effector cells in asthma. Red highlights CRTh2+ cells, green highlights cells where CRTh2 expression is yet to be determined. CRTh2 activation in response to PGD $_2$  on Th2 cells, ILC2 cells, eosinophils and basophils causes the release of pro-inflammatory cytokines which trigger further pro-inflammatory responses in surrounding cells.

### 1.20. CRTh2 expression and effects of activation on T-cells

Several T-cell subsets and the many cytokines they secrete influence asthma pathobiology. Th2 cells are thought to be important in asthma as they release IL-4, IL-5 and IL-13 cytokines which are responsible for humoral immune responses as well as the growth and differentiation of mast cells and eosinophils (180). IL-5 has a well-recognised role in promoting blood and tissue eosinophilia (181-183). IL-4 and IL-13 have the ability to induce isotype-switching leading to the production of

immunoglobulin E (IgE) (184,185). IL-13 has also been shown to act directly on bronchial epithelial cells to promote mucus production and airway responsiveness (186). Activated Th2 cells are the main cells expressing CD40 ligand (CD40L) and binding of CD40L to CD40 molecules promotes IgE production on B-cells and antigen presenting capability for dendritic cells (187). CD3 can be used as a marker to identify T-cells (188) and as such has been used to investigate the number of T-cells in both BAL and bronchial biopsies in asthma. In both the BAL (189) and the mucosa of severe asthmatics the number of T-cells and activated T cells (identified by expression of IL-2 receptor) has been shown to be elevated compared to normal subjects (36,190-192).

Th2 lymphocytes have been found to express CRTh2 (103,104), with CRTh2 considered to be a reliable marker of Th2 cells (193). CRTh2<sup>+</sup> CD4<sup>+</sup> T-cells have been found to be mostly positive for the activation marker CD25 (103). In addition, for CD4<sup>+</sup> T-cells the level of CRTh2 expression can also be increased by IL-4 and inhibited by IL-12 (103,194). Activation of CRTh2 via PGD<sub>2</sub> causes the release of IL-4, IL-5 and IL-13 (105,106), up-regulation of CD40L, and a delay in T-cell apoptosis (105,106). The effects of CRTh2 activation on Th2 cells, particularly the release of the cytokines IL-4, IL-5 and IL-13 are reported to have a significant impact on the pathobiology of asthma (195-197). T-cell activation through the T-cell receptor has been shown to decrease CRTh2 expression which could indicate a mechanism by which T-cells can re-circulate from the site of antigen presentation to the draining lymph nodes (178,198). Rapid and transient down-regulation of receptor expression which is restored following removal of the stimulant is also observed for other chemokine receptors such as CCR1 and CCR3 (199). These

findings demonstrate that the expression of CRTh2 on T-cells is influenced by activation state and the surrounding milieu of cytokines present.

### ***1.21. CRTh2 expression and effects of activation on eosinophils***

Eosinophils are important in the late phase inflammatory reactions in asthma. Human eosinophils encounter a variety of ligands for chemoattractant receptors in the asthmatic airways such as chemokines (200), and lipid derived mediators (201). These interactions cause eosinophil accumulation within the airways and stimulate their cytotoxic effector functions. In asthma, eosinophils have been found to be in an activated state and they secrete increased amounts of arginine rich proteins such as eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and major basic protein (MBP) (202). These proteins can have adverse effects on airway structural cells, such as causing epithelial injury (203,204), with deposits of MBP being found within areas of epithelial damage in asthma (204). Eosinophils have been found to be increased in the sub-mucosa and epithelium in asthmatic patients (192,205,206).

CRTh2 expression has been described on the surface of eosinophils (174,207). CRTh2 activation via PGD<sub>2</sub> has been found to cause shape change, actin polymerisation and CD11b up-regulation and to moderate eosinophil chemotaxis (168,169,173,208-210). PGD<sub>2</sub> has also been found to prime eosinophils for other chemoattractants such as eotaxin through CRTh2 activation (211). These effects are considered to contribute to the pathogenesis of asthma (212). IL-5 has been shown to influence CRTh2 expression on eosinophils by down-regulating CRTh2 expression causing a desensitisation to PGD<sub>2</sub> (139), and eosinophils isolated from



IL-5 transgenic mice can't bind to PGD<sub>2</sub> (172). These studies suggest that CRTh2 activation on eosinophils depends on the surrounding cytokines.

### ***1.22. CRTh2 expression and effects of activation on basophils***

Basophils are crucial in initiating allergic inflammation through the binding of antigen-specific IgE antibodies at the high affinity IgE receptor, FcεRI (213). They also drive Th2 cell differentiation of activated naïve CD4<sup>+</sup> T-cells via production of IL-4 and direct cell-cell contact (214). Basophils are required for Th2 differentiation (215) and they can release IL-4 and IL-6 in the presence of activated CD4<sup>+</sup> T-cells which can lead to B-cell proliferation and antibody generation (216).

CRTh2 expression has been determined for basophils (103,170). CRTh2 activation on basophils has been shown to cause mobilisation of intracellular Ca<sup>2+</sup>, CD11b up-regulation, CD203c expression and enhancement of IgE mediated basophil degranulation and to moderate basophil chemotaxis (170). Activation of CRTh2 therefore activates basophils, contributing to the pro-inflammatory processes in asthma.

### ***1.23. CRTh2 expression and effects of activation on innate lymphoid type 2 cells (ILC2)***

The term innate lymphoid cells (ILCs) unify a group of cells that are developmentally related and lack most lineage markers. They share morphological feature with lymphocytes, but, in contrast to B and T cells, they do not express recombined antigen receptors and can therefore be classified as innate immune cells (217). Based on their cytokine and transcription factor expression profile ILCs have been given a uniform nomenclature, dividing ILCs in three main groups (218). The

first subset (ILC1s) depends on the expression of T-bet, leading to the expression of IFN $\gamma$  (219,220). The second subset (ILC2s) expresses GATA-3 and is characterised by the secretion of type 2 cytokines such as IL-13 and IL-5 (221,222). Group 3 (ILC3s) depends on the expression of the transcription factor retinoid acid receptor-related orphan receptor (ROR) $\gamma$ t for their development as well as their function and secrete IL-22 and IL-17 (223). ILC2 cells when activated by cytokines such as IL-25 or IL-33 can influence some of the features of asthma including BHR, goblet cell hyperplasia and eosinophilia through the production of IL-5, IL-9 and IL-13 (224-226).

There is emerging evidence for a role of CRTh2 on ILC2 cells (227). CRTh2 positive ILCs have been found to be present in fetal and adult lung and gut and more CRTh2 positive ILCs were found in nasal polys of chronic rhinosinusitis patients (227). CRTh2 expressing ILCs were found to respond *in vitro* to IL-2, IL-25 and IL-33 by producing IL-13. Activation of ILC2 cells with PGD<sub>2</sub> and activated mast cell supernatant has been found to cause cell migration and increase the production of IL-4, IL-5 and IL-13 (228). CRTh2 positive ILC2 cells are likely therefore to contribute to the number of positive inflammatory cells within asthmatic airways and to the production of pro-inflammatory cytokines.

#### ***1.24. Absent/low levels of CRTh2 expression have been reported on circulating neutrophils***

In acute, severe exacerbations of asthma, there are increased eosinophils and neutrophils within the airway, with the increase in neutrophils proportionately higher than that of eosinophils (229). Some asthmatics have predominantly

neutrophilic airway inflammation and the presence of airway neutrophils is correlated with a worse outcome. Neutrophil elastase acts as a potent proteolytic agent capable of degrading a wide range of proteins. Elastase is important in the killing of microorganisms and initiation of tissue injury in inflammation (230). Neutrophil counts have been seen to increase in asthma compared to healthy (231).

To date, three studies have demonstrated a lack of CRTh2 expression on peripheral blood neutrophils (104,174,232), with an additional study demonstrating barely detectable expression (124). These data suggest that CRTh2 is unlikely to have a role on circulating neutrophils, however, expression has not been explored on neutrophils within the tissue compartment of the airways. Effects of CRTh2 antagonists on neutrophils with animal models have been reported, with CRTh2 activation causing neutrophil migration (124,125,233). However, it is important to note that the expression profile for CRTh2 is different in mouse compared to humans, with expression being reported in the mouse on neutrophils and Th1 cells (132,234).

### ***1.25. Smooth muscle cells***

Airway smooth muscle (ASM) mass is increased in the asthmatic airway (235), with the percentage of the airway wall area occupied by ASM being increased in subjects with severe disease (236). Airway smooth muscle cells can be induced to secrete mediators that may promote mast cell chemotaxis, proliferation, and survival, while cell-cell interaction between airway smooth muscle cells and mast cells enhances activated complement-induced mast cell degranulation (237-239).

To date the expression of CRTh2 on airway smooth muscle has not been described.

#### ***1.26. Potential for the expression of CRTh2 on human mast cells***

Mast cells are critical in mediating the acute response in asthma. While classically, mast cell activation occurs following the binding of antigens to FCεRI-bound, antigen specific IgE, they may also be activated through other mechanisms, including stimulation of complement receptor, FCγRI, and via Toll-like receptors (TLRs) (240). Captured allergens and IgE bound to FCεRI on the cell surface of mast cells causes signal transduction and the release of cytokines, chemokines, histamine and leukotrienes including PGD<sub>2</sub> (241). Anti-IgE dependent activation of rat and human mast cells results in preferential generation of PGD<sub>2</sub> and PGI<sub>2</sub>, in addition to the secretion of granule associated mediators (241). *In vivo* allergen challenge leads to mast cell activation and release of histamine, associated with a rise in mast cell tryptase (242). Mast cell tryptase is a trypsin like neutral serine protease which is predominantly expressed in mast cells. Mast cell tryptase is capable of degrading vasoactive intestinal peptide and activating prekallikrein as well as generating kinins, all important mediators involved in bronchoconstriction and airway hyperresponsiveness (243). In asthma, mast cells show extensive changes in their granules, associated with their activation (206). In bronchial biopsies tryptase positive mast cells have been found to be highest in mild asthmatic patients with no inhaled corticosteroid (ICS) therapy, and lowest in severe asthma (112). The majority of positive mast cells in the severe asthmatic groups have been found to be of a tryptase positive chymase positive phenotype (MC<sub>TC</sub>). These MC<sub>TC</sub> cells are thought to be steroid resistant and T-cell independent (244). Mast cells also

promote the recruitment and activation of Th2 lymphocytes within allergic tissue, a process which can occur independently of antigenic-specific T cell activation (245). In a model of allergic asthma in mice mast cells have been shown to be essential for the accumulation of lymphocytes in BAL fluid in addition to a number of other features of airway inflammation (246).

To date the expression of CRTh2 has not been investigated on human lung mast cells. In contrast, murine bone marrow derived mast cells (BMMC) have been found to express CRTh2 (247). Activation of CRTh2 in these cells causes an increase in intracellular calcium and mast cell chemotaxis but only at relatively high levels of agonist (247). Interestingly, activation of murine BMMC via polyvalent antigen cross-linking of IgE bound to FCεRI receptors when combined with PGD<sub>2</sub> activation did not induce histamine release, and CRTh2 mRNA expression levels within the BMMC dramatically decreased upon IgE binding. **These observations suggest that CRTh2 could be expressed on human lung mast cells and the current study aims to investigate CRTh2 expression on mast cells within asthmatic biopsies and human mast cells isolated from lung tissue to aid in the overall understanding of CRTh2 expression on inflammatory cells in asthma.**

#### ***1.27. Mast cells, PGD<sub>2</sub> and CRTh2***

Supernatants from immunologically activated mast cells display potent chemotactic activity for Th2 lymphocytes, an activity that is mediated by PGD<sub>2</sub> acting on CRTh2 (248). Additionally, supernatants from IgE activated mast cells have been reported to cause greater chemotaxis of CRTh2<sup>+</sup> CD4<sup>+</sup> Th2 cells than un-activated mast cell supernatants demonstrating enhanced release of PGD<sub>2</sub> from

activated cells (249-251). Activation of mast cells through the FCεRI has been found to increase H-PGDS expression levels and the release of PGD<sub>2</sub> (252). These data suggests that increased levels of H-PGDS could result in increased levels of PGD<sub>2</sub>. In severe asthmatic subjects MC<sub>TC</sub> mast cells have been found to produce higher levels of PGD<sub>2</sub> (241,253).

#### ***1.28. Potential for the expression of CRTh2 on bronchial epithelial cells in asthma***

Although the expression of CRTh2 has not been investigated on bronchial biopsies from asthmatic patients there is some limited evidence that demonstrates that CRTh2 is expressed on epithelial cells of the nose (254), lung (255), skin (138), and retina (256). More recently CRTh2 expression has been described on epithelial cells of COPD patients (128) and colon (124). These investigations would suggest that CRTh2 could be expressed within bronchial epithelial cells of asthmatic patients. **To date no studies have investigated the expression of CRTh2 on epithelial cells from asthmatic patients. In addition no functional studies have been carried out for CRTh2 activation on bronchial epithelial cells. The current study therefore aims to investigate these gaps in knowledge about CRTh2 expression and activation in asthma.**

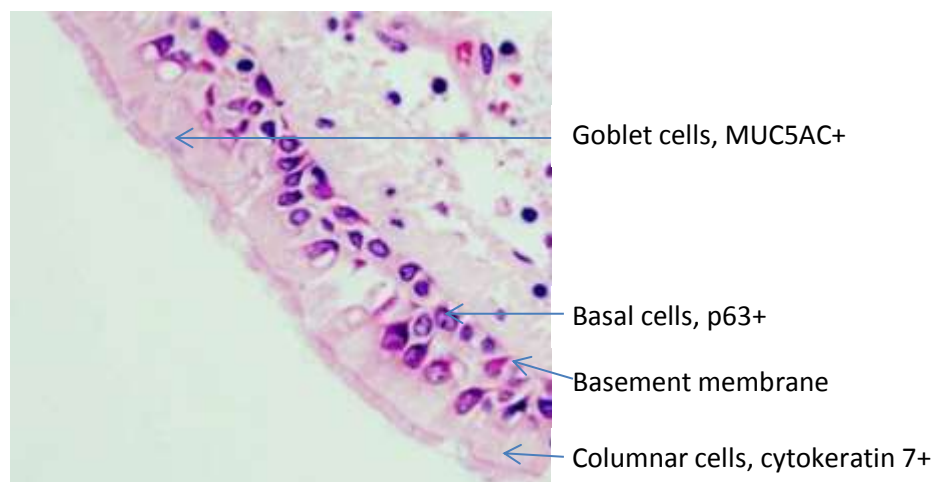
#### ***1.29. Bronchial epithelial cells***

Epithelial cells form the interface between the external environment and the host. Mucosal surfaces encounter non-toxic proteins continually, and vigorous immune responses do not generally develop. The respiratory tract must maintain its essential gas exchange function and therefore has evolved to limit access of proteins to the immune system with barriers like the mucus layer and intracellular tight

junctions. To understand the changes that epithelial cells undergo within the remodelling process in asthma it is important to appreciate the functions of the various components of the upper airway bronchial epithelium, as described in the following section.

### ***1.30. Components of the upper airway bronchial epithelium***

The human bronchial epithelium is composed of three main cell types, which together form a pseudostratified ciliated layer, **Figure 1.5** (257).



**Figure 1.5.** H&E image to show components of bronchial epithelium for a healthy control biopsy (x400 magnification).

1) **Goblet cells** are the mucus-producing cells of the upper airways. The characteristic gel-like property of mucus is believed to be attributable mainly to the presence of high molecular weight, polydisperse glycoproteins, or mucins (258-260). The mucin family of glycoproteins is classified into those that are secreted by epithelial cells and form the mucus gel and those that are embedded in the epithelial cell membrane. Five major mucins are expressed in the airways: MUC1, MUC4,

MUC5AC, MUC5B and MUC16 (261). The predominant mucin component of the goblet cells is MUC5AC (258-260,262), which is located inside the airway epithelium (263). For the membrane associated mucins, MUC1, MUC4, and MUC16, their role involves the activation of intracellular signal transduction pathways, control of inflammation and immune responses to infectious agents, and regulation of cell differentiation and proliferation (264). Airway mucus maintains the hydration in the airways and traps particulates, bacteria and viruses. Both the quality and quantity of mucin production determines the viscoelastic property of mucus, which is critical for efficient muco-ciliary clearance. Viscoelasticity refers to the combined viscous (resistance to flow) and elastic (returning to original shape) characteristics of mucus (265). The other important functions of gel-forming mucins are the capture, retention, and release of biologically active molecules (266). Among the molecules that mucins have been shown to be associated with are cytokines, growth factors and trefoil factors (TFFs). These associations may allow mucins to regulate inflammation and immune responses, and to influence post-injury repair. For example mucins interact with interleukins such as IL-1, IL-4, IL-6 and IL-7. Because mucins can bind to pathogens, these associations may allow mucins to serve as bridges between inflammatory mediators and microorganisms, thereby facilitating the resolution of inflammation.

2) **Ciliated cells** are terminally differentiated columnar cells which express proteins such as cytokeratin 7. Their main function in combination with the goblet cells is to remove particulate matter by means of the mucociliary stairway (257).

3) **Basal cells**, which can be identified by p63 (267) are considered as the stem cells of the bronchial epithelium (268). Basal cells are differentiated with respect to



junctional adhesion mechanisms and their main role is to attach columnar epithelium to the basal lamina (269-271). They are capable of binding to extra-cellular matrix components found in early development inflammation and wound healing (272), and following epithelial wounding a number of studies have found that basal cells flatten out to cover the basement membrane (273-275). Basal cells may also be important in determining the composition of the epithelial barrier. Under normal conditions there is a tight balance between basal cell self-renewal and generation of physiologically appropriate proportions of secreting and ciliated cells (276). However, under disease settings where excessive stimulants are present in the surrounding milieu of basal cells there is the potential of excessive self-renewal at the expense of differentiation, leading to basal cell hyperplasia or metaplasia and lack of ciliated cells.

**4) Adherens junctions** together with the cell types described above provide a highly regulated barrier which helps to prevent entry of external factors such as allergens and viruses. Epithelial tight junctions and adherens junctions establish cell-cell contact and cell polarity and also regulate the paracellular movement of anions and macromolecules (277). Tight junctions are belt-like structures surrounding each cell, providing a physical barrier (278). Cadherins are involved in cellular architecture and in cell-cell adhesion (279) and they effectively couple the cytoskeletons of adjacent cells so that they can undergo co-ordinated movement (277). E-cadherin is required for the maintenance of stable junctions as demonstrated by the effects of anti E-cadherin antibodies which can disrupt epithelial contact and induce a mesenchymal phenotype (280). Numerous regulatory proteins can affect these structural components such as epidermal growth factor

receptor (EGFR), kinases and regulators of actin dynamics. If disruption of the epithelium occurs via changes to the polarity of the cells, alterations in the differentiation status or breakdown of tight junctions, then easier access of external factors to underlying tissue may occur. This is demonstrated by E-cadherin knock-down experiments, where a decrease in expression, decreases epithelial resistance in epithelial monolayers (281).

### ***1.31. Epithelial cell models***

In order to investigate changes that may occur within the epithelium in asthma different *in vitro* models have been used.

#### ***1.31.1. Bronchoscopic brushings***

Using a bronchial cytology brush, bronchial epithelial cells can be removed using a well-tolerated methodology (282). These cells can then be maintained in culture either as submerged or differentiated as ALI (283).

#### ***1.31.2. Submerged epithelial cell cultures***

Bronchial epithelial cells can be maintained in media in submerged monocultures (283,284), however these cell cultures lack some of the features of *in vivo* bronchial epithelium such as goblet cells and ciliated cells (285).

#### ***1.31.3. Air Liquid Interface (ALI) cultures***

ALI cultures are airway epithelial cells grown on a porous membrane on which they form a continuous epithelial sheet with the basal aspect exposed to media and the apical surface exposed to the air. Epithelial cells from passage 2 to 4

have been found to be competent to differentiate into mucus and ciliated cells (285). Epithelial cells grown in an ALI format can reach full differentiation whereas, cells that have been grown immersed in culture media and attached to plastic or matrix tend to lose normal differentiated muco-ciliary features (285). Therefore epithelial cells grown as an ALI will be more similar to the *in vivo* mucosal barrier. The media components that ALI cultures are grown in can influence its morphology, for example if cells are grown without retinoic acid the ALI is characterised by cells which are multi-layered and regions of cells will be of a squamous metaplastic morphology (285). ALI cultures of fully differentiated bronchial epithelium from endobronchial specimens of asthmatics maintain inherent phenotypic differences specifically related to disease severity (286).

### ***1.32. Normal response of the epithelium to damage***

During normal repair of the epithelium an intrinsic repair pathway utilises epithelial growth factor (EGF), amphiregulin and heparin-binding EGF (HB-EGF) which act on epithelial growth factor receptors (EGFR) to create cell migration, proliferation and differentiation (287). Following epithelial injury, such as wounding, a transient release of mucus has been found followed by a rapid shedding of columnar, ciliated cells. Basal cells may be still attached to the basal lamina (288). Spreading and migration of basal epithelial cells neighbouring the wound has been shown (289). This is followed by the emergence of pre-ciliated cells, ciliogenesis and regeneration of a pseudostratified muco-ciliary epithelial barrier. However at any point this process can be affected by other interacting cells and molecules which influence this dynamic process of repair and regeneration.

### ***1.33. Epithelial changes in asthma***

The epithelial barrier is considered to be disrupted in asthma, thereby allowing more allergens and soluble proteins to pass through the epithelium and interact with underlying cells. By the time asthma is diagnosed, the epithelium is bathed in inflammatory mediators from eosinophils, mast cells, and lymphocytes, many of which increase release of cytokines from and cause damage to epithelial cells (290).

#### ***1.33.1. Expression of stress-related proteins***

Evidence proposes that epithelial cells are more stressed in asthma, expressing stress markers such as activated transcription factors (NFκB), activator proteins (AP1), signal transducer and activation of transcription 1 (STAT1) (291) and heat shock proteins (284).

#### ***1.33.2. Evidence of increased fragility of asthmatic epithelial cells***

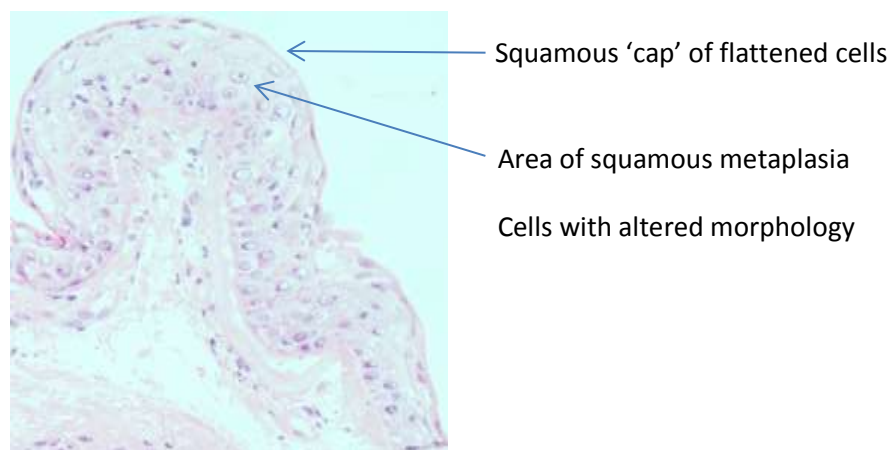
Increased loss of epithelial cells has been reported in asthma (292,293), however, arguments have been made that this is an artefact of the bronchoscopy procedure and is an unreliable end-point (63,294). There is still a wealth of literature which suggests that the epithelium is more fragile in asthma in both established asthmatics and children (77,287,295).

#### ***1.33.3. Continued activated epithelial repair response in asthmatic epithelial cells***

Up-regulation of the EGFR in asthma (287,296) indicates a continuous activation of epithelial repair processes. CD44 expression has been demonstrated to be increased in asthmatic epithelium (287,295,297). CD44 has been shown to link

the extracellular environment with the intracellular machinery and actin cytoskeleton (298). As this protein is associated with cell migration and wound repair (298-300) this may suggest an ongoing activated repair process in asthma. Damaged cultured epithelial cells, such as in scrape wound assays have been shown to release EGF (287) IL-33 (301), and express higher levels of the migratory protein, CD44 (298). Cellular damage to the epithelium in asthma may occur by a number of mechanisms. Allergic airway inflammation may cause damage to the epithelial layer. Epithelial integrity may be affected by allergens themselves. Preparations of enzymes from pollen have been shown to detach mouse epithelial cells from ECM in a dose-dependent manner (302). Other domestic allergens such as house dust mite may have proteolytic actions (303). Epithelial cell damage may also occur from interaction of enzymatic secretions from inflammatory cells such as eosinophils (244,304). It has been proposed that asthmatic epithelial cells respond differently to damage, with incomplete repair occurring leading to a chronic wound scenario with the secretion of a range of growth factors capable of driving structural changes linked to airway remodelling (295,305). The time to repair mechanical wounds has been found to be significantly decreased in children with asthma compared to healthy control and atopic children (306), which could suggest abnormal repair processes even without a number of years of disease. The repair process in the airway epithelium is structurally characterised by the formation of multiple layers of polygonal and flat poorly differentiated basal cells (epithelial reparative metaplasia) followed by the development of normal differentiated epithelium (307,308). Squamous metaplasia has been reported to occur within 48 hours after mechanical tracheal injury (309). Therefore epithelial metaplasia in the airways of asthmatic

biopsies may be interpreted as an ongoing repair process (310). **Figure 1.6** demonstrates an area of squamous metaplasia for a severe asthmatic biopsy. The area of squamous metaplasia is characterised by cells at the apical surface of a flattened morphology, forming a 'cap' of tessellate cells. Involucrin is frequently used as a marker of squamous metaplasia within bronchial epithelium (311,312).



**Figure 1.6.** H&E image to show an area of squamous metaplasia within bronchial epithelium for a severe asthmatic biopsy (x400 magnification).

#### ***1.33.4. Epithelial Mesenchymal Trophic Unit (EMTU)***

The interaction between airway epithelial cells and mesenchymal cells within the airway is essential for development and normal repair (273). Epithelial-mesenchymal transition is a highly conserved and fundamental process that governs morphogenesis in multicellular organisms (313). This epithelial mesenchymal trophic unit (EMTU) has been hypothesised to be activated in the airways of asthmatics (314), and may result from a primary defect of epithelial repair which causes prolonged activation of the EMTU and ultimately tissue remodelling (315). It describes the interactions between the bronchial epithelium and mesenchymally derived cells immediately below in the bronchial wall (316). It has also been

hypothesised that epithelial cells themselves can undergo epithelial transition into a mesenchymal-like cells (EMT), hence losing their epithelial functionality and characteristics (317-319). EMT occurs during early development where there is a seamless plasticity between epithelial and mesenchymal cells and this may also occur in some adult tissues during carcinoma cell invasion and metastasis or following wound repair or organ remodelling in response to injury (320). EMT has also been observed in cell culture systems and in this environment cells lose polarity, adherence to adjacent cells and the extracellular matrix, and gain mesenchymal cell properties such as motility and the expression of  $\alpha$ -SMA (321). Expression of  $\alpha$ -SMA is characteristic of myofibroblasts which are laying down an excessive amount of extracellular matrix (322). An early molecular sign of EMT in epithelial cells is the down-regulation of E-cadherin (317,320), and this has been demonstrated in the epithelial cells of asthmatics (323,324). Treatment of ALI cultures with cytokines and growth factors that are known to be released from inflammatory cells within the asthmatic airways have contributed to our understanding of potential remodelling changes of the epithelium in asthma. The growth factor, TGF $\beta$ 1 has been shown to induce a range of changes within ALI culture that are thought to have a role in epithelial remodelling in asthma. TGF $\beta$ 1 has been shown to induce epithelial mesenchymal transition (EMT) in ALI cultures derived from healthy control and asthmatics (323), and to induce squamous metaplasia (325). However, to date within asthmatic biopsies no epithelial cells have been found to express mesenchymal markers (323). In addition, although reduced junctional protein expression during EMT might result in greater epithelial cell permeability, the net effect on airway permeability will also be affected by sub-

epithelial fibrosis and other compensatory structural changes that might occur over time during remodelling processes.

***1.33.5. Enhanced mediator release from asthmatic epithelial cells and increased expression of inflammatory markers***

Epithelial cultures from asthmatic patients have been found to release increased levels of IL-1 $\beta$ , IL-6, IL-8, TGF $\beta$ 1, and lipid mediators 15-HETE and PGE<sub>2</sub> (326-330) with this altered mediator release being present in cultured epithelial cells from children (331,332). Activation of epithelial cells in mice has been shown to lead to the release of thymic stromal lymphopietin (TSLP), GM-CSF, IL-1 $\alpha$ , IL-25 and IL-33 (88,315,333). These cytokines have been found to activate dendritic cells, promote Th2 responses, and activate mast cells (334). Increased expression of MCP-1 (335), eotaxin (336) and GM-CSF (337) are observed within asthmatic epithelium and have been suggested to contribute to homing of inflammatory cells to the epithelium. These findings suggest that activation of the epithelium in asthma further contributes to the overall pathogenesis of the disease by causing additional activation of both inflammatory cells and surrounding airway structural cells.

***1.33.6. Altered composition and integrity of the epithelium in asthma***

Numerous studies suggest that the overall structure and differentiation status of the epithelium may be altered in asthma, compromising barrier function (82,338-341). An increase in cytokeratin 5/14 (basal epithelial cell marker) expression was found for epithelial cells taken from non-bronchoscopic brushings of atopic children with mild asthma compared to healthy controls. This phenotype was



maintained throughout culture (332). In addition, epithelial tight junctions have been shown to be disrupted in asthma (305,342). These studies suggest that the overall architecture of the epithelium may be different in asthma.

ALI cultures from asthmatic patients have been compared to those from healthy control donors, and a number of differences have been found. Asthmatic ALI cultures have been found to contain cells that are less differentiated, having increased numbers of basal cells identified by cytokeratin 5. In addition, they have been described as having disrupted tight junctions, containing less E-cadherin and occludin expression (343). Other studies have shown increased mucin expression in asthmatic ALI cultures (286) and that the cells release increased TGF $\beta$ 2, VEGF, periostin and the pro-inflammatory cytokines, IL-6, IL-8 and GM-CSF compared to healthy control ALI cultures (343,344).

#### ***1.33.7. Goblet cell hyperplasia***

Excess mucus production is considered part of pathophysiological features of asthma (83), with mucus plugs being a feature of asthma (345). Infectious agents and host inflammatory mediators activate mucin gene expression in asthma. Microbial pathogens and host response molecules also drive airway remodelling through goblet cell hyperplasia, which refers to increased goblet cell numbers and goblet cell metaplasia, the reversible differentiation of non-goblet airway epithelial cells into goblet cells. The precise mechanisms that regulate goblet cell hyperplasia and metaplasia are unknown. Mucus can become pathological due to hyper-secretion during asthma and associated with the loss of clearance and breakdown of the muco-ciliary escalator, allows more mucus to accumulate and obstruct the

airways causing respiratory distress and sometimes mortality (346). Epithelial cell metaplasia and hyperplasia of the mucus-secreting goblet cells in asthma is thought to contribute to luminal narrowing (347). Goblet cell hyperplasia and sub-mucosal gland hypertrophy are thought to substantially increase the amount of mucus producing tissue in asthma, and goblet cell increase may contribute to asthma deaths (62,348). Goblet cell numbers have been described as increasing 2.5-fold in asthmatic subjects compared to normal subjects (63), with the mucin MUC5AC accounting for the increase in mucin stores in asthma (63). A larger sub-mucosal gland area is characteristic of fatal asthma (349) and sub-mucosal gland area was found to be significantly higher in patients with mild to moderate and severe, persistent asthma compared to controls (350). It is possible that the airway epithelium in asthma has an inherent capacity to generate goblet cells. Airway epithelial side population cells (stem) readily generate *in vitro* a multi-layered and differentiated epithelium layer including MUC5AC producing cells, and these side population cells are increased in asthmatics (351). Release of inflammatory mediators during asthma has been shown to lead to excessive mucus production via activation of various intracellular signalling pathways (83). A number of different cytokines have been implicated in the increased production of mucus in asthma. TNF $\alpha$ , IL-1 $\beta$ , IL-13 have been found to up-regulate MUC5AC (352). IL-13 is thought to be a critical driver for mucus production (353-356). IL-13 signals through STAT6 and FOXA2 to induce transcription of MUC5AC (357). In ALI cultures treated with repeated IL-13 pre-treatment (1ng/ml basolaterally applied for 3 days, replaced daily) there was a two-fold increase in MUC5AC found in pooled washes and MUC5AC cellular content was increased by 25% (358). Trypsin

proteins and reactive oxygen species have also been found to increase MUC5AC expression *in vitro* (359,360). IL-9 is thought to influence MUC5AC through calcium activated chloride channels (CLCAs) (357). IL-1 $\beta$  has been found to be released by asthmatic bronchial epithelial cells (361). Indirectly IL-1 $\beta$  has been shown to up-regulate MUC5AC via the activation of CD4<sup>+</sup> cells which release IL-4, IL-9, IL-13, GM-CSF and TNF $\alpha$  (362). IL-1 $\beta$  has been found to directly influence MUC5AC through COX2 and PGE<sub>2</sub> leading to MUC5AC up-regulation (363).

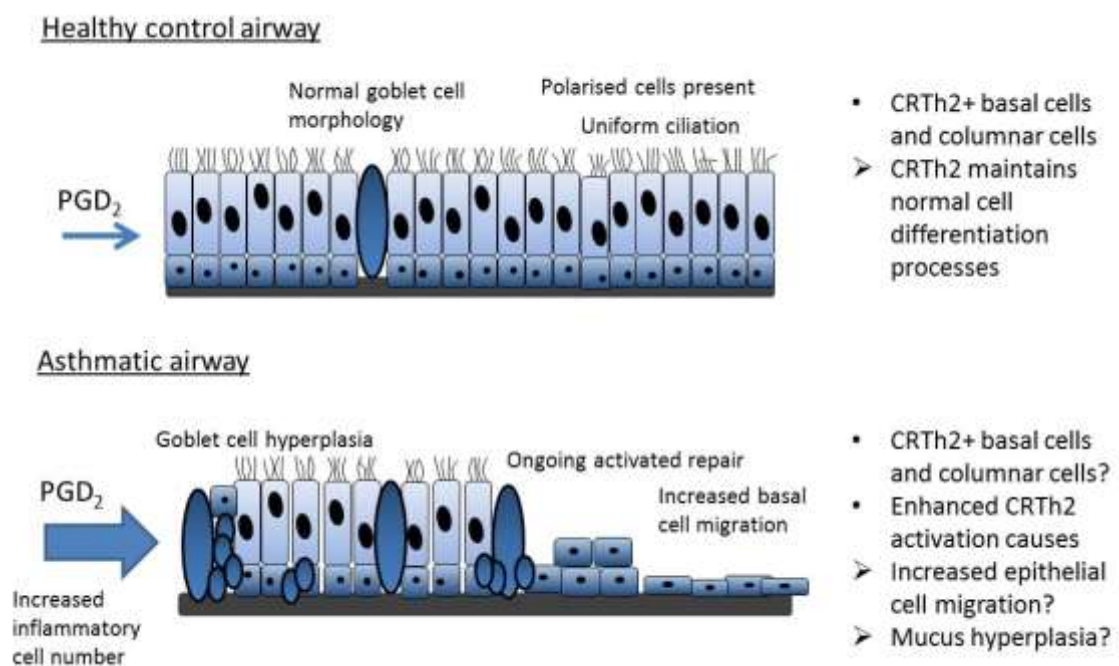
These studies identify that the epithelium in asthma is significantly altered and likely to contribute to the pathogenesis of asthma. Some of these features such as levels of MUC5AC and MUC5AB have been shown to be unaffected by glucocorticosteroid treatment (364), although others studies have shown some efficacy thought to be due to a reduction in numbers of inflammatory cells (365). This highlights that currently there is no pharmacotherapy to address these key pathophysiological features of asthma aimed at maintaining muco-ciliary clearance, biochemical protection of epithelium, trafficking, polarisation of membrane receptors, or protection of tight junctions.

### *1.34. HYPOTHESIS*

To date there is a lack of data describing the protein expression of CRTh2 in bronchial biopsies in asthma. Considerable investigation of the expression of CRTh2 on inflammatory cells in peripheral blood have been described, however, currently no studies have described CRTh2 expression on inflammatory cells within the tissue compartment of the airways. In addition, CRTh2 expression has not been explored in airway structural cells such as bronchial epithelial cells and airway smooth muscle cells. In contrast, the expression of CRTh2 on epithelial cells has been described from a variety of tissues including the nose (254), skin (138), retina (256), colon (124) and bronchial epithelial cells in patients with COPD (128). These studies highlight the potential that CRTh2 may be expressed on epithelial cells within asthmatic airways. Whether CRTh2 expression and activation on bronchial epithelial cells regulate epithelial cell migration as reported in T-cells, eosinophils and basophils (103,169,170,366) remains unknown. The possibility of a role of CRTh2 in driving airway remodelling is indirectly supported by studies in mouse challenge models (134,367), where CRTh2 antagonists caused a reduction in goblet cell hyperplasia. This observation re-enforces the concept that CRTh2 activation on epithelial cells may play a key role in the pathogenesis of asthma. This is a relevant hypothesis as increased levels of the CRTh2 agonist, PGD<sub>2</sub> are present within asthmatic airways (116) compared to healthy control airways. Comparative studies looking at the expression of CRTh2 on asthma and healthy control biopsies would provide useful data to indicate possible target cells located within the airways for CRTh2 antagonism in study read-outs and patient populations.

The hypotheses for this study are that;

- CRTh2 is expressed on sub-mucosal inflammatory cells and the bronchial epithelium within bronchial biopsies.
- CRTh2 expression on inflammatory cells and bronchial epithelial cells is increased in disease relative to health and for asthmatic patients, related to disease severity.
- The central hypothesis (summarised in figure 1.7) states that increased CRTh2 activation on bronchial epithelial cells in asthma causes increased epithelial cell migration and induces mucus cell hyperplasia, contributing to the remodelling processes of the epithelial barrier in asthma.



**Figure 1.7.** Diagram to show the hypothesis for the proposed effects of CRTh2 activation on epithelial remodelling under asthmatic conditions in patients with increased levels of PGD<sub>2</sub> in the airways.

**The specific aims of the PhD are therefore:**

- To undertake detailed immunohistochemical analysis for CRTh2, inflammatory and epithelial phenotype markers on bronchial biopsies from subjects with asthma and healthy controls.
- To investigate the expression and function of CRTh2 in primary epithelial cells in submerged culture from healthy control and asthmatic subjects.
- To study the effects of CRTh2 activation on epithelial cells grown in a differentiated format as an ALI culture.
- To study the effects of CRTh2 activation in mast cell and air-liquid interface co-cultures.

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# **Chapter 2**

## **Materials and Methods**

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## **2.1. MATERIALS**

The materials and reagents that have been used for each technique are listed below. Each table contains the materials and reagents for a particular methodology. Detailed methodology is described in the subsequent section.

### ***Immunohistochemistry***

**Table 2.1.** List of reagents used for immunohistochemistry

<b>Reagent</b>	<b>Order details</b>
EnVision FLEX+, Mouse, High pH (Link)	Dako (Cambridge) K8002
EnVision G2 Doublestain system rabbit/mouse	Dako K5361
Microscope slides Colourfrost PLUS	Fisher Scientific (Leicestershire) SD999102
Coverslips 22x50mm	VWR International (Leicestershire) 631-0137
10% neutral buffered formal	Pioneer research chemicals (Colchester) PRC/R/4
Gill's 2 haematoxylin	Pioneer research chemicals PRC/13/1
Eosin Y	Acros Organics (New Jersey, USA)
DPX mountant	Sigma (Dorset) 44581
Xylene	Fisher Scientific X/0200/17
IMS	Genta medical industrial denatured (York) alcohol 01320913
Antibody diluent (PBS with BSA pH7.4, 0.01M PBS, 0.138M NaCl, 0.0027M KCl, 1% w/v BSA)	Sigma P3688
Tween 20	Dako 2013-09
Immedge pen	Vector Labs (Leicestershire) H-4000

### ***Antibodies***

**Table 2.2.** List of antibody reagents used for studies.

<b>Reagent</b>	<b>Order details</b>	<b>Concentration used at</b>
Rabbit polyclonal CRTh2 AZ	Antibody produced at AstraZeneca using rabbit immunisation of peptide: CAASPQTGPLNRALSSTSS	1 µg/ml
Mouse monoclonal mast cell tryptase	Dako IR640	Ready to use



Mouse monoclonal CD3	Dako M7254	5µg/ml
Mouse monoclonal CD4	Dako M7310	5µg/ml
Mouse monoclonal CD8	Dako M7103	1µg/ml
Mouse monoclonal CD68	Dako IR609	Ready to use
Alexa fluor 647 rat anti human CD294	BD Biosciences (Oxford) 558042	10µg/ml
Alexa fluor 647 rat IgG2a isotype control	BD Biosciences 557906	10µg/ml
Mouse monoclonal mast cell chymase	Abcam (Cambridge) Ab2377	2µg/ml
Mouse monoclonal CD34	Abcam Ab8536	20µg/ml
Mouse monoclonal Collagen I	Abcam Ab90395	2µg/ml
Mouse monoclonal CD44H	R&D Systems (Abingdon) BBA10	5µg/ml
Normal rabbit IgG	Santa cruz (Insight Biotechnology, Middlesex) sc-3888	1µg/ml
Mouse monoclonal MU5AC	Abcam Ab24070	1µg/ml
Mouse monoclonal pan cytokeratin	Dako M0821	1µg/ml
Mouse monoclonal cytokeratin 7	Dako M70187	0.02µg/ml
Mouse monoclonal cytokeratin 14	Abcam ab7800	1µg/ml
Mouse monoclonal neutrophil elastase	Dako M0752	0.04µg/ml
Rabbit polyclonal G-Protein-Coupled receptor 44 polyclonal (CRTh2 commercial)	Fisher Scientific OPA1-15328	5µg/ml
Mouse monoclonal p63	Abcam Ab735	1µg/ml
Mouse monoclonal p63/High Molecular Weight cytokeratin	Abcam ab58514	Ready to use
Mouse monoclonal involucrin	Abcam ab68	0.75µg/ml
Mouse monoclonal α-SMA	Dako M0851	0.02µg/ml
Mouse monoclonal MBP	Monosan (Newmarket) MON 6008	1.3µg/ml
Rabbit polyclonal Occludin	Zymed (Fisher Scientific) 71-1500	5µg/ml
Mouse monoclonal E-cadherin	BD Biosciences 610182	5µg/ml

Goat polyclonal PGD synthase (D-17)/H-PGDS	Santa Cruz sc-14816	1µg/ml
Swine anti-goat secondary antibody	Dako E0466	0.02µg/ml
Mouse IgG1 negative control	Dako X0931	Used at matching [protein] to primary antibody
Rabbit IgG negative control	Dako X0936	Used at matching [protein] to primary antibody
Goat IgG negative control	Sigma I9140	Used at matching [protein] to primary antibody

### *Cell culture*

**Table 2.3.** List of reagents used for cell culture

Reagent	Order details
HMC-1 media (IMDM GlutaMAX-1)	Invitrogen (Paisley) 31980022
ALI media (MucilAir culture medium)	Epithelix SaRL (Switzerland) EP04MM
Bronchial epithelial cell media (hAEC culture medium)	Epithelix SaRL EP09AM
Human airway bronchial epithelial cells	Epithelix SaRL EP08AM
Accutase	Invitrogen A11105
MucilAir normal ALI cultures	Epithelix SaRL EP01MD
MucilAir asthmatic ALI cultures	Epithelix SaRL EP03MD
Purecol purified bovine collagen solution	Advanced Biomatrix (Cell systems, Germany) 5005-B
DMEM (+ HEPES, + D-Glucose) for HLMC	Invitrogen 32430-027
Collagenase	Invitrogen 17018-029
FBS	Invitrogen 16000036
Hyaluronidase	Sigma H1115000
Disposable scapel 10A	Fisher scientific SCA-353-050E
10% neutral buffered formal	Pioneer research chemicals PRC/R/4
Recombinant human IL-13	R&D Systems 212-ILB-005
TGFβ1	Miltenyl Biotech (Surrey) 130-095-067
FGF	R&D Systems 233-FB
13, 14-dihydro-15-keto prostaglandin D <sub>2</sub> DK-PGD <sub>2</sub>	Cayman Chemicals (Cambridge) 10007208
CRTh2 antagonist AZD6430	AstraZeneca, tested in >100 assays at MDS Pharma with only TP and AT2 showing as low affinity hits

Human IgE purified	Millipore (Nottingham) AG30P
Goat anti-human IgE, affinity purified polyclonal antibody	Millipore AP175

### *Flow cytometry*

**Table 2.4.** List of reagents used for flow cytometry.

Reagents	Order details
Human IgG	Sigma I4506
Albumin (BSA powder)	Sigma A7906
Dulbecco's Phosphate Buffered Saline	Sigma A11105
Saponin from quillaja bark	Sigma S7900
Alexa fluor 647 rat anti human CD294	BD Biosciences 558042
Alexa fluor 647 rat IgG2a isotype control	BD Biosciences 557906

### *Quantitative RT-PCR*

**Table 2.5.** List of reagents used for quantitative RT-PCR.

Reagent	Order details
CRTh2 Taqman primer/probe	Applied Biosystems (Invitrogen) 4331182_Hs01867513_s1 FAM
HPGDS Taqman primer/probe	Hs00183950_m1 FAM
DP receptor Taqman primer/probe	Hs00235003_m1 FAM
18S Taqman primer/probe	Hs03928985_g1 FAM
IL-13 Taqman primer/probe	Hs00174379_m1 FAM
MUC5AC Taqman primer/probe	Hs00873651_mH FAM
Involucrin Taqman primer/probe	Hs00846307_m1 FAM
IL-1 $\beta$ Taqman primer/probe	Hs01555410_m1 FAM
TGF $\beta$ Taqman primer/probe	Hs00998133_m1 FAM
Taqman master mix	Fisher Scientific VY4369016
RNAqueous-4PCR kit	Invitrogen AM1914
Retroscrip kit	Invitrogen AM1710
Polypropylene 96-well tube plates	Agilent Technologies (Cheshire) 410088
Mx3000P/Mx3005P optical strip caps	Agilent Technologies 401425
Spleen total RNA	Invitrogen AM7970
Placenta total RNA	Invitrogen AM7950
Brain total RNA	Invitrogen AM7962

### *Fluorescent cell staining*

**Table 2.6.** List of reagents used for fluorescent cell staining.

Reagents	Order details
Nunc Labtek II chamber slides RS glass slide sterile	Nalgene 154534
Purecol purified bovine collagen solution	Advanced Biomatrix (Cell systems, Germany) 5005-B
Albumin (BSA powder)	Sigma A7906
Vectashield Mounting medium for fluorescence with DAPI	Vector Laboratories (Fisher Scientific H-1200
Normal goat serum	Dako X0907

### *Western Blot*

**Table 2.7.** List of reagents used for western blot.

Reagents	Order details
1% Nonidet P-40 (NP-40)	Sigma 74385
Sodium deoxycholate	Sigma D6750
SDS	Sigma L3771
PMSF	Sigma P7626
Na <sub>3</sub> VO <sub>4</sub>	Sigma S6508
NaF	Sigma S7920
EDTA	Sigma E6758
Leupeptin	Sigma L8511
Aprotinin	Sigma A1153
TBS	Sigma T5912
Tween	Sigma P1379
Hybond C Super nitrocellulose membrane	GE Healthcare (Buckinghamshire) 10402495
Anti-rabbit-HRP antibody	Santa Cruz sc-2313

### *Viability assays*

**Table 2.8.** List of reagents used for MTT assay and Annexin-V/PI assay.

Reagents	Order details
MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)	Sigma M2128
10mM HCl	Sigma H1758
Isopropanol	Sigma W292907
0.1% Nonidet P-40 (NP-40)	Sigma 74385
Apoptosis Detection kit	Sigma APOAF-20TST

### *Cell migration assay*

**Table 2.9.** List of reagents used for cell migration assay.

Reagents	Order details
Biocoat Poly-D-Lysine 96-well plate Black/Clear	BD Biosciences 354640
Oris cell migration assembly kit-FLEX	Platypus (Peterborough) CMAUFL4
Hoescht 33342	Molecular Probes (Life technologies, Paisley)
10% neutral buffered formal	Pioneer research chemicals PRC/R/4
Calcein	Invitrogen C3099

### *Calcium assay*

**Table 2.10.** List of reagents used for calcium assays.

Reagents	Order details
Ionomycin	Sigma I3909
Fura2 AM made up in DMSO+5% pluronic acid	Fisher Scientific 11524766
Coverslips	Fisher Scientific 12392128
Tyrodes solution (2mM Ca <sup>2+</sup> Tyrode)	Contains Sigma: NaCl 135mM, KCl 6mM, NaH <sub>2</sub> PO <sub>4</sub> 0.33mM, Na pyruvate 5mM, Glucose 10mM, HEPES 10mM, CaCl <sub>2</sub> 2mM, pH7.4.

### *Prostaglandin D<sub>2</sub> ELISA*

**Table 2.11.** List of reagents used for Prostaglandin D<sub>2</sub> ELISA.

Reagents	Order details
Prostaglandin D <sub>2</sub> -MOX EIA kit	Cayman Chemicals 512011

## **METHODS**

### ***2.2. Biopsies***

Bronchial biopsies from healthy control and asthmatic subjects were utilised for immunohistochemical staining. Mucosal biopsy specimens were processed into glycol methacrylate (Polysciences), using methodology as described in Hanstede (1982) (368).

### ***2.3. Subjects***

Healthy control and asthmatic subjects were recruited from Glenfield Hospital, Leicester, UK. Asthma severity was defined according to the Global Initiative for Asthma treatment steps (1). Subjects were characterised in terms of demographics, smoking history, spirometry, sputum cell counts, and atopic status defined as either one or more positive skin prick test or blood specific IgE to common aeroallergens. Healthy subjects had no history of respiratory or allergic disease and had normal spirometry. The study was approved by the Leicestershire Research Ethics committee. Informed consent was obtained from all subjects.

## ***2.4. IMMUNOHISTOCHEMISTRY STAINING***

### ***2.4.1. Overview of technique***

Immunohistochemistry is used to identify protein expression of targets of interest within tissue sections. The advantage of this technique is that it allows for identification of protein in context to surrounding cells and tissue components. The

technique employs a primary antibody directed to the protein of interest, followed by detection of where the antibody is bound using reagents such as secondary-labelled antibodies, or polymer technology. The location of the antibody complex is identified using a chromagen reaction which produces a coloured product. Immunohistochemistry for this study was used to identify CRTh2, inflammatory phenotype marker and epithelial cell phenotype marker expression in bronchial biopsies. An EnVision™ kit was used for the immunohistochemistry staining as this has been found to be sensitive for detecting a number of antigens (369). For co-localisation experiments on bronchial biopsies two techniques were investigated to identify CRTh2+ cells with phenotype marker+ cells. Dual staining utilises two detection stains to co-localise two separate antibodies on the same cell. However, problems can occur with this technique as competition for similar binding sites on the same cell can interfere with the antibody binding. Serial staining enables two separate antibodies to identify the same cell but on two serial sections. As the sections from GMA blocks can be cut at a thin depth of 2 microns, the same cell can be identified on two separate sections. However, for some cells such as T-cells which are of a relatively small size it can be difficult to see the same cell on two different slides. It is therefore important to try to find a 'locating' feature within the biopsy to identify the same regions.

For the immunohistochemistry procedure for some antibodies heat mediated antigen retrieval was used as fixation methods such as formalin fixation and glycol methacrylate (GMA) embedding processes can mask some antigens (370,371). Using heat mediated antigen retrieval has been shown to unmask epitopes allowing binding to primary antibodies (372-374).

#### ***2.4.2. Haematoxylin and Eosin Staining (materials used described in table 2.1 and 2.2)***

Paraffin slides were dewaxed in xylene and taken through graded alcohols and into distilled water. Dewaxed slides or GMA slides were stained with Gills Haematoxylin (Pioneer Research Chemicals) for approximately 10-20 minutes. Slides were then washed in running water and then stained with eosin Y (Acros Organics) for approximately 2 minutes, washed briefly in running water, cleared through graded alcohols to xylene, then mountant and a coverslip added.

#### ***2.4.3. Dual staining optimisation (materials used described in table 2.1 and 2.2)***

2 micron sections were cut from GMA blocks (processed as described in (375) of asthmatic and healthy control biopsy material and floated onto Superfrost positively charged slides (ThermoScientific). Sections were left to dry at room temperature (rtp) overnight in a glass dish to allow sections to fully adhere to slides. Antigen retrieval was applied to the slides (slides placed in 400mls antigen retrieval solution high pH (Dako) in microwave at 700W for 5minutes), then cooled for 1min in water. Slides were stained as per manufacturer's instructions using EnVision dual stain kit (Dako) using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and permanent red as chromagens.

#### ***2.4.4. Serial staining method (materials used described in table 2.1 and 2.2)***

Serial sections were taken as described above before being stained. When sections were floated onto slides care was taken to orientate them in the same way on each slide. Staining was carried using an EnVision FLEX™ immunohistochemical kit (Dako) as per manufacturer's instructions. In brief, for the



immunohistochemistry procedure antigen retrieval was applied to appropriate slides. EnVision™ peroxidise blocking solution was applied to each section for 10 minutes at rtp. The slides were washed in EnVision™ wash buffer. For primary antibodies raised in goat, e.g. H-PGDS (see table 2.2) an additional blocking step was applied using 3% BSA/PBS for 30minutes. The appropriate primary antibody was incubated on the slides for 1 hour at rtp (see table 2.2 for details) or isotype controls (Dako) matched to the primary antibody protein concentrations. The slides were washed in EnVision™ wash buffer. For appropriate slides an EnVision™ rabbit linker or EnVision™ mouse linker was added to the sections for 15 minutes at rtp, this step is an amplification step and used for antibodies for which a more sensitive detection system is required. For primary antibodies raised in goat, a swine anti-goat antibody (Dako) was incubated on the sections for 15 minutes. The slides were washed in EnVision™ wash buffer. EnVision FLEX/HRP™ was added to all sections for 20 minutes at rtp. The slides were washed in EnVision™ wash buffer. The slides were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for an optimised time at rtp. Sections were counterstained with Gill's II haematoxylin (Pioneer), left to dry overnight at rtp then mounted with DPX mountant (Sigma) and a coverslip was added.

#### ***2.4.5. Immunohistochemistry control experiments for CRTh2 AZ antibody (materials used described in table 2.1 and 2.2)***

To help to validate the CRTh2 AZ antibody for immunohistochemistry a number of control experiments were incorporated into the biopsy work. The CRTh2 AZ antibody is a rabbit polyclonal antibody and as such a rabbit immunoglobulin fraction antibody (Dako) was used at a matched protein concentration for each

biopsy. This control is used to identify any non-specific binding of the antibody due to it being a rabbit protein. For a sub-set of biopsies, the CRTh2 AZ antibody was incubated with the peptide that was used to produce the antibody. This blocking peptide control is used to identify any non-specific staining due to the CRTh2 AZ peptide sequence. In addition, the staining of the CRTh2 AZ antibody was compared to that of a commercially available CRTh2 antibody (Fisher Scientific). This control was to ascertain if similar expression profiles were obtained for biopsies, therefore giving more confidence in the expression profile obtained for the CRTh2 AZ antibody.

#### ***2.4.6 ALI culture immunohistochemistry (materials used described in table 2.1 and 2.2)***

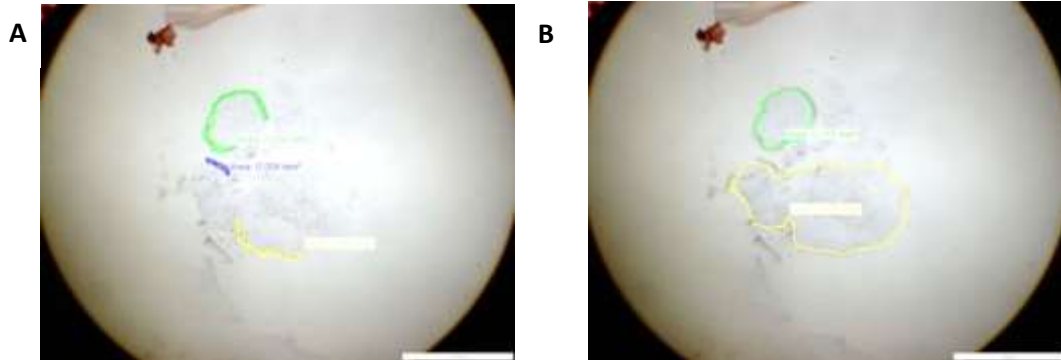
4µm sections were cut from paraffin blocks and floated onto Superfrost positively charged slides. Sections were dried overnight at 37°C. Sections were dewaxed using xylene and IMS. Antigen retrieval was applied to appropriate slides as described above. Slides were stained as per manufacturer's instructions using EnVision™ dual stain kit (Dako) using 3,3'-diaminobenzidine tetrahydrochloride (DAB), counterstained as described above. Slides were taken back through IMS to xylene before mountant and coverslip were added.

### ***2.5. IMMUNOHISTOCHEMISTRY ANALYSIS***

#### ***2.5.1. Area measurements for bronchial biopsies***

For each patient three separate area measurements were made using CellF software, Olympus BX50 microscope and Olympus U-DA camera. Area measurements recorded included total biopsy area, area of epithelium (**Figure 2.1A**)

and area of sub-mucosa (**Figure 2.1B**). Area measurements were taken at x20 magnification. The drawing tool was used to draw round the area of interest and measurements were calculated per mm<sup>2</sup>.



**Figure 2.1.** Examples of area measurements taken using drawing tool of Cell F software (Olympus) for bronchial biopsies. Using the drawing tool areas of interest are drawn round to give area measurement in mm<sup>2</sup>. A) Example of epithelial area measurement (x20 magnification) B) Example of sub-mucosal area measurement (x20 magnification).

#### **2.5.2. Membrane length measurements for ALI cultures**

For each ALI culture the length per mm of the membrane covered with epithelial cells was measured on 2 separate occasions by a blinded observer (**Figure 2.2**).



**Figure 2.2.** Example of ALI culture length measurement taken using drawing tool of Cell F software (Olympus). Using the drawing tool the length of the ALI culture is assessed in mm. A) Example of ALI culture length measurement (x20 magnification)

### ***2.5.3. Cell count and grading analyses***

Co-localisation was undertaken using sequential sections as described previously (376). Positively stained nucleated cells were enumerated per mm<sup>2</sup> of sub-mucosal area (36), per 10mm<sup>2</sup> of total epithelial area (231), or per mm length of ALI culture by a blinded observer. The percentage area of MUC5AC<sup>+</sup> staining within the epithelium of the biopsies was assessed using methods similar to (77,377-379). Grading criteria were derived for the intensity of CRTh2<sup>+</sup> staining, histology of biopsies, and area of involucrin<sup>+</sup> staining (similar to (380)). The percentage area of basement membrane covered with epithelium was calculated by assessing the percentage of basement membrane that was covered by epithelial cells for each biopsy. For the ALI cultures, the same grading criteria as for the area of involucrin<sup>+</sup> staining for the biopsies were used for the area of involucrin<sup>+</sup> staining and area of H-PGDS<sup>+</sup> within the ALI. The same grading criteria devised for the assessment of the intensity of staining for CRTh2 on the biopsies were used for E-cadherin<sup>+</sup> staining and CD44<sup>+</sup> staining of the ALI cultures. Grading was carried out on two separate occasions by a blinded observer.

## ***2.6. CELL CULTURE***

Bronchial epithelial cells can be cultured in submerged conditions for a number of passages (381). Bronchial epithelial cells cultured under these conditions allow for the investigation of protein expression and functional assays using a number of techniques. Epithelial cells were derived from bronchial brushings (382) of asthmatic subjects. Healthy control cells were derived either from bronchial brushings of healthy control subjects or bought from Epithelix (Switzerland).

Submerged bronchial epithelial cells can be seeded onto collagen-coated inserts and grown at an air liquid interface (ALI) to allow differentiation of the epithelial cells (383). ALI cultures are epithelial cells that are fully differentiated and so contain features such as mucus producing cells and ciliated cells that are not present for epithelial cells in submerged conditions (285). Reagents can be added to the basal (media) compartment of the ALI to investigate effects on features such as goblet cell number and the differentiation status of the ALI. Fully differentiated epithelial cells were purchased as MucilAir-ALI cultures and grown in bronchial epithelial media (Epithelix).

***2.6.1. Culture conditions for epithelial cells taken from bronchial brushings (materials used described in table 2.3)***

For Glenfield samples, bronchial brushings taken from asthmatic and healthy control subjects were incubated overnight at 4°C in bronchial epithelial growth medium (BEGM, Lonza, supplemented with a SingleQuot bullet kit (Lonza), 1.5ml fungizone (Gibco; solution containing amphotericin B with sodium deoxycholate) and 1% antibiotic-antimycotic (Sigma; solution containing 100 units/ml penicillin, 100mg/ml streptomycin sulphate, and 0.25µg/ml amphotericin). After the overnight incubation, brushings were shaken vigorously to dislodge cells, and to allow cells to become suspended in the medium. Brushes were then removed from the medium and the cells were centrifuged. The resulting cell pellet was resuspended in fresh BEGM and seeded into 4 wells of a 12-well plate pre-coated with 1% PureCol (Inamed; solution containing collagen type I, diluted in PBS to give working concentration of 0.03mg/ml). Upon reaching confluence, cells were passaged into culture flasks (T75) pre-coated with 1% PureCol and cultured using bronchial

epithelial cell media (Epithelix). Cells were removed from flasks using accutase to avoid cleavage of receptors which may occur when using a harsher enzyme such as trypsin. For all expression experiments cells were used at passage 1 and 2 in order for the cells to remain as similar as possible to freshly isolated cells.

#### ***2.6.2. Healthy control epithelial cells (Epithelix) (materials used described in table 2.3)***

Epithelial cells were delivered as frozen vials (approximately 1 million cells). Cells were defrosted quickly in a 37°C water bath. Cells were put into 1% PureCol pre-coated flasks in 10mls normal bronchial epithelial cell media (Epithelix).

#### ***2.6.3. CRTh2 selective reagents (materials used described in table 2.3)***

DK-PGD<sub>2</sub> (Cayman chemicals) was used as a selective CRTh2 agonist (104) for functional assays. AZD6430 (AstraZeneca) was used as a selective CRTh2 antagonist. AZD6430 has excellent selectivity. It has been tested in over 100 assays and the only significant, very low affinity activities were observed at the following receptors and enzymes: DP1 (pIC<sub>50</sub> = 5.5), TP (pIC<sub>50</sub> = 5.4), AT<sub>2</sub> (pIC<sub>50</sub> = 5.4), Aldose reductase (pIC<sub>50</sub> = 5.2), COX1/2 (pIC<sub>50</sub> = 5.5). AZD6430 therefore showed at least 1500-fold selectivity over all other targets tested.

#### ***2.6.4. MucilAir® Airway Liquid Interface (ALI) cultures (Epithelix) (materials used described in table 2.3)***

ALI cultures were delivered on agar in 24-well plates. Upon receipt each culture was transferred to a fresh 24-well plate containing 700µl MucilAir medium (Epithelix), as per manufacturer's instructions. ALI cultures were cultured

overnight in an incubator. The following treatments (final concentrations) were added to the basal media of duplicate cultures for 24hours, 48hours or 72hours: CRTh2 agonist, 13, 14-dihydro-15-keto prostaglandin D<sub>2</sub> (DK-PGD<sub>2</sub>) 100nM (Cayman chemicals), DMSO (vehicle control) 1μM (Sigma), AZD6430 1μM (AstraZeneca, concentration 500 fold above its K<sub>i</sub> for human CRTh2, IL-13 100ng/ml (R&D Systems), TGFβ1 10ng/ml (Miltenyl Biotech).

Following treatment filters were removed from their insert and cut in half with a scalpel. For all of the samples half of the filter was placed between thin pieces of foam (to stop the filters rolling up) and then placed into a cassette. The remaining filter was processed for RNA isolation (see methods section 2.7). Cassettes were then fixed in 10% neutral buffered formalin (Pioneer) for a minimum of 4 hours. The filters were then processed through standard conditions in a processor (Shandon) overnight. The filters were removed from the cassettes and embedded in paraffin, taking care to orientate the filter so that it was on its end, with a flat surface upper-most in the block (filters were kindly embedded by Hilary Marshall). The blocks were trimmed into and 4 micron sections were taken.

#### ***2.6.5. HMC-1 cells (materials used described in table 2.3)***

HMC-1 cells are a mast cell line. These cells are considered to be an immature mast cell phenotype (384) as they lack native expression of the high affinity IgE receptor, FCεRI (385,386). However, as these cells are fast growing they are useful to work up methodologies for which human lung mast cells cannot be used due to lack of availability of cells. HMC-1 cells were grown in IMDM + GlutaMAX-1, Iscove's Modified Dulbecco's media 1x (GIBCO) with added 10% FCS (iron supplemented), NEAA, antibiotics, monothioglycerol.

#### ***2.6.6. Human lung mast cells (HLMC) (materials used described in table 2.3)***

Human lung mast cells were isolated using a method based on Sanmugalingam, (2000) (387). In brief, lung resection material was cut into small pieces in 2% DMEM media to stop the tissue from drying out. Blood and mucus was removed by washing and leaving the tissue pieces overnight at 4°C. Collagenase and hyaluronidase were used to remove connective tissue. The cell suspension was resuspended with 2% FCS DMEM. Magnetic beads were used to isolate the mast cells.

#### ***2.6.7. HLMC and ALI co-culture (materials used described in table 2.3)***

HLMC were co-cultured with ALI cultures as they have been shown to release significant amounts of the CRTh2 agonist, PGD<sub>2</sub> (251). Upon activation through the high affinity IgE receptor the release of PGD<sub>2</sub> has been shown to be elevated (248,252,388). The co-culture of HLMC in the basal compartment of ALI cultures, and the addition of activated HLMC supernatant to the basal compartment of ALI cultures can therefore assess the effects of an endogenous CRTh2 agonist on the features of the ALI cultures. 4 separate HLMC donors were combined in mast cell media (DMEM, 10% FBS, no cytokines). For incubation with ALI cultures 350µl HLMC in media was incubated with 350µl of ALI culture media, in the basal compartment of the ALI culture for 24 hours.

#### ***2.6.8. HLMC activation (materials used described in table 2.3)***

4 separate HLMC donors were combined and centrifuged. 1ml of supernatant was taken as an un-activated supernatant sample. This was stored in aliquots at -80°C. The remaining cells were resuspended in 5mls HLMC media



(DMEM, 10% FBS, no cytokines) and placed in a 6-well plate. 1 µg/ml human IgE (Millipore) was incubated with the HLMC for 24 hours at 37°C. 1 µg/ml goat anti-human IgE was added to the HLMC and incubated for 30 minutes at 37°C. The cells were centrifuged; the activated supernatant was removed and stored at -80°C in aliquots. For incubation with ALI cultures 350 µl activated supernatant was incubated with 350 µl of ALI culture media, in the basal compartment of the ALI culture for 24 hours.

## **2.7. FLOW CYTOMETRY (*materials used described in table 2.4*)**

Flow cytometry allows for the assessment of the number of positive cells that express a particular protein and the amount of expression associated with that protein. It is a useful technique to quantitate the expression levels of a protein for a particular cell population. A cell population can be identified by the forward scatter and side scatter characteristics of the cells. Extracellular expression can be assessed in the absence of a permeabilise step and intracellular staining, when cells are treated with a paraformaldehyde and saponin step to allow entry of the antibody into the cell.

### **2.7.1. Flow cytometry method**

Extracellular and intracellular (4% paraformaldehyde, 0.1% saponin) CRTh2 expression was assessed with CRTh2-Alexa 647 (BD Pharmingen) antibody relative to isotype control (Rat-Alexa 647 isotype, BD Pharmingen), or CRTh2 AZ antibody with Alexa 488 secondary antibody (Invitrogen), Rabbit IgG (Sigma) with Alexa 488 secondary antibody (Invitrogen) using FACS Aria (BD Biosciences). Before antibodies were applied to the cells blocking steps were performed; for bronchial

epithelial cells 5% BSA was used, for HMC-1 and human lung mast cells 10% FCS (Sigma) and human IgG (Sigma) were used. The effects of corticosteroids on CRTh2 expression was investigated as described above by incubating healthy control cells with 1 $\mu$ M fluticasone propionate (Sigma) for 24 hours. For flow cytometry data, CRTh2 expression was expressed relative to the isotype control. The percentage positive of CRTh2 cells and geometric mean fluorescence units was assessed. The expression histograms were also analysed using FlowJo software.

## **2.8. QUANTITATIVE RT-PCR**

For the current study, quantitative RT-PCR was carried out using Taqman® primers and probes. This allows for the investigation of gene expression of tissue and cell samples. This PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold™ DNA polymerase to cleave a TaqMan® probe during PCR, releasing a fluorescent reporter dye. Accumulation of PCR products can be detected directly by monitoring the increase in fluorescence of the reporter dye. Peak normalised reporter values are averaged for each cycle of PCR and plotted versus cycle number to produce amplification plots. This fluorescence detection system allows the threshold cycle (Ct) to be observed when PCR amplification is still in the exponential phase and so is a more reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product, as none of the reagents are limited in the exponential phase.

### ***2.8.1. Sample preparation***

To prepare samples for quantitative RT-PCR analyses, RNA was extracted from cell samples followed by cDNA preparation before setting up the reaction mixes for the quantitative RT-PCR (see details below);

### ***2.8.2. RNA preparation (materials used described in table 2.5)***

An RNAqueous-4PCR kit (Ambion) was used to prepare RNA as per manufacturer's instructions. In brief, cell pellets were re-suspended in 100µl lysis/binding solution and then the RNA was isolated on columns as per kit instructions. RNA was eluted in 40µl elution solution followed by 10µl elution solution. RNA concentrations were assessed using a nanodrop. All RNA samples had an  $A_{260}/A_{280}$  ratio of approximately 2.0 suggesting good quality of RNA. Samples were stored at -80°C.

### ***2.8.3. cDNA preparation (materials used described in table 2.5)***

A RetroScript cDNA synthesis kit (Ambion) was used to prepare cDNA. Where possible 2µg RNA was added to a cDNA reaction, alternatively the maximum volume of RNA that could be added to a reaction was 10µl. cDNA was prepared at per kit instructions. cDNA was diluted with DEPC-treated water to a final concentration of 5ng/µl. cDNA was also prepared for a standard curve containing RNA from brain, placenta and testis and a mixture of epithelial samples. The standard curve was used to define the relative amounts of target RNA species present in cDNA samples. Quantitation of the amount of target in unknown samples was accomplished by producing a plot of the log of these relative dilutions against the  $C_T$ . This gives an equation of the line that can be used to relate  $C_T$  to the initial

copy number for the samples of interest. 18S was used as a normalising gene to correct for sample to sample variation in the amount of RNA converted to cDNA within each sample. 18S is considered to be a good normalisation gene as it has an advantage of being less likely to fluctuate under conditions that affect the expression of mRNAs since they are transcribed by a distinct polymerase (389).

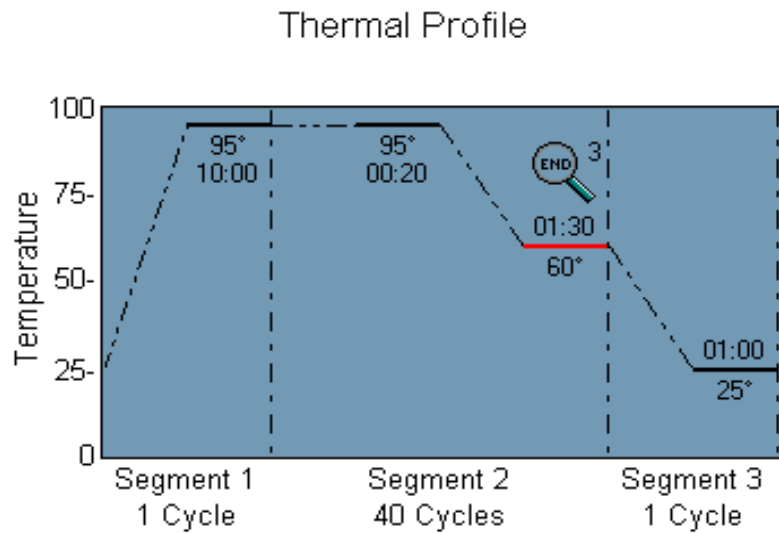
#### ***2.8.4. Taqman® reactions (materials used described in table 2.5)***

Taqman® primer and probe reagents were obtained from Applied Biosystems. All probes for genes of interest were labelled with FAM (6-carboxy-fluorescein) reporter dye.

##### *PCR reaction mix components for a single reaction*

20x Taqman gene expression assay	1µl
2x Taqman gene expression master mix (Invitrogen 4369016)	10µl
cDNA template	5µl
RNase-free water	4µl

Samples were loaded onto a PCR plate (Agilent) and lids added (Agilent). –RT reactions were used for each sample to control for DNA contamination within the RNA samples and no template controls were used to control for DNA contamination on the plate, equipment or within the atmosphere. Quantitative RT-PCR was carried out on a Stratagene Mx3000P. Each gene was set up as FAM and ROX. The thermal profile as shown below was used;



**Figure 2.3.** Cycle details for gene expression experiments

## 2.9. *Fluorescent cell staining (materials used described in table 2.6)*

Fluorescent cell staining on cultured cells enables protein expression to be observed on individual cells. Bronchial epithelial cells are adherent so can be grown on chamber slides. Human lung mast cells and HMC-1 cells are non-adherent and so cytopins can be used where a centrifuge is used to spin cells onto a slide.

Bronchial epithelial cells were seeded onto collagen coated chamber slides (Nalgene). The cells were left to settle overnight at 37°C in an incubator. Cells were fixed in 10% neutral buffered formalin for 10minutes, washed in PBS, blocked in 3% BSA/PBS for 30minutes at rtp and CRTh2 AZ antibody was applied to the cells for 1hour. Cells were washed in PBS and a secondary Alexa Fluor 488 antibody was applied (Invitrogen rabbit) for 30 minutes. Cells were washed and mounted in Vectamount with DAPI (Vector Labs). Alexa 488 Fluor (green) and DAPI (blue) staining were visualized using a Leica fluorescence microscope, Leica DM 2500 camera and Leica LAS Core software.

Isolated human lung mast cells were spun onto Superfrost positively charged slides using a cytospin (Shandon). Slides were fixed in 10% neutral buffered formalin for 10 minutes, washed in PBS for 10 minutes, allowed to air dry then placed in a glass dish with lid and stored at -20°C. For immunohistochemical staining the glass dish was removed from the freezer and allowed to come up to rtp before the lid was removed to prevent any condensation on the slides. The immunohistochemistry method applied to the slides was as described for the chamber slides.

#### ***2.10. Western blot (materials used described in table 2.7)***

Western blot procedures allow for protein expression to be investigated based on the size of the protein that is being interrogated. For the current study a western blot was used to validate the specificity of the CRTh2 AZ antibody by investigating positive control cells (CRTh2 transfected HEK cells) compared with negative control cells (wild-type HEK cells). Protein lysates were prepared from CRTh2-HEK cells and wild-type HEK cells using RIPA buffer [1xPBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 200µM Phenylmethanosulphenylfluoride (PMSF), 2mM sodium orthovanadate  $\text{Na}_3\text{VO}_4$ , 10mM sodium fluoride (NaF), 2mM ethylenediaminetetracetic acid (EDTA) and protease inhibitors 10µg/ml leupeptin, 10µg/ml aprotinin]. Samples were placed on ice for 30 minutes, centrifuged at 12,000rpm for 20 minutes at 4°C. The supernatants containing total proteins were collected and stored at -80°C.

Total protein was separated on 8% SDS-PAGE. After the transfer of proteins into a Hybond C super nitrocellulose membrane they were blocked in 5% non-fat powdered milk in 1xTBS/0.1% Tween 20 for 1 hour, washed briefly and incubated

with the CRTh2 AZ antibody at 4°C overnight. The membranes were washed 3x 10minutes with 1xTBS/0.1% tween and then incubated with anti-rabbit HRP conjugated secondary antibody for 1 hour at rtp and washed again. Protein bands were visualised by incubation of membranes with chemiluminescence reagents (ECL) and exposed to X-ray film.

### **2.11. MTT assay (materials used described in table 2.8)**

The MTT assay can be used to assess the mitochondrial activity for cell samples (390). As total mitochondrial activity is linked to the number of viable cells, the MTT assay can indicate whether cellular toxicity has occurred within cells, as these cells will have low mitochondrial activity. Bronchial epithelial cells were plated into 96-well plates (BD Biosciences) and left to adhere overnight in an incubator at 37°C. 100nM DK-PGD<sub>2</sub>, 500nM DK-PGD<sub>2</sub>, 100nM DK-PGD<sub>2</sub> with 1µM AZD6430 and 1µM AZD6430 were added to triplicate wells and incubated overnight. Media only wells and untreated bronchial epithelial cells were used as controls. 20µl MTT reagent was added to each well and incubated for 3.5 hours at 37°C. Media was removed and 150µl MTT solvent was added. The plate was covered with foil to protect from light and put on a plate shaker for 15 minutes. The absorbance of each well was read at 590nm with a reference filter of 620nm using an Enspire plate reader.

### **2.12. Annexin V-FITC Apoptosis assay (materials used described in table 2.8)**

The annexins are a group of homologous proteins which bind phospholipids in the presence of calcium (391,392). During the early stages of apoptosis there is a

loss of phospholipid asymmetry within cells with phosphatidyl-serine translocating from the internal part of the plasma membrane to the external portion of the membrane. The phosphatidyl-serine becomes available to bind to the annexin V-FITC conjugate in the presence of calcium. Annexin V-FITC is detected as a green fluorescence. Healthy control cultured bronchial epithelial cells were incubated for 2 hours with either 1µg/ml staurosporine (apoptosis positive control), 100nM DK-PGD<sub>2</sub>, or 1µM AZD6430. Cells were stained as per manufacturer's instructions using Annexin V-FITC apoptosis detection kit (Sigma), and fluorescence was determined using a flow cytometer.

### ***2.13. Cell migration (materials used described in table 2.9)***

Cell migration in response to CRTh2 activation for bronchial epithelial cells was assessed using an Oris cell migration kit (tebu-bio). This procedure utilises stoppers, which when applied to the centre of a well of a 96-well plate create a migration zone. Cells are seeded around the stopper and left to adhere overnight. Stoppers are removed then treatments are added for 24 hours to enable cellular migration to take place. To date, this assay has not been used for bronchial epithelial cells. This assay format has advantages over alternative assays such as scrape-wound migration assays (331,393), as no damage is being applied to the cells and therefore the migratory activity is based on the agonist activation of migration. Optimisation of the assay for bronchial epithelial cells was carried out. Cells adhered sufficiently to 96-well plates without the requirement for collagen coating which could affect migration activity. For this assay, the manufacturer suggests staining with a fluorescent dye such as calcein (Invitrogen) to assess the degree of



migration into the migration zone, then read the level of fluorescence in each migration zone (using a detection mask fitted onto the well which only shows the migration zone within the well) with a plate reader. However, optimisation of this assay within the current study highlighted that debris within some wells created non-specific fluorescence readings. It was determined in the current study that the best way to assess migration was by visualising and counting the number of cells within the migration zone using a fluorescence based microscope. To ensure that bronchial epithelial cells resembled as close as possible to *in vivo*, early passage cells were used (passage 1 and 2). However, this did limit the number of cells available for the assay. Triplicate repeats for vehicle control (1 $\mu$ M DMSO), 100nM DK-PGD<sub>2</sub> or 100nM DK-PGD<sub>2</sub> and 1 $\mu$ M AZD6430 were added for 24 hours in 5 healthy control donors and 5 asthmatic donors. The concentrations of 500nM and 1 $\mu$ M DK-PGD<sub>2</sub> were tested in cells from 5 healthy control donors. TGF $\beta$ 1 and FGF are growth factors that have been shown to induce cell migration in wound repair assays (394-396). TGF $\beta$ 1 10ng/ml and 25ng/ml fibroblast growth factor (FGF) (R&D Systems) were used as positive controls in cells from 5 healthy control donors and 2 asthmatic donors. Following treatment, cells were fixed, and labelled with 0.05 $\mu$ g/ml Hoescht nuclear dye (Invitrogen) for 15 minutes. The number of cells migrated into the migration zone was counted by a blinded observer.

#### ***2.14. Measurement of intracellular calcium for bronchial epithelial cells (materials used described in table 2.10)***

The measurement of intracellular calcium can be monitored using a number of systems, with high through-put assays such as fluorometric imaging plate reader

(FLIPR) assays routinely used (397). However, calcium responses for primary cells can be technically challenging and alternative small-scale assay formats can be more appropriate. For the current study calcium responses were investigated using a Hamamatsu 1394 perfusion system and a Nikon Eclipse microscope system. Fura-2 was used to investigate the change in intracellular calcium in response to DK-PGD<sub>2</sub> for healthy control bronchial epithelial cells. Fura-2 is a dual excitation, single emission, Ca<sup>2+</sup> ratiometric indicator and can be used with fluorescent microscopy to measure intracellular calcium. Fura-2(AM) ester is an acetoxymethyl (AM) ester, which is a cell permeable derivative of Fura-2. In combination with pluoric acid (mild, non-ionic detergent) Fura-2(AM) crosses cell membranes and once inside the esters are cleaved by endogenous intracellular esterases, regenerating the fluorescent Fura-2 salt in a cell impermeable state. Dual excitation of Fura-2 was carried out at 340/380nm whilst monitoring at 510nm. Fura-2 emits fluorescence at 510nm regardless of calcium binding; however, upon binding to calcium there is an increase in the excitation peak intensity. Therefore, when Fura-2 is excited in quick succession at 340/380nm whilst monitoring at 510nm a ratio of the emission signals allow for monitoring of calcium concentrations (398). Healthy control bronchial epithelial cells were grown on collagen-coated glass coverslips to improve the adherence of the cells to the coverslips. Once seeded onto the coverslips the cells were incubated for a minimum of 3 days to allow for the cells to further adhere to the coverslips. However, the adherence of the epithelial cells was still limited. Sufficient cells were present for limited calcium response analysis, but the technical challenges of the assay prevented thorough testing such as dose-response analysis. Calcium responses to 1µM DK-PGD<sub>2</sub>, 1µM DK-PGD<sub>2</sub> and 1µM AZD6430, 1µM

ionomycin (Sigma) were assessed in fura-2 (Invitrogen) loaded cells. Data was visualised using Perkin Elmer Velocity 6.1.1 software.

### **2.15. *PGD<sub>2</sub> ELISA (materials used described in table 2.11)***

ELISA assays allow for the detection and quantitation of proteins within solutions. PGD<sub>2</sub> is a CRTh2 agonist. It is known to be released from mast cells and for increased levels to be released from IgE activated mast cells (248,251,388). To establish levels of PGD<sub>2</sub> that ALI cultures were exposed to for the HLMC:ALI co-cultures, a prostaglandin D<sub>2</sub>-MOX EIA kit (Cayman Chemicals) was used to assess levels of PGD<sub>2</sub> in supernatant samples. In addition, apical washes from ALI cultures were also assessed for levels of PGD<sub>2</sub>. Apical washes were performed using PBS gently pipetted onto the apical side of the ALI culture for 30 seconds before being removed and stored at -80°C. The PGD<sub>2</sub> ELISA is based on the competition between sample PGD<sub>2</sub> and a PGD<sub>2</sub>-acetylcholinesterase conjugate (PGD<sub>2</sub> tracer) for a limited number of PGD<sub>2</sub> monoclonal antibody binding sites. Sample extraction procedures for protein removal and eicosanoid stabilisation were performed according to the manufacturer's instructions (MOX reactions). PGD<sub>2</sub> concentrations were determined as per manufacturer's instructions. The assay has a detection range between 250 and 2pg/ml and intra- and inter-assay co-efficient of variation was <10%.

### **2.16. *Analysis***

Statistical analysis was performed using PRISM software, version 6 (GraphPad Software, La Jolla, CA). Parametric data analysed with one-way or two-

way analysis of variance (ANOVA), Tukey post-test correction for intergroup comparison, or unpaired and paired t-test. Non-parametric data analysed using the Kruskal-Wallis tests, Dunn's test for post-hoc comparison and Mann-Whitney t-test. Spearman correlation test used for correlation analysis.  $P < 0.05$  was considered significant.

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## **Chapter 3**

### **Results**

# **Expression of human CRTh2 in healthy control and asthmatic bronchial biopsies**

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### 3.1. Chapter Overview

**The aim of this work was to characterise the protein expression profile of human CRTh2 on clinically well-characterised biopsies from healthy control and asthmatic bronchial biopsies.**

An increase in the number of CRTh2 positive inflammatory cells in allergic disease (116) has highlighted a potential role for this receptor in allergy (127,130) and asthma (119,123). In addition, considerable interest in the development of CRTh2 antagonists for both allergic conditions (127,130) and asthma (119,120,123) have strengthened the linkage between CRTh2 and inflammatory related disorders. To date, limited efficacy has been demonstrated for CRTh2 antagonists in asthma (119,123) however, it remains to be determined whether CRTh2 antagonism is more effective in a sub-set of patients. A thorough understanding of CRTh2 expression within the airways and whether changes in receptor expression correlates with disease severity, may aid in identifying a responsive asthmatic group. Unfortunately, there is currently a lack of data describing the protein expression of CRTh2 in bronchial biopsies in asthma, which has limited the cell types that CRTh2 function has been explored within. Comparative studies looking at the expression of CRTh2 on asthma and healthy control biopsies would provide useful data to indicate possible target cells located within the airways thus helping to focus future CRTh2 antagonist study read-outs and patient populations.

In order to investigate the expression of CRTh2 on bronchial biopsies an antibody developed at AstraZeneca (CRTh2 AZ antibody) was used for the immunohistochemistry analysis. The antibody was validated before and during the immunohistochemistry analysis. The CRTh2 immunohistochemistry staining was

quantified to evaluate expression on inflammatory cells and epithelial cells between both healthy control and asthmatic biopsies and across asthma disease severity. To establish which cells expressed CRTh2, CRTh2 staining in relation to phenotype markers was completed using serial staining methods as co-staining methods were not successful. Differential CRTh2 expression was observed for inflammatory cells and epithelial cells in the healthy control compared to the asthmatic biopsies.

### ***3.2. Biopsy clinical characteristics***

The clinical characteristics of subjects with mild, moderate or severe asthma and healthy controls are as shown in **Table 3.1**. Asthmatic biopsies were grouped according to disease severity as mild (GINA I, II), moderate (GINA III) and severe (GINA IV, V). Groups were well matched for age and smoking history. Subjects with asthma had impaired lung function and evidence of eosinophilic airway inflammation. Healthy subjects had no history of respiratory or allergic disease and had normal spirometry.



**Table 3.1.** Clinical characteristics for biopsies used for immunohistochemical analysis.

	<b>Healthy Control (n=10)</b>	<b>Mild asthma (n=8)</b>	<b>Moderate asthma (n=7)</b>	<b>Severe asthma (n=7)</b>	<b>P value</b>
<b>Age</b>	50 (5)	48 (5)	53 (6)	52 (4)	0.94
<b>Gender Male (Female)</b>	7 (3)	3 (5)	2 (6)	2 (5)	0.19
<b>Atopy (n)</b>	4	5	4	6	0.31
<b>Smoking history (ex, current, never)</b>	(2, 0, 8)	(2, 0, 6)	(1, 0, 6)	(2, 0, 5)	0.92
<b>ICS dose mcg BDP eq/day</b>	0	75 (53)	800 (0)	1565 (148)	<0.001
<b>LABA use (%)</b>	0	0	100	100	<0.001
<b>Oral corticosteroid use (n)</b>	0	0	0	3	0.01
<b>FEV<sub>1</sub> (% predicted)</b>	99.9 (4.4)	78.6 (6.8)	79.2 (3.7)	82.0 (8.4)	0.01
<b>FEV<sub>1</sub>/FVC (%)</b>	78.6 (2.9)	72.5 (3.8)	72.1 (3.7)	68.7 (3.3)	0.22
<b>Bronchodilator reversibility (%) #</b>	0.5 (5.2)	10.9 (24.6)	12.4 (20.3)	8.9 (8.9)	0.04
<b>Total cell count(10<sup>6</sup> cells/g sputum) #</b>	ND	1.7 (1.1)	5.5 (5.3)	4.2 (6.1)	0.06
<b>Sputum eosinophils (%) #</b>	ND	3.5 (18.7)	0.4 (1.1)	5.2 (30.8)	0.30
<b>Sputum neutrophils (%) #</b>	ND	47.5 (25.5)	57.4 (67.8)	57.9 (54.0)	0.60

Data expressed as mean (SEM). Comparisons across groups were done by means of ANOVA or the Kruskal-Wallis test. # median (IQR), BDP eq- beclomethasone dipropionate equivalent, ND-not done

### ***3.3. AstraZeneca antibody details used to investigate CRTh2 protein expression***

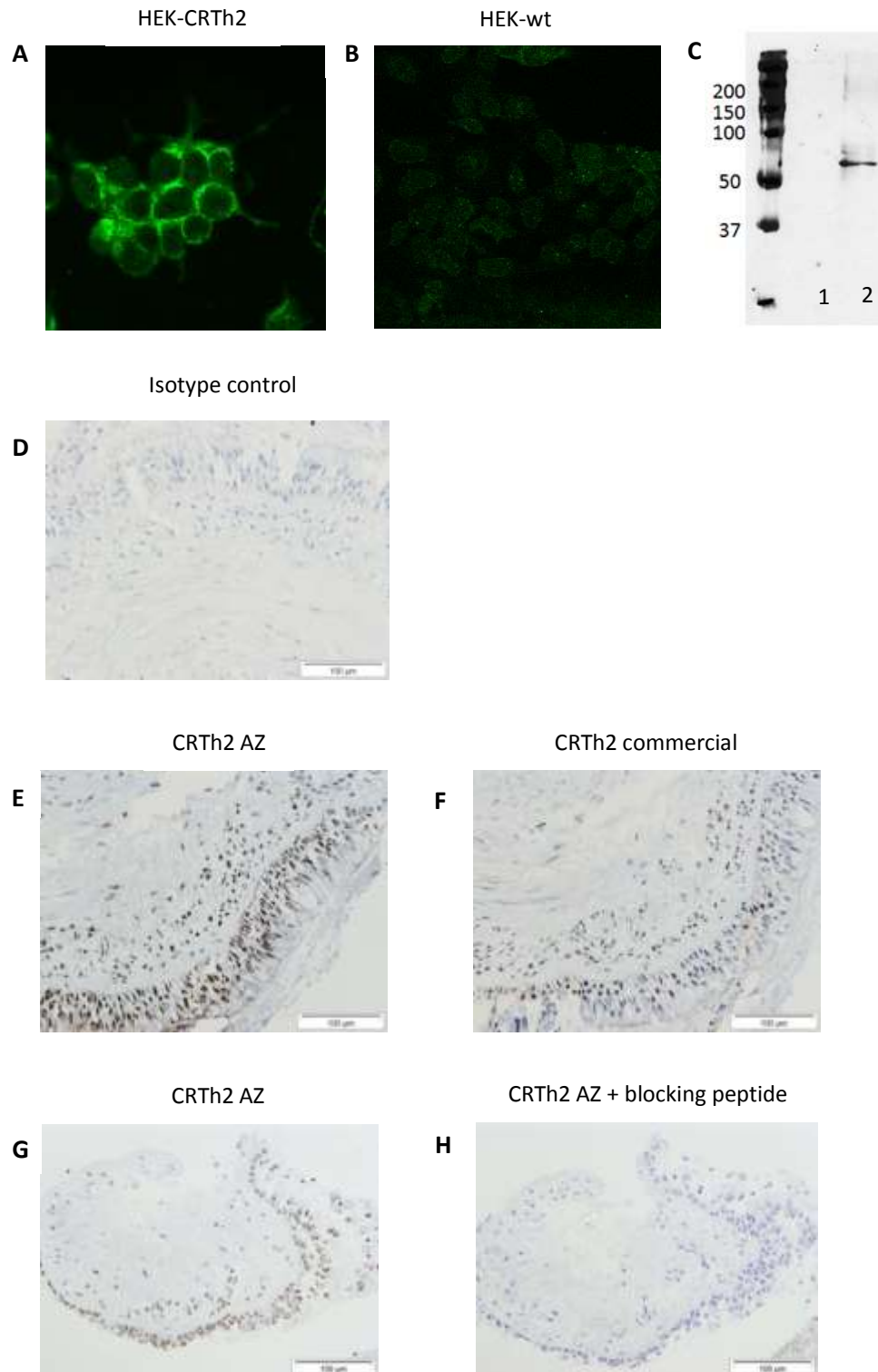
The CRTh2 antibody used for immunohistochemistry was produced at AstraZeneca (CRTh2 AZ, AstraZeneca). This rabbit polyclonal antibody was made using a peptide in the extreme C-terminus region of the human CRTh2 protein. A comparison of the peptide sequence with a peptide database was made and showed no other hits with other peptide sequences except for CRTh2 (data not shown).

### ***3.4. Validation of AstraZeneca antibody used to investigate CRTh2 immunohistochemistry***

The aim of the following experiments was to ascertain the specificity of the CRTh2 AZ antibody using a number of different validation assays. Human embryonic kidney cells (HEK) cells with and without transfected CRTh2 were used as positive (HEK-CRTh2) and negative (HEK-wild-type, HEK-wt) control cells to help to validate the specificity of the CRTh2 AZ antibody. An immunofluorescence assay showed clear membrane staining in HEK-CRTh2 cells, while no staining was found in HEK-wt cells (**Figure 3.1A, B**). A western-blot assay also showed that the CRTh2 AZ antibody recognized only a single band of around 70kDa, the appropriate size for the CRTh2 protein (103) for the HEK-CRTh2 cells, while no band was present for the HEK-wt cells (**Figure 3.1C**). These results using the positive and negative control cells helped to give confidence in the specificity of the CRTh2 AZ antibody before it was used for immunohistochemistry on tissue samples.

Additional validation of the CRTh2 AZ antibody was carried out during immunohistochemistry work on tissue. A rabbit immunoglobulin fraction isotype

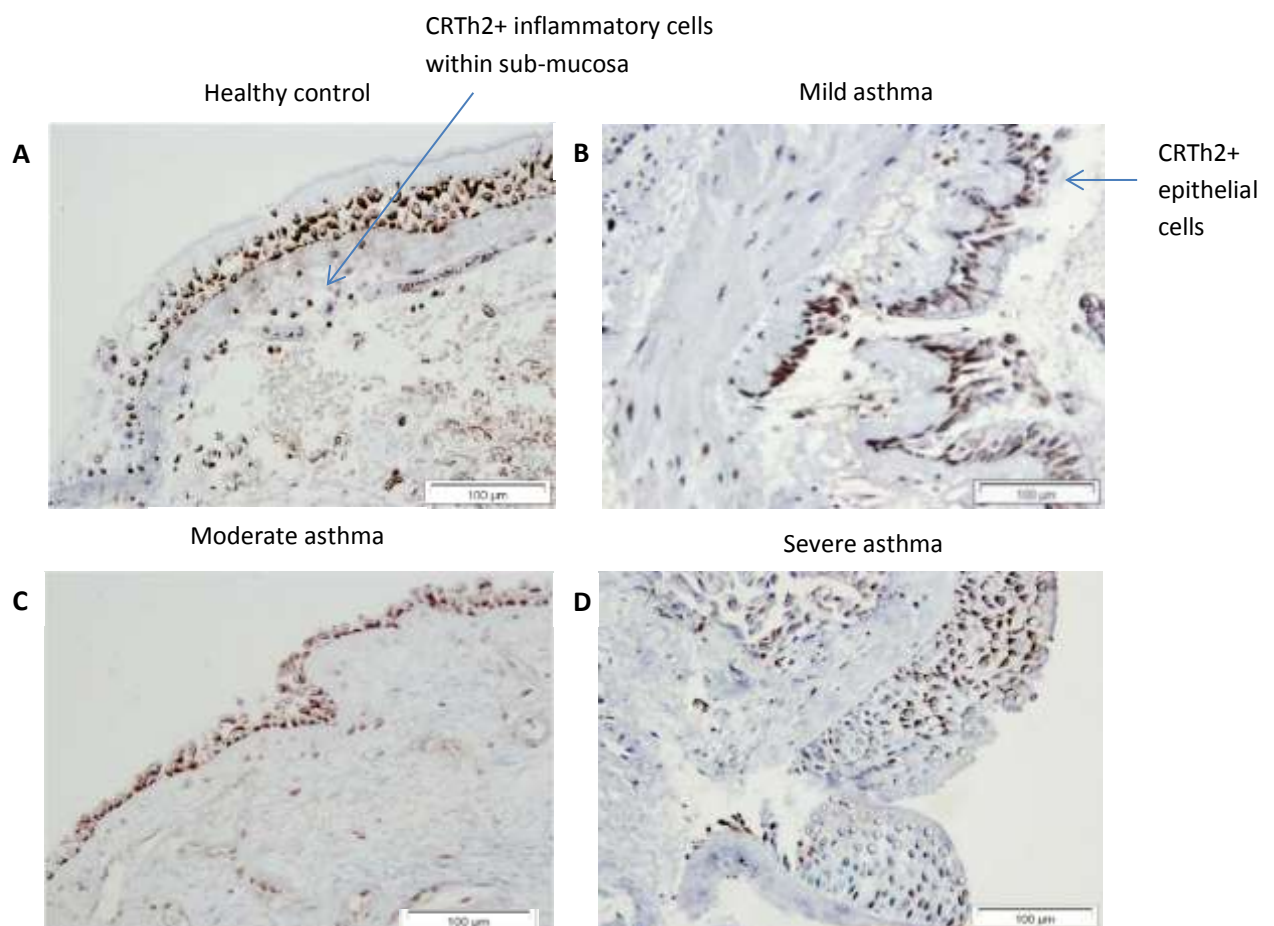
control was used on corresponding sections of bronchial biopsies to the CRTh2 AZ antibody. The protein concentration of the isotype control was matched to the protein concentration of the CRTh2 AZ antibody. No staining was observed for the isotype control suggesting that no non-specific rabbit proteins were binding to the tissue at the protein concentration that the CRTh2 AZ antibody was being used at (**Figure 3.1D**). An additional antibody validation step was to compare the protein expression profile achieved for the CRTh2 AZ antibody with a commercially available rabbit polyclonal antibody made to the N-terminus region of the human CRTh2 peptide (CRTh2 commercial). For both antibodies the same staining pattern was observed, although the staining with the commercial antibody was less intense (**Figure 3.1E, F**). These results help to give confidence in the staining profile for the CRTh2 AZ antibody. A further validation step was to incubate the CRTh2 AZ antibody with its blocking peptide (used to produce the antibody). The staining observed with the CRTh2 AZ antibody was absent when in the presence of the blocking peptide (**Figure 3.1G, H**). These studies help to validate the CRTh2 AZ antibody for immunohistochemistry studies and give increased confidence to the staining pattern that was observed for the bronchial biopsies.



**Figure 3.1. Antibody validation of human CRTh2 AZ antibody.** A) Confocal fluorescent cell staining of CRTh2 AZ antibody (green) on CRTh2 transfected HEK cells. B) Confocal fluorescent cell staining of CRTh2 AZ antibody (green) on wild-type HEK cells. Note lack of staining. C) Western blot for CRTh2 AZ antibody. Lane 1=Wild-type HEK cells. Note lack of band. Lane 2=CRTh2-HEK cells. Single band at 70kDa (appropriate size for CRTh2). D) Rabbit immunoglobulin fraction isotype control. Note lack of staining. E) CRTh2 AZ antibody on asthmatic biopsy (x200 magnification) F) Commercial antibody (ThermoScientific) on same biopsy as E (x200 magnification). G) CRTh2 AZ antibody on asthmatic biopsy (x200 magnification). H) CRTh2 AZ antibody which has been pre-incubated with blocking peptide on same biopsy as G (x200 magnification). Note lack of staining.

### 3.5. Immunohistochemistry for CRTh2 on healthy control and asthmatic bronchial biopsies

Positive CRTh2 expression (using CRTh2 AZ antibody) was observed on healthy control and the asthmatic bronchial biopsy material (**Figure 3.2**). CRTh2 expression was observed on inflammatory infiltrate within the sub-mucosa and on bronchial epithelial cells for all biopsies. Positive expression was also observed on sporadic epithelial cells of the sub-mucosal glands.



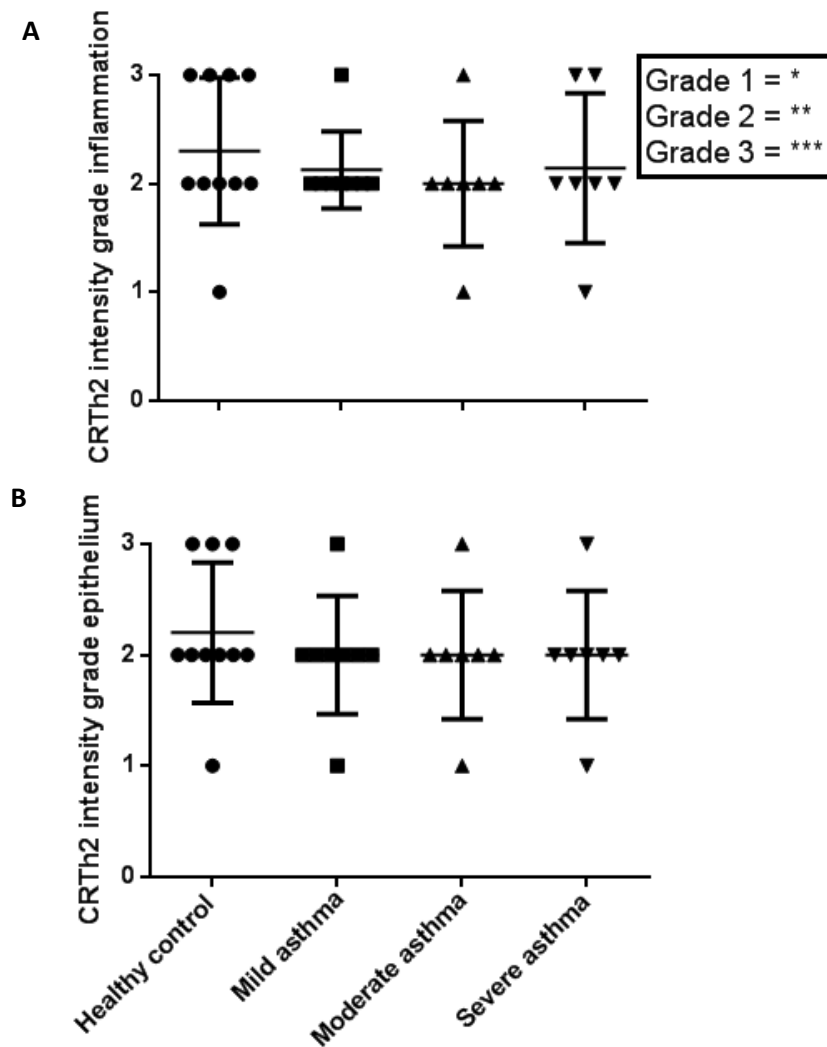
**Figure 3.2. Immunohistochemistry of CRTh2 on healthy control and asthmatic biopsies.** CRTh2 AZ antibody staining (brown) on: A) healthy control biopsy B) mild asthmatic biopsy C) moderate asthmatic biopsy D) severe asthmatic biopsy (all images x200 magnification).

### ***3.6. Intensity gradings for CRTh2 immunohistochemical staining***

To establish if there were any differences in the intensity of CRTh2 staining across the bronchial biopsies a grading system was devised as shown in table 3.2. The intensity of staining was assessed for both the CRTh2 positively stained inflammatory cells and the epithelial cells. All slides were initially screened to be able to identify the range of intensity of CRTh2 staining from which table 3.2 was devised. The intensity grading ranged from grade 1, low level intensity of staining, to grade 3, a high level intensity of staining. No significant difference was observed between groups for the intensity of the CRTh2 staining for the inflammatory cells or the epithelial cells (**Figure 3.3**). These data suggests that the intensity of CRTh2 staining is not different in healthy control biopsies compared to asthmatic biopsies. However, the number of CRTh2+ cells and the CRTh2+ phenotype may be different in healthy control compared to asthmatic biopsies so the aim of the next section of work was to investigate if the numbers of CRTh2+ cells were different in health compared to disease.

**Table 3.2.** Intensity of staining grading criteria

Grade	Description of intensity of staining
1	* Low level intensity of CRTh2+ staining
2	** Medium level intensity of CRTh2+ staining
3	*** High level intensity of CRTh2+ staining



**Figure 3.3.** CRTh2 intensity staining assessment of healthy control and asthmatic biopsies. A) Intensity grades of CRTh2 staining on inflammatory cells for healthy control and asthmatic biopsies. p-value based on Kruskal-Wallis. Overall p-value 0.730 B) Intensity grades of CRTh2 staining on epithelial cells for healthy control and asthmatic biopsies. p-value based on Kruskal-Wallis. Overall p-value 0.827.

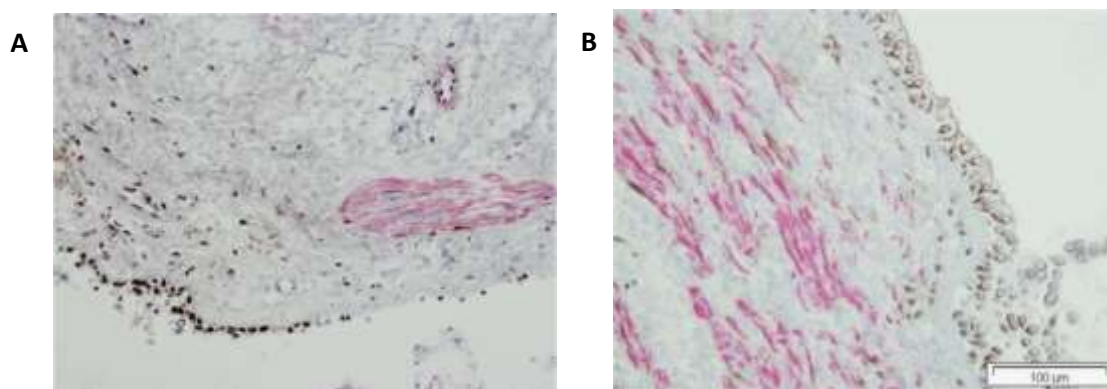
### ***3.7. Phenotyping of CRTh2+ cells***

The aim of this section of work was to try to phenotype the CRTh2+ cells that had been observed in the initial CRTh2 immunohistochemistry work (**Figure 3.2**), to help to understand which cells CRTh2 may have a role on within the tissue compartment of the airways. Co-localisation of CRTh2 protein expression and the phenotype marker of interest could be established using a dual staining method, where both stains are applied to the same section, or on serial sections. Dual staining is routinely carried out using fluorescent-based immunohistochemistry (254). However, this technique could not be applied to the GMA blocks used in this study as the tissue is fixed in acetone which causes high levels of autofluorescence. Therefore a chromagen dual stain was instead used.

### ***3.8. Validation of dual staining for CRTh2 and phenotype markers***

A commercially available dual staining kit was used. CRTh2 and a number of phenotype antibodies were tested on the same sections, these included mast cell tryptase, CD3, MBP and  $\alpha$ -SMA. However, only CRTh2 and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) could be optimised (**Figure 3.4**). It is likely that this pair of antibodies work together as a dual stain as CRTh2 was absent from the smooth muscle cell bundles so it was easy to identify the CRTh2 staining localised by DAB (brown) in comparison to smooth muscle actin identified by permanent red (red).





**Figure 3.4. Dual staining optimisation of CRTh2 and phenotype markers.**

CRTh2 expression (brown staining) on epithelial cells and inflammatory cells within the sub-mucosa and smooth muscle actin (red staining) on smooth muscle bundle for A) healthy control biopsy B) an asthmatic biopsy (x200 magnification).

As the dual stain method did not work for CRTh2 and the phenotype markers, the CRTh2 positive cells were phenotyped using the serial stain technique (376). In addition, quantifying the CRTh2<sup>+</sup> cell numbers and the phenotype<sup>+</sup> cell numbers, provided data to show how inflammatory phenotype, epithelial phenotype and CRTh2 phenotype numbers differed in healthy control versus asthmatic bronchial biopsies. A summary of these findings are described below.

### ***3.9. Expression of CRTh2 on inflammatory cells***

For inflammatory cells, cell counts were calculated per mm<sup>2</sup> area of sub-mucosa for inflammatory cells. Quantification of CRTh2<sup>+</sup> inflammatory cells within the sub-mucosa demonstrated a significant increase in the severe asthmatic biopsies compared to the healthy control biopsies (mean [SEM] 78[5] versus 22[3] cells/mm<sup>2</sup> submucosa,  $p < 0.001$ . **Figure 3.5A**). This data suggests that the number of CRTh2<sup>+</sup> inflammatory cells is increased in asthma, and related to disease severity.

### 3.9.1. CRTh2 and T-cells

T-cells were identified using CD3, which highlights the total T-cell population. Staining for CRTh2 in relation to CD3 staining indicated that CRTh2 was expressed on some T-cells within the sub-mucosa compartment of biopsies from healthy control and asthmatic patients (**Figure 3.5B**). The number of CRTh2<sup>+</sup>CD3<sup>+</sup> cells was significantly increased in the mild, moderate and severe asthmatic biopsies compared to healthy control (mean [SEM] 25[5] versus 14[2] mm<sup>2</sup>submucosa p=0.011; 33[3] versus 14[2] mm<sup>2</sup>submucosa p<0.001; 47[4] versus 14[2] mm<sup>2</sup>submucosa p<0.001. **Figure 3.5F**) and in the severe compared to the mild and moderate asthmatic biopsies (47[4] versus 25[5] mm<sup>2</sup>submucosa p<0.001; 47[4] versus 33[3] mm<sup>2</sup>submucosa p=0.030. **Figure 3.5F**). In a sub-set of asthmatics (n=12) and healthy controls (n=5) patients the CRTh2<sup>+</sup>CD3<sup>+</sup> phenotype was investigated further using CD4 and CD8 markers (**Figure 3.5C**). The number of CRTh2<sup>+</sup>CD4<sup>+</sup> was significantly increased in asthma compared to healthy controls (mean [SEM] 15[3] versus 4[1] mm<sup>2</sup>submucosa p=0.002). CRTh2 expression was also observed for CD8<sup>+</sup> cells but only a small proportion of cells and no significant differences between healthy control (mean [SEM] 0.6[0.4] mm<sup>2</sup>submucosa) and mild (4[1] mm<sup>2</sup>submucosa), moderate (6[3] mm<sup>2</sup>submucosa) and severe (6[2] mm<sup>2</sup>submucosa) asthmatic biopsies were found. These findings suggest that CRTh2 is expressed on T-cells within the tissue compartment of bronchial biopsies and that in asthmatic patients increased numbers of CRTh2<sup>+</sup> T-cells are present, and these cells are predominantly of the phenotype CD4<sup>+</sup>.

For total population of CD3<sup>+</sup> counts across the healthy control and asthmatic biopsies, the numbers of CD3<sup>+</sup> cells were significantly increased in the moderate

and severe asthmatic biopsies compared to healthy control (mean [SEM] 71[12] versus 26[3] mm<sup>2</sup>submucosa p=0.002; 69[7] versus 26[3] mm<sup>2</sup>submucosa p=0.003. **Figure 3.5G**). These findings suggest that T-cell numbers are elevated in the sub-mucosa of moderate-severe asthmatic airways compared to healthy control.

### **3.9.2. CRTh2 and Eosinophils**

Eosinophils were identified by major basic protein (MBP). Eosinophils secrete increased amounts of arginine rich proteins such as eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and major basic protein (MBP) (202) in asthma so it was thought that MBP would be a good marker to identify eosinophils in this study. Deposits of MBP have also been found in areas of epithelial damage of asthmatic lungs (204). It was hoped in addition to identifying eosinophils the MBP marker would highlight areas of MBP aggregates within the epithelium and therefore any association of CRTh2 and these deposits could be investigated. Staining for CRTh2 in relation to MBP staining indicated that CRTh2 was expressed on eosinophils within the sub-mucosa compartment of biopsies from normal and asthmatic patients (**Figure 3.5D**). The numbers of CRTh2+MBP+ cells was found to be significantly increased in the moderate asthmatic biopsies compared to healthy control, mild and severe asthmatics (mean [SEM] 20[7] versus 2[0.5] mm<sup>2</sup>submucosa p<0.001; 20[7] versus 5[3] mm<sup>2</sup>submucosa p=0.003; 20[7] versus 4[2] mm<sup>2</sup>submucosa p=0.002. **Figure 3.5F**). As only 2 asthmatic biopsies of all the biopsies investigated had MBP deposits present within the epithelium it was not possible to identify any correlation between MBP deposits in the epithelium and CRTh2 expression. These data suggest that CRTh2 is expressed on eosinophils

within the tissue compartment of bronchial biopsies and that increased numbers of CRTh2+ eosinophils are present in moderate asthmatic biopsies.

For the total MBP+ population across healthy control and asthmatic biopsies, the total numbers of MBP+ cells were not significantly different across biopsies (**Figure 3.5H**). The moderate severe asthmatic group overall contained the highest MBP+ counts, but due to a high variance across the group this did not reach significance (**Figure 3.5H**).

### **3.9.3. CRTh2 and Mast cells**

Mast cells were identified by mast cell tryptase. Staining for CRTh2 in relation to mast cell tryptase staining indicated that CRTh2 was expressed on a small population of mast cells within the sub-mucosa compartment of biopsies from healthy control and asthmatic patients (**Figure 3.5E**). There were no significant differences in the percentages of CRTh2+tryptase+ cells for healthy control versus asthmatic groups (**Figure 3.5F**). Due to lack of CRTh2 staining on some mast cells, to identify if there was a sub-population of CRTh2+ mast cells, mast cell chymase was also investigated in relation to mast cell tryptase and CRTh2. There was no pattern of CRTh2+ staining in relation to mast cell tryptase or chymase, cells were identified that were tryptase<sup>+</sup>chymase<sup>-</sup>CRTh2<sup>+</sup> and tryptase<sup>+</sup>chymase<sup>+</sup>CRTh2<sup>+</sup> (data not shown). These data suggest that CRTh2 is expressed on a sub-set of mast cells within the tissue compartment of both healthy control and asthmatic bronchial biopsies. As not all mast cells were found to be CRTh2+ this finding suggests that CRTh2 may be expressed on a sub-set of mast cells. However, serial staining

experiments with the mast cell phenotype sub-group marker chymase did not demonstrate CRTh2 expression specific to this mast cell sub-set.

For the total mast cell tryptase+ population across healthy control and asthmatic biopsies, the total numbers of tryptase+ cells were not significantly different across biopsies (**Figure 3.5I**). This data suggests that the number of tryptase+ mast cells are not significantly altered within the sub-mucosa for the asthma compared to healthy control biopsies used in the current study.

#### ***3.9.4. CRTh2 and Neutrophils***

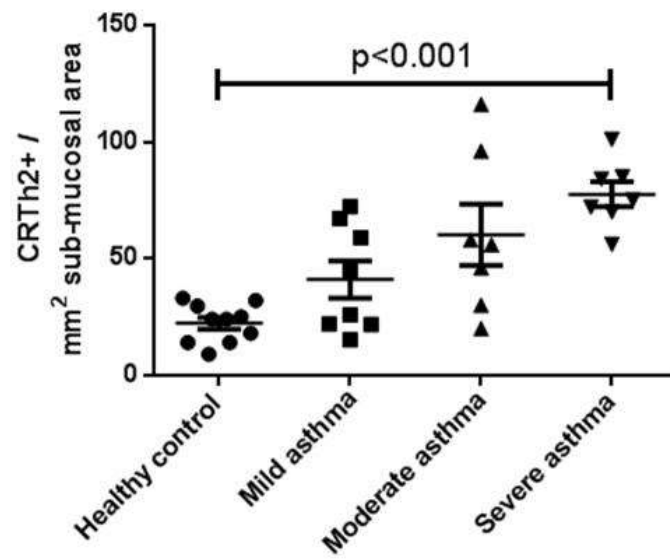
Neutrophils were identified by neutrophil elastase. There was no co-localisation of CRTh2 and neutrophil elastase (data not shown). This finding demonstrates for healthy control and asthmatic bronchial biopsies CRTh2 is not present on neutrophils within the tissue compartment.

#### ***3.9.5. CRTh2 and Macrophages***

Staining for CRTh2 in relation to CD68 (macrophage marker) was inconclusive due to the low number of CD68 positive cells present. For the majority of biopsies no CD68 staining was present.

Taken together, these results demonstrate that CRTh2 is expressed on a sub-set of T-cells, eosinophils and mast cells within the sub-mucosa tissue of healthy control and asthmatic bronchial biopsies. The number of CRTh2+ inflammatory cells is significantly increased within the sub-mucosa of severe asthmatic patients, with the largest contribution of these cells being CRTh2+ T-cells.

A



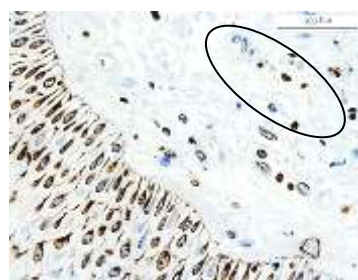
CD3

CRTh2

B



CD4



CRTh2

C

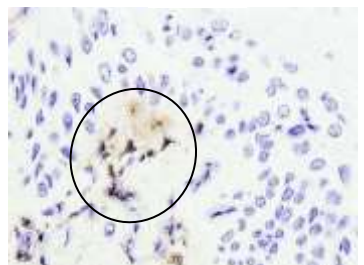


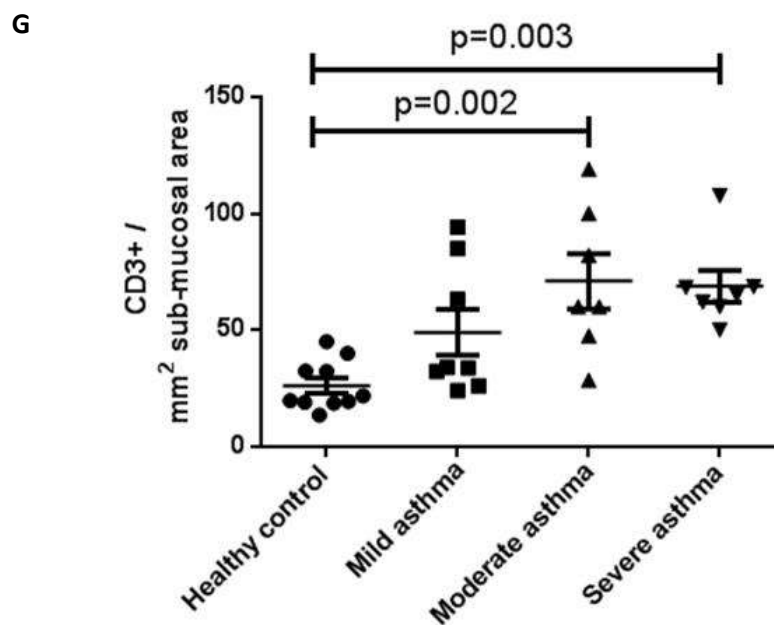
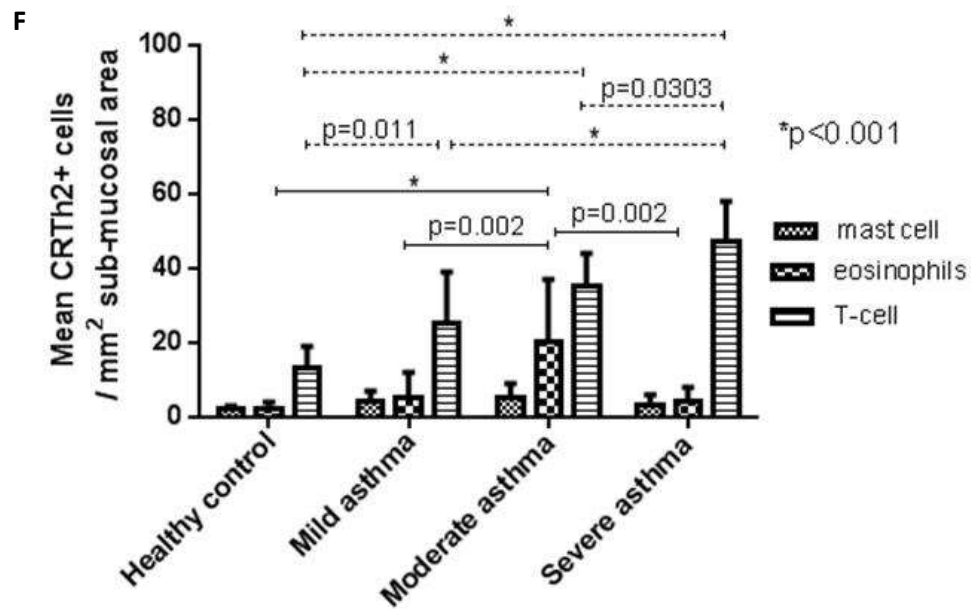
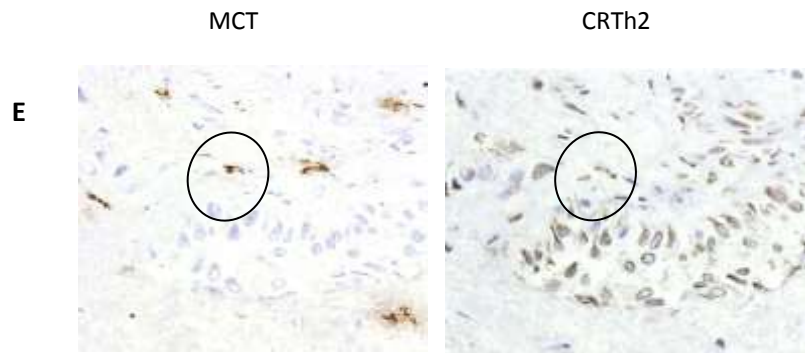
MBP



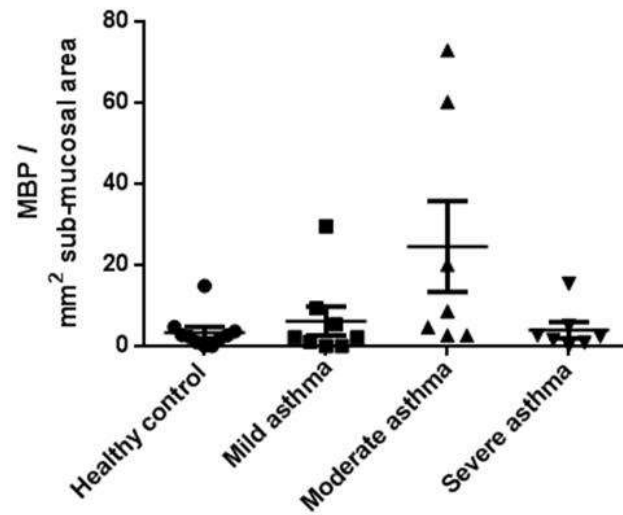
CRTh2

D

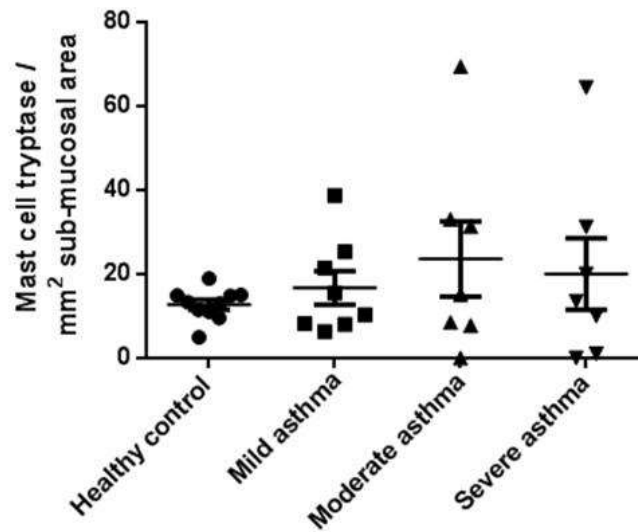




H



I



**Figure 3.5. Serial section staining for CRTh2 with inflammatory cell phenotype markers.** All images x400 magnification. A) Dot-plot of CRTh2+ inflammatory cells within the sub-mucosa for healthy control, mild, moderate and severe asthmatics. p value based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test. B) CD3 expression (brown staining) on T-cells within the sub-mucosa on a healthy control biopsy and CRTh2 expression (brown staining) on CD3+ cells within the sub-mucosa on same biopsy. C) CD4 expression (brown staining) on T-cells within the sub-mucosa on a severe asthmatic biopsy and CRTh2 expression (brown staining) on CD4+ cells within the sub-mucosa on same biopsy. D) MBP expression (brown staining) on eosinophils within the sub-mucosa on a moderate asthmatic biopsy and CRTh2 expression (brown staining) on MBP+ cells within the sub-mucosa on same biopsy. E) Mast cell tryptase expression (brown staining) on mast cells within the sub-mucosa on a moderate asthmatic biopsy and CRTh2 expression (brown staining) on MCT+ cells within the sub-mucosa on same biopsy. F) Numbers of mast cells (mast cell tryptase+), eosinophils (MBP+) and T-cells (CD3+) that are CRTh2+ as assessed by co-localisation of sequential sections. p value based on two-way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. G) Dot-plot of CD3+ T-cells for healthy control, mild, moderate and severe asthmatics. p value based on one-way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. H) Dot-plot of MBP+ cells for healthy control, mild, moderate and severe asthmatics I) Dot-plot of mast cell tryptase+ cells for healthy control, mild, moderate and severe asthmatics.



### **3.10. Expression of CRTh2 on epithelial cells**

Total counts were assessed per 10mm<sup>2</sup> of epithelial area. The number of CRTh2<sup>+</sup> epithelial cells was significantly reduced in moderate and severe asthma compared to the healthy control biopsies (mean [SEM] 30 [5] versus 72 [11] /10mm<sup>2</sup> epithelium, p=0.036; 21 [3] versus 72 [11] /10mm<sup>2</sup> epithelium, p=0.001), and in the severe asthma biopsies compared to mild asthma (21 [3] versus 54 [8] /10mm<sup>2</sup> epithelium, p=0.027. **Figure 3.6A**).

As described in the introduction the bronchial epithelium is composed of basal, columnar epithelial cells and mucus producing goblet cells. To identify if there were differences in the composition of these cells within the healthy control compared to the asthmatic biopsies, immunostaining for epithelial phenotype markers was carried out. In addition, serial staining of the epithelial cell markers with CRTh2 was used to identify if CRTh2 expression was associated with a particular epithelial phenotype. p63 which is essential for the correct differentiation of multi-layered epithelium (267), and has also been found to be critical for epithelial repair in wound healing assays (399), was used as a basal epithelial cell marker (400,401). Cytokeratin 7 was used as a mature epithelial cell marker (402). MUC5AC was used as a goblet cell marker (62).

p63 protein expression was observed on epithelial cells in healthy control and asthmatic biopsies. The p63<sup>+</sup> epithelial cells in areas of normal epithelial differentiation were found adjacent to the basement membrane, in agreement with previous data (400) (**Figure 3.6B**). CRTh2 was found to co-localise with the p63<sup>+</sup> cells showing that CRTh2 is present on basal epithelial cells (**Figure 3.6B**) however,

CRTh2 was clearly on additional p63- epithelial cells. p63+ cells were also evident in areas where epithelial repair processes may have been occurring. Here, flattened cells were present where there was a lack of a true epithelial barrier, suggesting that p63+ cells could be spreading to try to cover an area of basement membrane which has been stripped of epithelium, a previously reported function of these cell types (275). Co-localisation with CRTh2 demonstrated p63+CRTh2+ cells in areas of denuded epithelium (**Figure 3.6C, D**). These data suggests that CRTh2 is expressed on basal epithelial cells and on basal epithelial cells that have the appearance of migrating along the basement membrane.

No significant changes in the numbers of p63+ cells were observed for the healthy control compared to the asthmatic biopsies (**Figure 3.6G**). This data suggested that the number of basal epithelial cells was not significantly different for the healthy control compared to asthmatic bronchial biopsies used for the current study.

Cytokeratin 7 was co-localised with CRTh2 (**Figure 3.6E**), demonstrating that CRTh2 is also present on columnar epithelial cells. No significant changes in cytokeratin 7+ cells were observed between healthy control compared to asthmatic biopsies (**Figure 3.6H**). This data suggests that the number of columnar epithelial cells, identified by cytokeratin 7, is not significantly altered in asthma compared with the healthy control bronchial biopsies used for the current study.

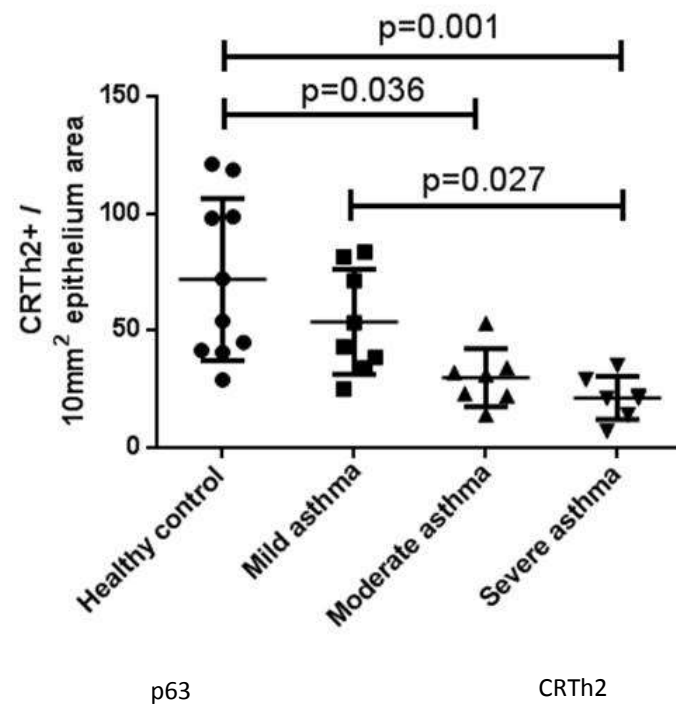
The percentage area of MUC5AC+ staining was calculated per area of fully differentiated epithelium only, rather than total epithelial area, as MUC5AC+ cells are normally only found in the higher layers of the epithelium (268). CRTh2 was

not expressed on the large MUC5AC+ goblet cells, but was expressed on surrounding epithelial cells (**Figure 3.6F**), suggesting that CRTh2 is not expressed on goblet cells.

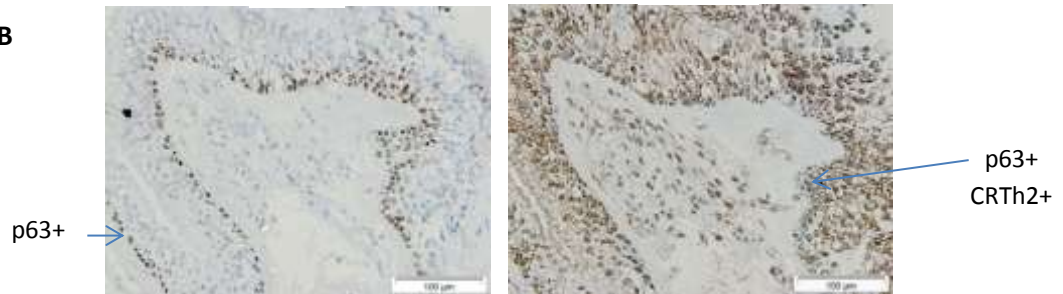
A significantly increased area of MUC5AC+ staining was observed for both the moderate and severe biopsies compared to healthy control (mean [SEM] 62[12]% versus 27[4]%,  $p=0.035$ ; 61[8]% versus 27[4]%,  $p=0.042$ . **Figure 3.6I**). These data suggests that in asthmatic airways the overall area of the mucus-producing, MUC5AC+ cells, is increased compared to healthy control.

Taken together, these data suggest that CRTh2 is expressed on both basal and columnar epithelial cells within healthy control and asthmatic bronchial biopsies. The number of CRTh2+ epithelial cells is significantly lower for moderate-severe asthmatic biopsies when compared to healthy control.

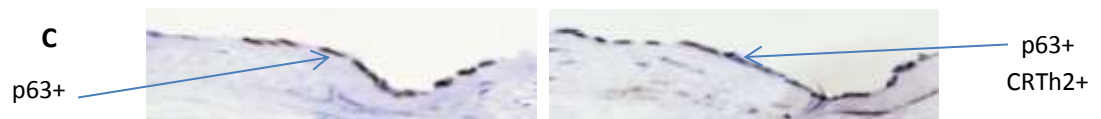
**A**



**B**



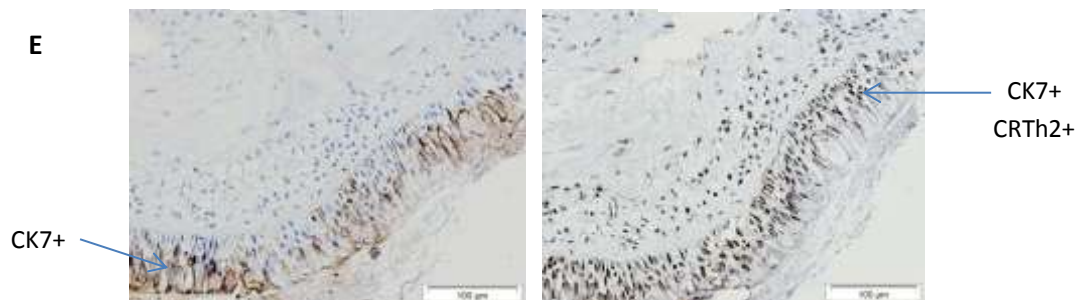
**C**

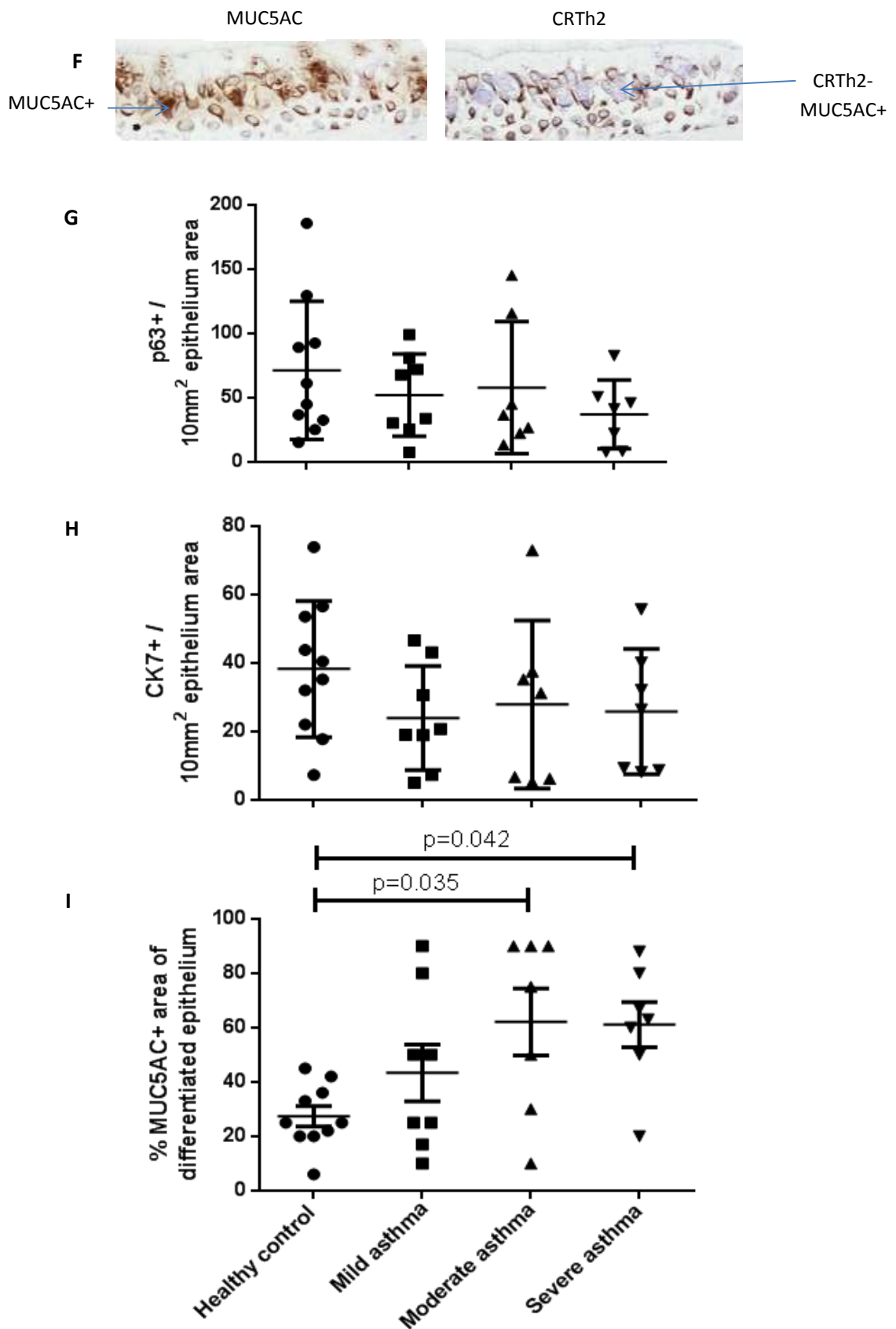


**D**



**E**



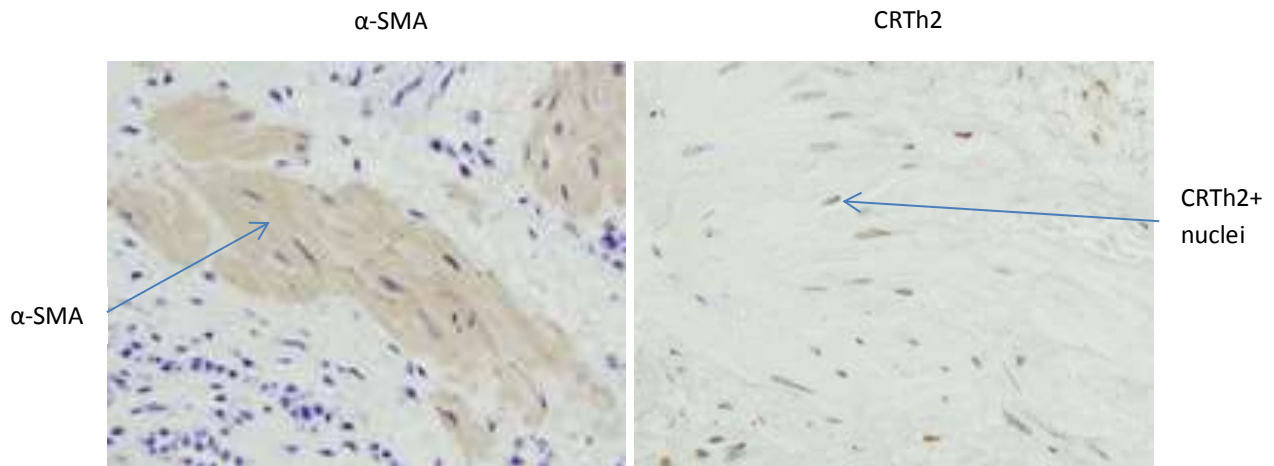


**Figure 3.6. Serial staining for CRTh2 and epithelial cell markers.** A) Dot-plot of CRTh2+ epithelial cells, in healthy control, moderate and severe asthmatics. p value

based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test. B) p63 expression (brown staining) on epithelial cells on an asthmatic biopsy and CRTh2 expression (brown staining) on epithelial cells on same biopsy (x200 magnification). C) p63 expression on flattened epithelial cells in an area of epithelial loss and CRTh2 expression on p63 positive cells (x400 magnification). D) p63 expression on flattened epithelial cells in an area of epithelial loss and CRTh2 expression on p63 positive cells (x400 magnification). E) Cytokeratin 7 expression (brown staining) on epithelial cells on an asthmatic biopsy and CRTh2 expression (brown staining) on epithelial cells on same biopsy (x200 magnification). F) MUC5AC expression (brown staining) on goblet cells on an asthmatic biopsy and lack of CRTh2 expression on MUC5AC cells on same biopsy (x400 magnification). G) Dot-plot of p63+ epithelial cells, in healthy control, moderate and severe asthmatics. H) Dot-plot of cytokeratin 7+ epithelial cells, in healthy control, moderate and severe asthmatics. I) Dot-plot of %MUC5AC staining in healthy control, moderate and severe asthmatics. p value based on one-way ANOVA. Overall p-value 0.019; p-values shown in figure based on Tukey post hoc test.

### ***3.11. CRTh2 and airway smooth muscle***

Smooth muscle actin ( $\alpha$ -SMA) was used to identify airway smooth muscle bundles within the bronchial biopsies. CRTh2 protein expression did not co-localise with the smooth muscle actin as CRTh2 protein expression was not found diffusely stained throughout the smooth muscle bundle. However, for 1-2 biopsies in each group, nuclei within the  $\alpha$ -SMA area were associated with CRTh2 protein expression (**Figure 3.7**). To address if the occasional positive nuclei were fibrocytes (403), CRTh2 was stained in relation to  $\alpha$ -SMA, CD34 and collagen I. There was no co-localisation of CRTh2 with CD34 or collagen I within the smooth muscle bundles (data not shown) suggesting the CRTh2+ nuclei were not fibrocytes within the smooth muscle bundle. These data suggest that CRTh2 is not expressed on airway smooth muscle cells. Occasional CRTh2 positive nuclei within the airway smooth muscle could be due to infiltrating inflammatory cells.



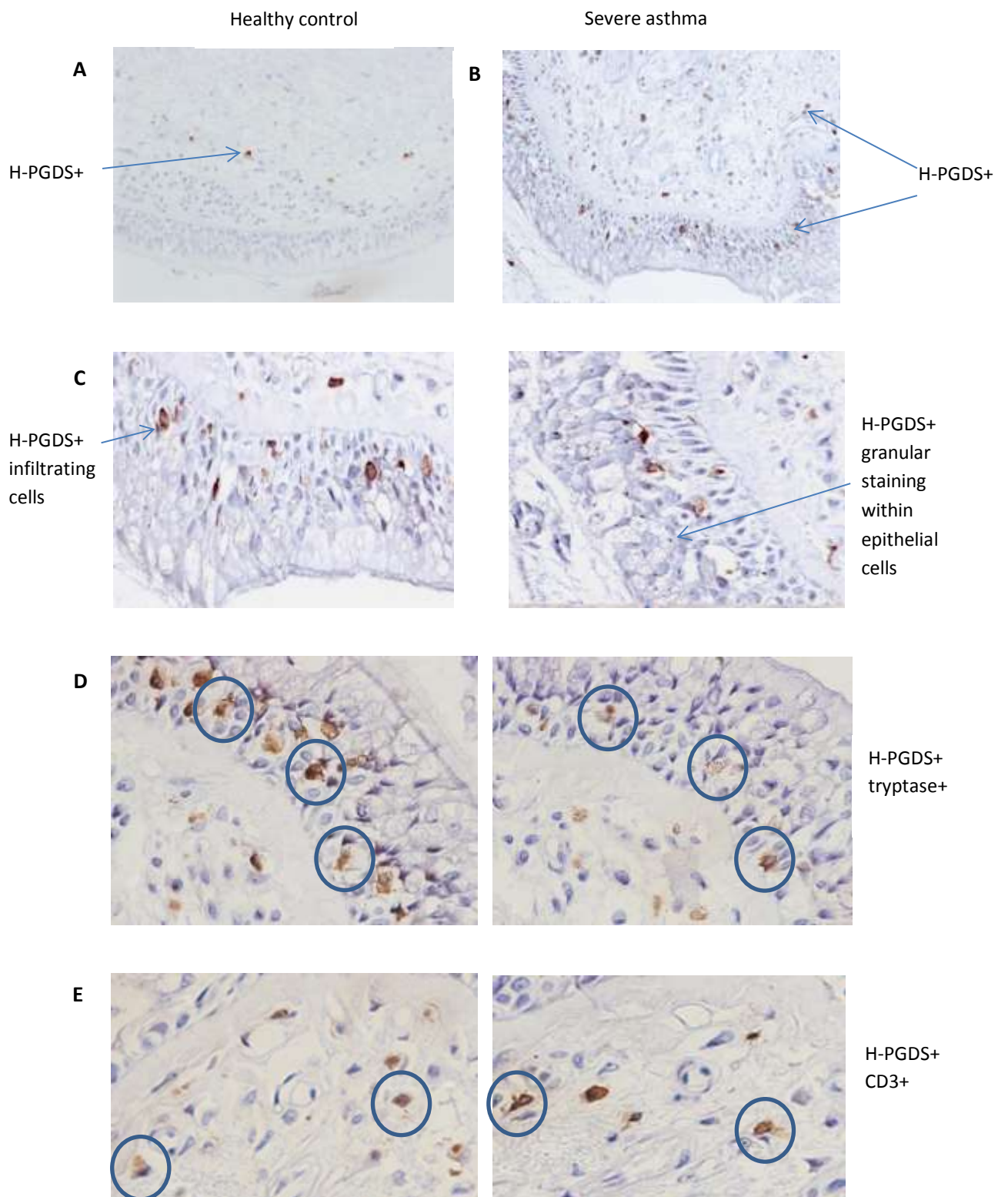
**Figure 3.7. Serial staining for CRTh2 and  $\alpha$ -SMA.** Representative images for 1-2 biopsies of n=10 healthy control, n=22 asthmatic biopsies.  $\alpha$ SMA staining on smooth muscle bundle of an asthmatic biopsy and CRTh2 expression (brown staining) occasional nuclei within a smooth muscle bundle on same biopsy (x400 magnification).

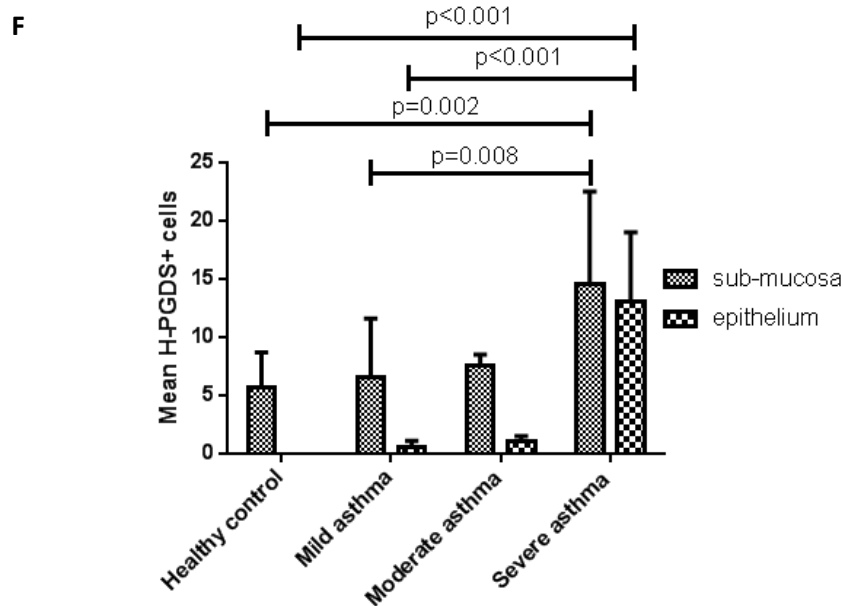
### 3.12. Hematopoietic prostaglandin synthase (H-PGDS) staining within biopsies

H-PGDS is an enzyme that is part of the pathway that liberates  $\text{PGD}_2$ , a major ligand for CRTh2, from oxidative metabolism of arachidonic acid (404). The expression of H-PGDS was investigated on the bronchial biopsies to assess if potential  $\text{PGD}_2$  producing cells were present in similar areas to where CRTh2+ cells were located. This information could therefore provide evidence for the potential for CRTh2 activation within the airways. There is also precedence for H-PGDS expression within the epithelium of the gastric mucosa and skin (405,406), so it was of interest to investigate if H-PGDS expression was present on bronchial epithelial cells. Immunohistochemical detection of H-PGDS demonstrated positive staining in cells found within the sub-mucosa for healthy control and asthmatic biopsies (**Figure 3.8A-D**). The number of H-PGDS+ cells within the sub-mucosa were

significantly increased in the severe asthmatic biopsies when compared to healthy control and mild asthmatics (14.5[8] versus 5.7[1] mean number of H-PGDS+ cells,  $p=0.008$ ; 14.5[8] versus 6.6[2] mean number of H-PGDS+ cells,  $p=0.008$ . **Figure 3.8F**). H-PGDS+ inflammatory cells were also present within the epithelium, but only for the asthmatic biopsies. The number of H-PGDS+ cells within the epithelium were significantly increased in the severe asthmatic biopsies when compared to the healthy control and mild asthmatic biopsies (13[6] versus 0[0] mean number of H-PGDS+ cells,  $p<0.001$ ; 13[6] versus 0.6[0.6] mean number of H-PGDS+ cells,  $p<0.001$ . **Figure 3.8F**). These data suggest that cells that contain the enzyme required to produce  $\text{PGD}_2$  are present within the sub-mucosa and epithelium of bronchial biopsies, and these cells are more frequent in severe asthma. For a subset of samples (healthy control  $n=8$ , asthmatic  $n=15$ ), serial staining of H-PGDS with mast cell tryptase and CD3 markers showed that some of the H-PGDS+ cells were mast cells and some were T-cells (**Figure 3.8D, E**), although additional H-PGDS+ cells were also present. These data suggest that in addition to H-PGDS+ mast cells and T-cells, other H-PGDS inflammatory cells are also present within bronchial biopsies. In addition, granular staining for H-PGDS was also observed within the epithelial cells, for the healthy control and the asthmatic biopsies, with this staining being more prominent in the asthmatic biopsies (**Figure 3.8C**). These data suggest that bronchial epithelial cells may express H-PGDS and therefore have the potential to produce  $\text{PGD}_2$ . Taken together this data suggests that  $\text{PGD}_2$  could be produced by cells both within the sub-mucosa and the epithelium, and that this could activate CRTh2, which in the current study has been found to be expressed on a subset of inflammatory cells and bronchial epithelial cells within the biopsies.





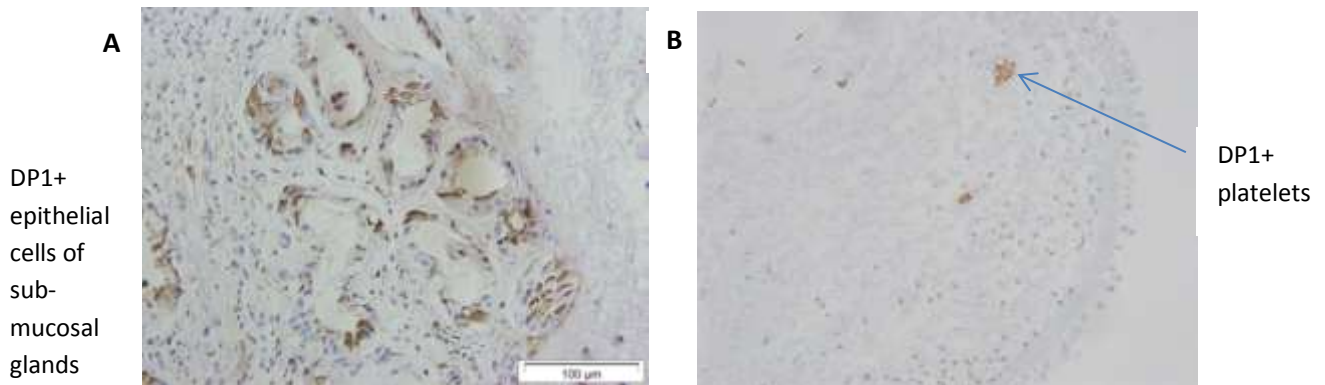


**Figure 3.8. H-PGDS staining for healthy control and asthmatic biopsies.** A) H-PGDS staining (brown staining) on a healthy control biopsy (x200 magnification). B) H-PGDS staining on a severe asthmatic biopsy (x200 magnification). C) H-PGDS staining on a severe asthmatic biopsy (x400 magnification). D) H-PGDS expression (brown staining) on cells within the sub-mucosa on a severe asthmatic biopsy and mast cell tryptase expression (brown staining) on cells on same biopsy (x400 magnification). E) H-PGDS expression (brown staining) on cells within the sub-mucosa on a severe asthmatic biopsy and CD3 expression (brown staining) on cells on same biopsy (x400 magnification). F) Dot-plot to show mean number of H-PGDS+ cells in the sub-mucosa and epithelium. p-value based on two way ANOVA, overall p-value <0.001, p-values on figure based on Tukey's post-hoc test.

### 3.13. *D prostanoid receptor (DP1) staining within biopsies*

The D-prostanoid receptor (DP1) is the other major PGD<sub>2</sub> receptor (407) that is thought to be expressed on inflammatory cells (408). The expression of DP1 was investigated to compare with that obtained for CRTh2. Within the healthy control and asthmatic bronchial biopsies, DP1 was expressed on platelets (identified by location within blood vessels and lack of nuclei) as would be expected from the literature (409), therefore helping to validate the staining pattern. DP1 was also

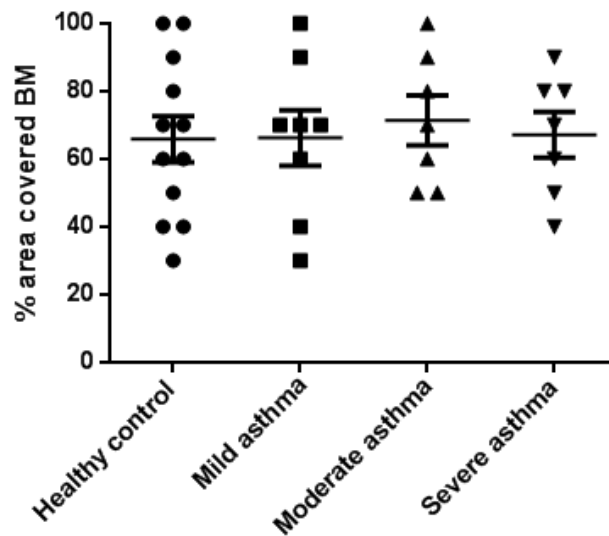
expressed on the epithelial cells of the sub-mucosal glands (**Figure 3.9A**) and occasional inflammatory cells and epithelial cells (**Figure 3.9B**). No differences were observed between healthy control and asthmatic biopsies. These data suggests that the predominant  $PGD_2$  receptor expressed within bronchial biopsies on inflammatory cells and epithelial cells is CRTh2.



**Figure 3.9. DP1 staining for healthy control and asthmatic biopsies.** A) DP1 staining (brown staining) on epithelial cells of sub-mucosal glands for a healthy control biopsy (x400 magnification). Representative images of n=10 biopsies B) DP1 staining on platelets for a moderate asthmatic biopsy (x200 magnification). Representative images of n=32 biopsies.

#### ***3.14. Investigation of reduced number of CRTh2+ epithelial cells in moderate-severe asthma***

Earlier in the current chapter results from the analysis of the number of CRTh2+ epithelial cells demonstrated that there was a significant reduction in the number of CRTh2+ epithelial cells in moderate-severe asthmatic bronchial biopsies compared to healthy control (see section 3.10). The aim of the following work was to investigate the reduced numbers of CRTh2+ epithelial cells within the moderate and severe asthmatic biopsies when compared to the healthy control biopsies. To establish if there was an overall loss of epithelial cells in the moderate and severe asthmatic biopsies the area of the basement membrane which was covered with epithelial cells was calculated (see methods). There was no significant difference found in the % area of the basement membrane that was covered with epithelial cells between the healthy control biopsies and asthmatic biopsies (**Figure 3.10**). This finding together with the finding of no significant differences in the number of immature (p63+) epithelial cells or mature (cytokeratin 7+) epithelial cells (**Figure 3.6G, H**), suggests that the reduction of CRTh2+ epithelial cells in moderate and severe asthma is not due to an overall loss of epithelium in the asthmatic biopsies.

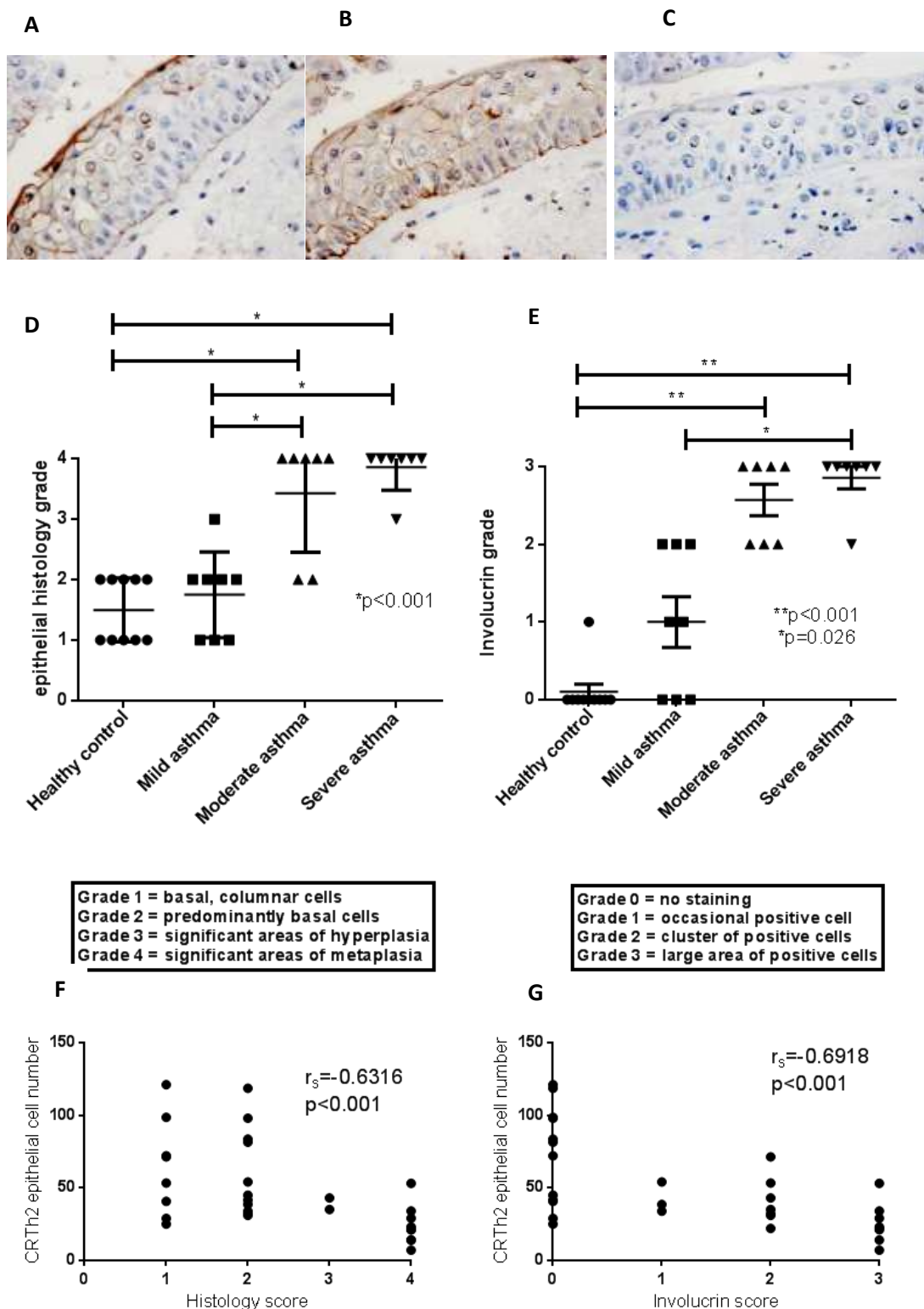


**Figure 3.10.** Dot-plot of % area of the basement membrane covered with epithelial cells for healthy control and asthmatic biopsies.

### ***3.15. CRTh2 expression and relationship to areas of squamous metaplasia***

It was noted that CRTh2 negative epithelial cells had an altered morphology when compared to CRTh2+ epithelial cells, with some cells of a flattened morphology, and that this was more evident for the moderate-severe asthmatic biopsies. To help identify if a change in phenotype had occurred in the CRTh2 negative epithelial cells in the moderate-severe asthmatic biopsies a squamous metaplastic phenotype marker, involucrin (312) was co-localised with CRTh2 and pan-cytokeratin (to confirm epithelial origin). Areas of squamous metaplasia are characterised by cells with a flattened, spreading morphology (69). Involucrin has been validated to identify cells of a squamous metaplastic phenotype (312). Analysis of CRTh2 expression alongside involucrin and pan-cytokeratin staining demonstrated a lack of CRTh2 staining on epithelial cells in areas expressing pan-cytokeratin and involucrin (**Figure 3.11A-C**). Many of the CRTh2 negative epithelial cells had a flattened, squamous morphology (**Figure 3.11C**). These

findings suggested that the reduction in CRTh2<sup>+</sup> epithelial cells was due to a change in phenotype of the epithelial cells in the moderate and severe asthmatic groups. To investigate this finding further, the epithelial histology for all biopsies were graded according to the criteria shown in **figure 3.11D**. These grading criteria provided a mechanism to assess the predominant histological description for the epithelial cells within the biopsy, ranging from grade 1, normal differentiation status with basal cells and columnar cells, to grade 4, significant areas of metaplastic epithelium, or epithelium with significantly altered morphology. Grading criteria was assessed on two separate occasions and intra-class correlation for all data was strong (Cronbach Alpha = 0.992,  $p < 0.001$ ). A significant increase in epithelial histology grade was observed for the moderate and severe asthmatic biopsy samples compared to the healthy control samples (median [IQR] grade 4 [2-4] versus grade 1.5 [1-2],  $p < 0.001$ ; grade 4 [3-4] versus grade 1.5 [1-2],  $p < 0.001$ ) and moderate and severe asthmatic samples compared to mild asthmatics (grade 4 [2-4] versus grade 2 [1-3],  $p < 0.001$ ; grade 4 [3-4] versus grade 2 [1-3],  $p < 0.001$ . **Figure 3.11D**). Quantification of a change in phenotype of some epithelial cells was achieved using involucrin, graded using the criteria described in **figure 3.11E**, ranging from grade 0, no staining to grade 3, a large area of involucrin+ cells. A significantly higher incidence of involucrin staining was observed for the moderate and severe asthmatic samples compared to the healthy control samples (grade 3 [2-3] versus grade 0 [0-1],  $p < 0.001$ ; grade 3 [2-3] versus grade 0 [0-1],  $p < 0.001$ . **Figure 3.11E**), and severe asthma compared to mild asthma (grade 3 [2-3] versus grade 1 [0-2],  $p = 0.026$ ). The number of CRTh2<sup>+</sup> epithelial cells were negatively correlated with both the histology grade and involucrin grade ( $r_s = -0.63$ ,  $r_s = -0.69$ ,  $p < 0.001$ . **Figure 3.11F, G**).



**Figure 3.11. Investigation of the phenotype of CRTh2 negative epithelial cells.** Serial sections of a severe asthma biopsy (x400) A) involucrin staining (brown), B) pan cytokeratin and C) CRTh2. D) Epithelial histology grades for healthy control, mild, moderate and severe asthmatic biopsies. p value based on Kruskal Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test. E) Grading of involucrin staining for healthy control, mild, moderate and severe asthmatic biopsies. p value based on Kruskal Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test. F) Spearman correlation graph for CRTh2+ epithelial cell number versus histology score. G) Spearman correlation graph for CRTh2+ epithelial cell number versus involucrin score.

### ***3.16. Correlations of CRTh2 positive inflammatory cell numbers and epithelial cell numbers with clinical characteristics***

To investigate if the expression of CRTh2 correlated with the clinical characteristics of the healthy control and asthmatic patients, correlation analyses of the number of CRTh2+ cells with clinical data was carried out. The aim of these analyses was to highlight any clinical measures that could be associated with the number of CRTh2+ inflammatory cells or epithelial cells.

A positive correlation was observed for the number of CRTh2+ inflammatory cells and the total sputum count ( $r_s=0.544$ ,  $p=0.003$ ) and ICS dose ( $r_s=0.758$ ,  $p<0.001$ ), see **table 3.3**. These data suggested that the number of CRTh2+ inflammatory cells was linked to the total sputum count of the patients and if the patients were receiving ICS treatment. A weaker correlation was observed between the number of CRTh2+ inflammatory cells and FEV<sub>1</sub> BDR ( $r_s=0.364$ ,  $p=0.048$ ).

Negative correlations were observed between the number of CRTh2+ epithelial cells and total sputum count ( $r_s=-0.422$ ,  $p=0.028$ ), atopy ( $r_s=-0.431$ ,  $p=0.014$ ), ICS dose ( $r_s=-0.719$ ,  $p<0.001$ ) and FEV<sub>1</sub> BDR ( $r_s=-0.433$ ,  $p=0.017$ ). These data suggested that the number of CRTh2+ epithelial cells was reduced in patients with a higher sputum count, in patients that had atopy and in patients that received ICS treatment. A positive correlation was observed between the number of CRTh2+ epithelial cells and PC<sub>20</sub> ( $r_s=0.546$ ,  $p=0.009$ ), see **table 3.3**.



**Table 3.3. Correlation data of patient clinical characteristics with CRTh2+ inflammatory cell and CRTh2+ epithelial cell numbers for healthy control and asthmatic biopsies.**

		<i>Spearman r value</i>	<i>p-value</i>
CRTh2+ inflammatory cell numbers	<b>Total sputum count</b>	<b>0.544</b>	<b>0.003</b>
	Sputum lymphocytes	0.155	0.480
	Sputum eosinophils	-0.0144	0.952
	Eosinophils/blood	-0.188	0.5104
	Atopy	0.307	0.088
	FEV <sub>1</sub> /FVC%	-0.160	0.382
	FEV <sub>1</sub>	-0.132	0.471
	<b>ICS dose</b>	<b>0.758</b>	<b>&lt;0.001</b>
	<b>PC<sub>20</sub></b>	<b>-0.586</b>	<b>0.0042</b>
	<b>FEV<sub>1</sub> BDR</b>	<b>0.364</b>	<b>0.048</b>
CRTh2+ epithelial cell numbers	<b>Total sputum count</b>	<b>-0.422</b>	<b>0.028</b>
	Sputum lymphocytes	-0.124	0.573
	Sputum eosinophils	-0.155	0.514
	Eosinophils/blood	0.263	0.364
	<b>Atopy</b>	<b>-0.431</b>	<b>0.014</b>
	FEV <sub>1</sub> /FVC%	0.319	0.075
	FEV <sub>1</sub>	0.239	0.187
	<b>ICS dose</b>	<b>-0.719</b>	<b>&lt;0.001</b>
	<b>PC<sub>20</sub></b>	<b>0.546</b>	<b>0.009</b>
	<b>FEV<sub>1</sub> BDR</b>	<b>-0.433</b>	<b>0.0017</b>

FEV<sub>1</sub> forced expired volume in 1 minute, FVC forced expired volume, ICS inhaled corticoid steroid, PC<sub>20</sub> concentration of methocholine which causes a 20% drop in FEV<sub>1</sub>, BDP beclomethasone dipropionate.

### **3.17. Chapter 3 Summary**

The aim of this section of work was to characterise the protein expression of human CRTh2 on clinically well-characterised biopsies from healthy control and asthmatic biopsies as this data is not currently described within the literature. This data may contribute to future CRTh2 antagonist studies as an understanding of where CRTh2 is expressed within the asthmatic airways could help to focus the clinical end-points assessed within these studies. The current study is the first to demonstrate differential CRTh2 expression on inflammatory cells and epithelial cells within bronchial biopsies of healthy control and asthmatic patients.

#### **3.17.1. Validation of the immunohistochemistry staining of bronchial biopsies**

The biopsies used for this study were acetone fixed and glycol methacrylate embedded (GMA), which is a type of plastic embedding medium. Areas of tissue analysed for cell counts for this study is comparable to previous GMA-based studies (191), with a mean sub-mucosal area of  $1.3\text{mm}^2$ . Areas smaller than  $0.25\text{mm}^2$  were eliminated from analyses as these have been found to associated with significantly larger variance (191). A limitation of the current study is that only one biopsy was assessed per patient. Previous studies however have described extremely low variability in cell counts between different biopsies taken from the main carcinoma (36,190), and it was concluded that the biopsy of one site is adequate for representative cell counts.

Biopsies in a plastic embedding medium generally contain lower cellular numbers when compared to frozen tissue because of the smaller section size ( $2\mu\text{m}$  for GMA compared to  $4\mu\text{m}$  for frozen) and cross-linking of proteins (410,411).

However, in the current study a more sensitive staining kit was used to identify positive cells for the first time on GMA biopsies (Dako EnVision™), and also antigen retrieval techniques were employed, so it is likely that a greater number of positive cells could be elucidated. Indeed, the counts for CD3 positive cells achieved in the current study for moderate and severe asthma (mean 71 [SEM 12], 69[7]) were comparable to those found on frozen tissue (70.6 [11.2], (410)) and asthmatic biopsies in formalin fixed resin embedded tissue (72 [19-192], (74)).

Further validation that the biopsy findings from the current study were comparable to previous studies is demonstrated with the findings that in moderate and severe asthmatic biopsies significantly increased numbers of T-cells were present when compared to healthy control biopsies. This are in agreement with previous findings on bronchial biopsies (36,190,191,231). Also in agreement with previous literature no significant differences in the number of mast cells were observed between healthy control and asthmatic biopsies (205). However, in contrast to previous literature the number of eosinophils was not significantly elevated in asthmatic biopsies compared to healthy control (74,231). However, different markers have been used to identify the eosinophils, with eosinophil cationic protein (EG2) being frequently used, so this may account for discrepancies. For the current study MBP was used as an eosinophil marker as it has been shown that MBP deposits are associated with asthmatic subjects (204), and it was of interest to investigate this for the current biopsy set, however, no convincing evidence was shown for the biopsies used in this study of MBP deposits within the epithelium.

### **3.17.2. CRTh2 is expressed on T-cells, eosinophils and mast cells in bronchial biopsies.**

The expression of CRTh2 on circulating eosinophils, Th2 cells and basophils has been well documented (103,207). The current study extends upon this data in that CRTh2 was found on T-cells and eosinophils within the tissue compartment of the airways. In addition the number of CRTh2 positive T-cells and eosinophils were found to be increased in moderate-severe asthmatic patients, again extending upon observations of how and where CRTh2 is expressed. A novel observation of the current study was that CRTh2 was expressed on some mast cells within bronchial biopsies, although the numbers of CRTh2 positive mast cells was not significantly different in asthma compared to healthy controls. Limited data to date has described CRTh2 expression on human mast cells (254), and the expression on mast cells with asthmatic airways has not been described previously. The expression of CRTh2 on human mast cells therefore requires further investigation. CRTh2 was found to be absent on neutrophils within bronchial biopsies. A lack of expression on neutrophils is in agreement with previous studies investigating circulating neutrophil expression (104). CRTh2 activation on circulating inflammatory cells has been found to have a range of pro-inflammatory effects. For eosinophils CRTh2 activation has been found to cause shape change, actin polymerisation, CD11b up-regulation and elicit chemotaxis (168,169,173,208-210), all of which can lead to the accumulation of eosinophils and activation of late-response inflammatory responses that have been described in asthma (212). For T-cells, activation of CRTh2 has been shown to cause chemotaxis and the release of the pro-inflammatory cytokines, IL-4, IL-5 and IL-13. These cytokines have been shown to contribute to the pathogenesis of

asthma (57,353). CRTh2 activation on T-cells has also been shown to cause a delay in apoptosis (105,106), which may contribute to the significantly increased number of CRTh2 positive T-cells in the asthmatic bronchial biopsies. The current study is the first to demonstrate that CRTh2 positive inflammatory cells are present within the sub-mucosa of bronchial biopsies and are increased in asthma. Given the above described effects of CRTh2 activation on these inflammatory cells it is likely that CRTh2 positive inflammatory cells play a role in the pathogenesis of asthma.

### **3.17.3. CRTh2 is expressed in bronchial epithelial cells in bronchial biopsies.**

In the current study CRTh2 was found to be expressed on bronchial epithelial cells of bronchial biopsies and detailed phenotyping of CRTh2 with epithelial markers provided additional information of its expression profile compared to previous studies (128,254,412). CRTh2 was found to be present on a range of bronchial epithelial cell phenotypes, including immature and mature cells, on cells in areas of epithelial loss, but not on goblet cells. In contrast to the number of CRTh2 positive inflammatory cells, the number of CRTh2 positive epithelial cells was significantly reduced in the moderate-severe asthmatic biopsies. Further investigation into this observation revealed that the CRTh2 negative epithelial cells had a squamous metaplastic phenotype. Fewer CRTh2 positive epithelial cells were observed in the moderate-severe asthmatic patients where there were significantly more epithelial cells of a metaplastic histology and more involucrin positive epithelial cells. Squamous metaplasia has been shown to be associated with severity of disease in COPD patients (312). The current study is the first to establish that there is an increased incidence of squamous metaplasia in patients with moderate-severe asthma, and that this phenotype expresses lower levels of CRTh2. Linkage

of CRTh2 expression with epithelial cells undergoing phenotypic change is a novel observation, which may suggest CRTh2 has an important role in the differentiation status of the epithelium. Further investigation on the role of CRTh2 on bronchial epithelial cells is therefore required.

#### **3.17.4. H-PGDS positive cells are located in similar regions to CRTh2 positive cells.**

H-PGDS positive cells were found to be present within the sub-mucosa and within the epithelium of bronchial biopsies. The presence of H-PGDS positive inflammatory cells within the biopsies demonstrates that there is the potential for the production of the CRTh2 ligand, PGD<sub>2</sub> in areas local to where CRTh2 expressing cells are present. More significantly, an increase in H-PGDS positive cells in asthma could suggest the potential for elevated levels of PGD<sub>2</sub> being produced locally to CRTh2 expressing cells within the airways. Previously elevated PGD<sub>2</sub> levels have been described in the bronchoalveolar lavage fluid (BAL) of asthmatics (110-112,116), but the current study is the first to describe H-PGDS positive cells within healthy control and asthmatic bronchial biopsies. The punctate, granular H-PGDS staining observed within the bronchial epithelial cells, with an increased presence within asthmatic epithelial cells is intriguing. This suggests that epithelial cells may be able to produce PGD<sub>2</sub>, as well as expressing CRTh2. This co-existence of ligand and receptor has previously been reported for circulating inflammatory cells as a proportion of CRTh2 positive Th2 cells were shown to co-express H-PGDS and abundantly produce PGD<sub>2</sub> in response to antigenic stimuli (161,366). Eosinophils have also been shown to express H-PGDS (147). The investigation of the effects of CRTh2 activation on bronchial epithelial cells and whether these

effects are pro- or anti-inflammatory, would therefore give an insight into whether CRTh2 antagonists have the potential to affect the epithelium in asthma.

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## **Chapter 4**

### **Results**

# **Exploring the expression of human CRTh2 and effects of its activation on cultured human bronchial epithelial cells**

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## 4.1. Chapter Overview

**The aims of this chapter were to explore the expression and function of CRTh2 on bronchial epithelial cells in culture. This information was to confirm and extend upon the novel observations made in chapter 3 that CRTh2 is expressed on bronchial epithelial cells within bronchial biopsies.**

Using a number of different techniques the expression of CRTh2 was explored on human bronchial epithelial cells grown in culture. Human bronchial epithelial cells were cultured from bronchial brushings from healthy control and asthmatic patients. To try to link the findings of the biopsy work with this work, expression of CRTh2 was explored on isolated bronchial epithelial cells from both healthy control subjects and asthmatic subjects. Cells were utilised in early passage to try to minimise effects of culture on the expression and phenotype of the epithelial cells. This did however limit the number of epithelial cells that were available for experimentation. After confirming expression of CRTh2 on human bronchial epithelial cells *in vitro*, the effects of CRTh2 activation within functional assays was explored. The activation of human CRTh2 was achieved using 13, 14-dihydro-15-keto prostaglandin D<sub>2</sub> (DK-PGD<sub>2</sub>), which is a CRTh2 selective agonist (104). To further validate responses were via CRTh2 activation, a CRTh2 selective antagonist was used, AZD6430 (AstraZeneca compound). This compound has been thoroughly characterised within selectivity assays at AstraZeneca and found to have excellent selectivity for CRTh2.

To extend upon the novel observation that CRTh2 was expressed on a sub-population of mast cells within the bronchial biopsies, CRTh2 expression was

explored on a human lung mast cell line (HMC-1) and human lung mast cells isolated from areas of normal morphology of cancer resection tissue.

#### ***4.2. Human CRTh2 protein expression on cultured bronchial epithelial cells***

To confirm and extend upon the novel findings of CRTh2 expression on healthy control and asthmatic epithelial cells in bronchial biopsies the expression of CRTh2 was explored on cultured bronchial epithelial cells derived from healthy control and asthmatic patients (moderate-severe asthmatics, GINA treatment steps 3-5). Flow cytometry allows for both the number of positive cells expressing CRTh2 as well as the level of CRTh2 expression associated with the cells to be assessed. Flow cytometry data currently in the literature for CRTh2 on circulating T-cells and eosinophils has employed the investigation of both extracellular and intracellular CRTh2 expression (103,143,161,194,198,412), therefore for the current study CRTh2 expression within both compartments was investigated. A well characterised antibody (BD Biosciences monoclonal BM16 antibody) that has been used in the literature for flow cytometry was used for this work (103,143,161,194,198,413). CRTh2 protein expression was found on both healthy control and asthmatic epithelial cells using flow cytometry. The data for CRTh2 extracellular expression demonstrated a bi-phasic profile (**Figure 4.1A, B**). This finding suggested that there could be cells of different phenotypes within the epithelial population that express different levels of CRTh2 expression. To address this, dual fluorescent staining was carried out for both an immature epithelial cell marker (cytokeratin 14 (414)) and a mature epithelial cell marker (cytokeratin 7 (402)). CRTh2 was found to be expressed on both cytokeratin 14+ and cytokeratin 7+ cells, with no differential on these phenotypes between healthy control and

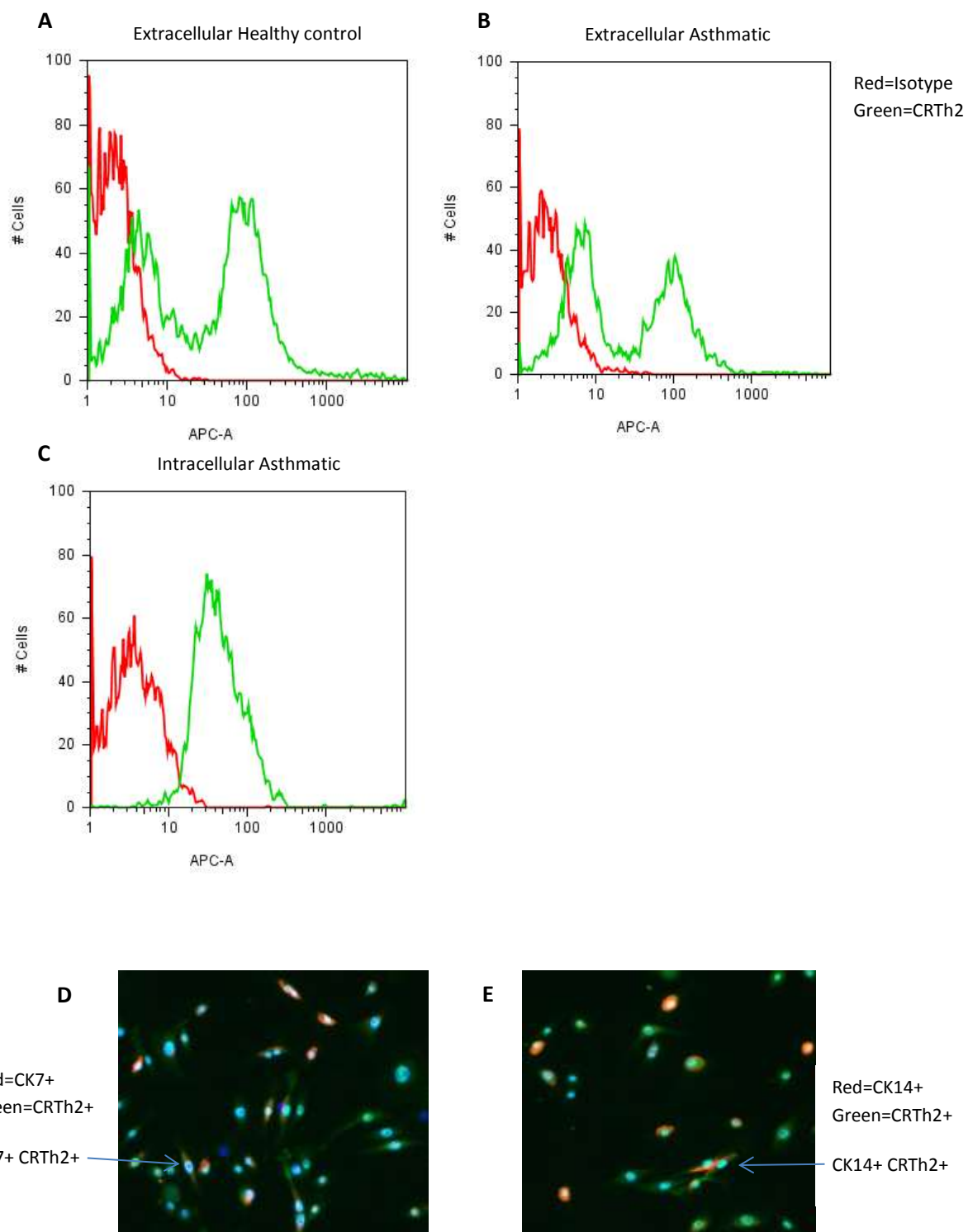
asthma (**Figure 4.1D, E**). This suggested that the bi-phasic profile was not due to CRTh2 expression being different on immature and mature epithelial cells. Alternatively a bi-phasic profile could suggest internal pools of receptor. When the intracellular expression of CRTh2 was investigated a single peak of expression was observed (**Figure 4.1C**) for both healthy control and asthmatic epithelial cells which could suggest that the bi-phasic peak is due to some cells having internal pools of receptor.

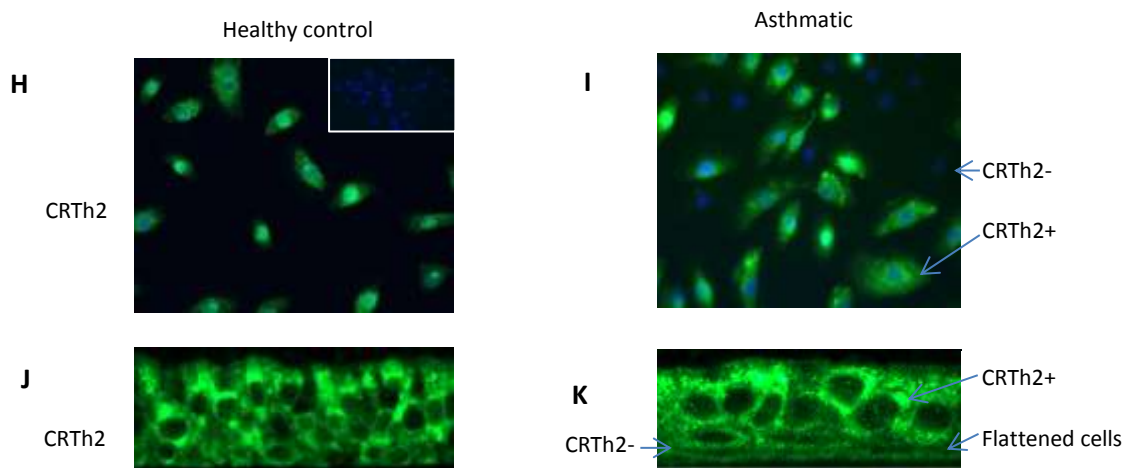
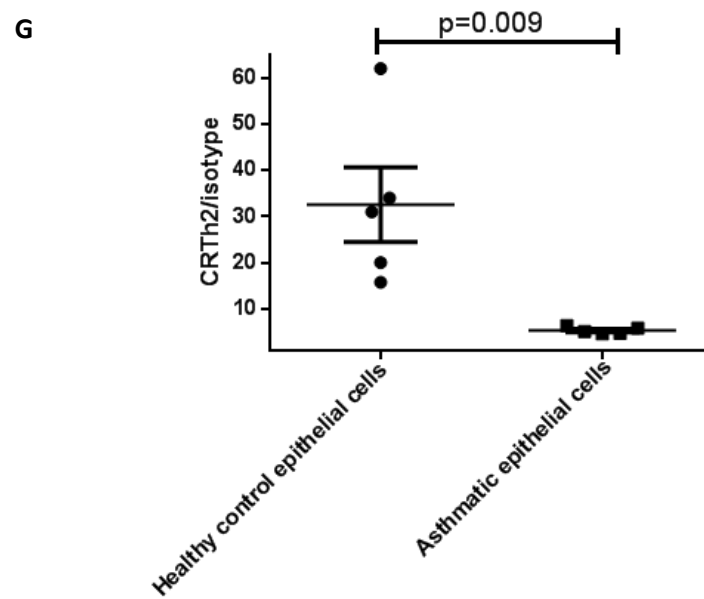
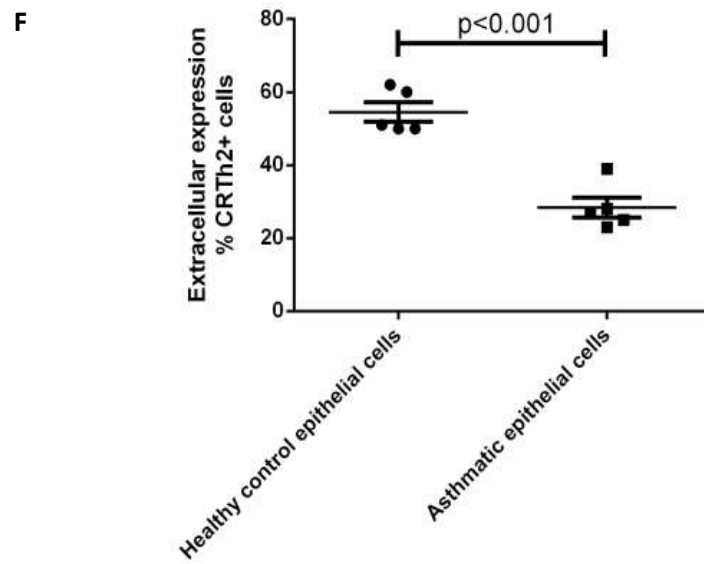
Comparisons of the expression of CRTh2 using flow cytometry for cultured healthy control bronchial epithelial cells compared to asthmatic epithelial cells demonstrated higher expression associated with the healthy control cells. A significantly higher percentage of CRTh2+ cells were found for the healthy control cells when compared to the asthmatic cells (mean [SEM] 54[7]% versus 28[6]%,  $p < 0.001$ . **Figure 4.1F**). CRTh2 expression was also higher for the healthy control cells as assessed by geometric mean fluorescence (29[15-55] versus 5[4-6],  $p = 0.009$ . **Figure 4.1G**). The higher percentage of CRTh2 positive bronchial epithelial cell for the healthy controls is in agreement with the bronchial biopsy data (chapter 3). The amount of CRTh2 expression as assessed by geometric mean fluorescence for the epithelial cells extends upon the biopsy data showing differences in the levels of expression, an observation that could not be elucidated with the immunohistochemistry staining in the biopsies. To investigate protein expression using an alternative technique the expression of CRTh2 was also assessed using fluorescent cell staining of bronchial epithelial cells grown on chamber slides. CRTh2+ expression was associated with both the healthy control and the asthmatic epithelial cells (**Figure 4.1H, I**). For the healthy control cells the majority of cells

within the field of view were found to be CRTh2+, however, for the asthmatic epithelial population there were cells that did not express CRTh2 (DAPI+CRTh2-), see **figure 4.1I**. No CRTh2 staining was observed with the isotype control (**Figure 4.1H, insert**), showing that no background fluorescent staining was present. Similar fluorescent cell staining results were found using a commercial antibody (data not shown), validating findings for the CRTh2 AZ antibody. These data add evidence to the observations that CRTh2 is expressed on a greater population of bronchial epithelial cells from healthy control subjects compared to cells from moderate-severe asthmatic subjects. CRTh2 expression was also present on epithelial cells grown in an ALI format from healthy donors (**Figure 4.1J**) and asthmatic donors (**Figure 4.1K**), suggesting CRTh2 expression remains when epithelial cells are differentiated into ALI. The staining pattern for the ALI cultures derived from the healthy control subjects was different from that found with the asthmatic ALI cultures. For the healthy control ALI cultures CRTh2 staining was uniform across the epithelial cells, whereas for the asthmatic ALI cultures CRTh2 staining was brighter for some cells and weaker or absent on other cells (**Figure 4.1J, K**). The morphology of the asthmatic ALI cultures was also different compared to the healthy control ALI cultures. The asthmatic cultures contained frequent epithelial cells with a flattened morphology, particularly the basal cells that are located in the lower layer of the ALI culture, next to the insert membrane (**Figure 4.1K**). These data suggest that the differences observed for CRTh2 expression in the healthy control compared to asthmatic biopsies is maintained in culture.

Taken together the CRTh2 expression data for healthy control compared to moderate-severe asthmatic bronchial epithelial cells suggests a differential in the

numbers and levels of CRTh2 expression. Within this study moderate-severe asthma was defined using GINA guidelines (1), where classification of moderate-severe asthma incorporates that patients are receiving corticosteroids. To determine if the reduced CRTh2 expression on the bronchial epithelial cells of the moderate-severe asthmatic subjects compared to the healthy control subjects was due to corticosteroid treatment affecting the levels of CRTh2 expression, changes in CRTh2 expression with the corticosteroid, fluticasone propionate (FP) was assessed using flow cytometry. No significant changes were observed in CRTh2 expression at the extracellular or the intracellular level with FP treatment. An increase in the percentage of CRTh2+ cells was observed with FP treatment (Extracellular 7.8-fold[4-16]% increase, intracellular 2.7-fold [0-5]% increase). An increase in CRTh2 expression was also observed in response to FP treatment as assessed by geometric mean fluorescence (Extracellular 1.2-fold[0.7-2] increase, intracellular 1-fold[0.8-1.4] increase). This data suggest that levels of expression of CRTh2 are not reduced in response to corticosteroid treatment. It also suggests that the differences observed in the number of CRTh2+ bronchial epithelial cells found within the moderate-severe asthmatic biopsies and the cultured asthmatic epithelial cells is not due to corticosteroid treatment.





**Figure 4.1. CRTh2 expression on isolated human bronchial epithelial cells.** A) Flow cytometry profile to show extracellular CRTh2 expression on healthy control epithelial cells (Red=isotype, Green=CRTh2), and B) asthmatic epithelial cells (Red=isotype, Green=CRTh2). C) Flow cytometry profile to show intracellular CRTh2 expression on asthmatic epithelial cells (Red=isotype, Green=CRTh2). D) Dual fluorescent staining for cytokeratin 7 (red) and CRTh2 (green) on healthy control cells, DAPI nuclear dye (blue). E) Dual fluorescent staining for cytokeratin 14 (red) and CRTh2 (green) on healthy control cells, DAPI nuclear dye (blue). F) Dot-plot to show %CRTh2+ for healthy control and asthmatic epithelial cells. p value based on unpaired two-tailed t-test. G) Dot-plot to show levels of CRTh2 expression (geometric mean fluorescent units) for healthy control and asthmatic bronchial epithelial cells. p value based on unpaired two-tailed t-test. H) CRTh2 expression characterisation on cultured, submerged epithelial cells. Fluorescent cell staining, Green staining for CRTh2, blue DAPI nuclear stain (healthy control cells). Inset rabbit isotype control with lack of any green staining. I) Green staining for CRTh2, blue DAPI nuclear stain (Asthmatic cells). Note cells with absence of CRTh2+ (green) staining J) Green staining for CRTh2 on healthy control ALI culture. K) Green staining for CRTh2 on asthmatic ALI culture.

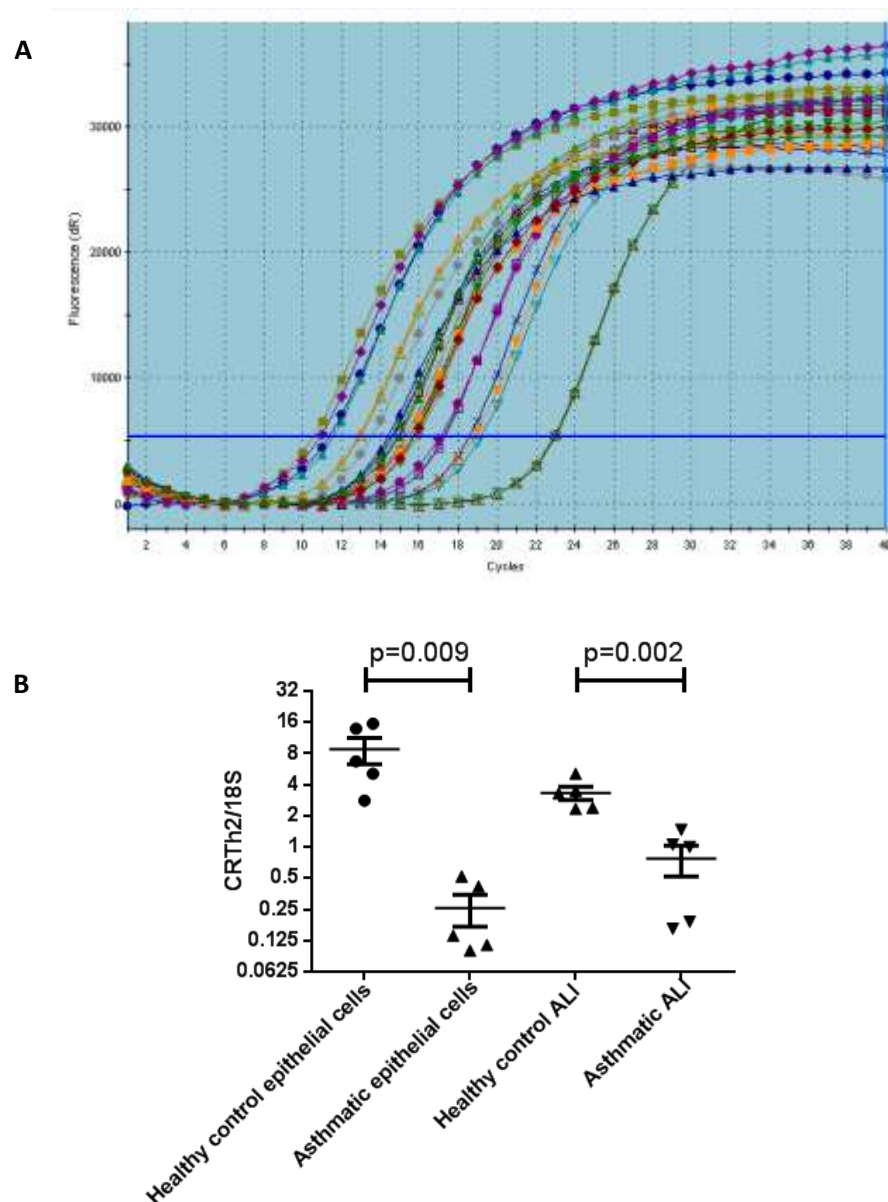
#### ***4.3. Human CRTh2 mRNA expression on cultured bronchial epithelial cells***

CRTh2 mRNA expression was assessed for bronchial epithelial cells using Taqman® primer/probe based quantitative RT-PCR. The standard curve method was used to quantify the levels of mRNA expression. All samples were normalised to 18S housekeeping gene, to account for variability in the amount of mRNA between samples. A typical Taqman amplification plot for 18S is shown in **figure 4.2A**. The threshold cycle ( $C_T$ ) value was established from where the amplification plot of the sample crossed the threshold line on the plot.

CRTh2 mRNA expression in epithelial cells was also detected for both healthy and asthmatic cells grown in a submerged or ALI culture format, with significantly more CRTh2 expression associated with healthy epithelial cells (mean [SEM] 9 [3] versus 0.3 [0.1];  $p=0.009$ ; 3 [1] versus 0.8 [0.3];  $p=0.002$ . **Figure 4.2B**). No DP1 mRNA expression was detected on epithelial cells grown in



submerged or ALI culture formats (data not shown), suggesting that no mRNA expression for the other main prostaglandin D<sub>2</sub> receptor was present for *in vitro* bronchial epithelial cells.



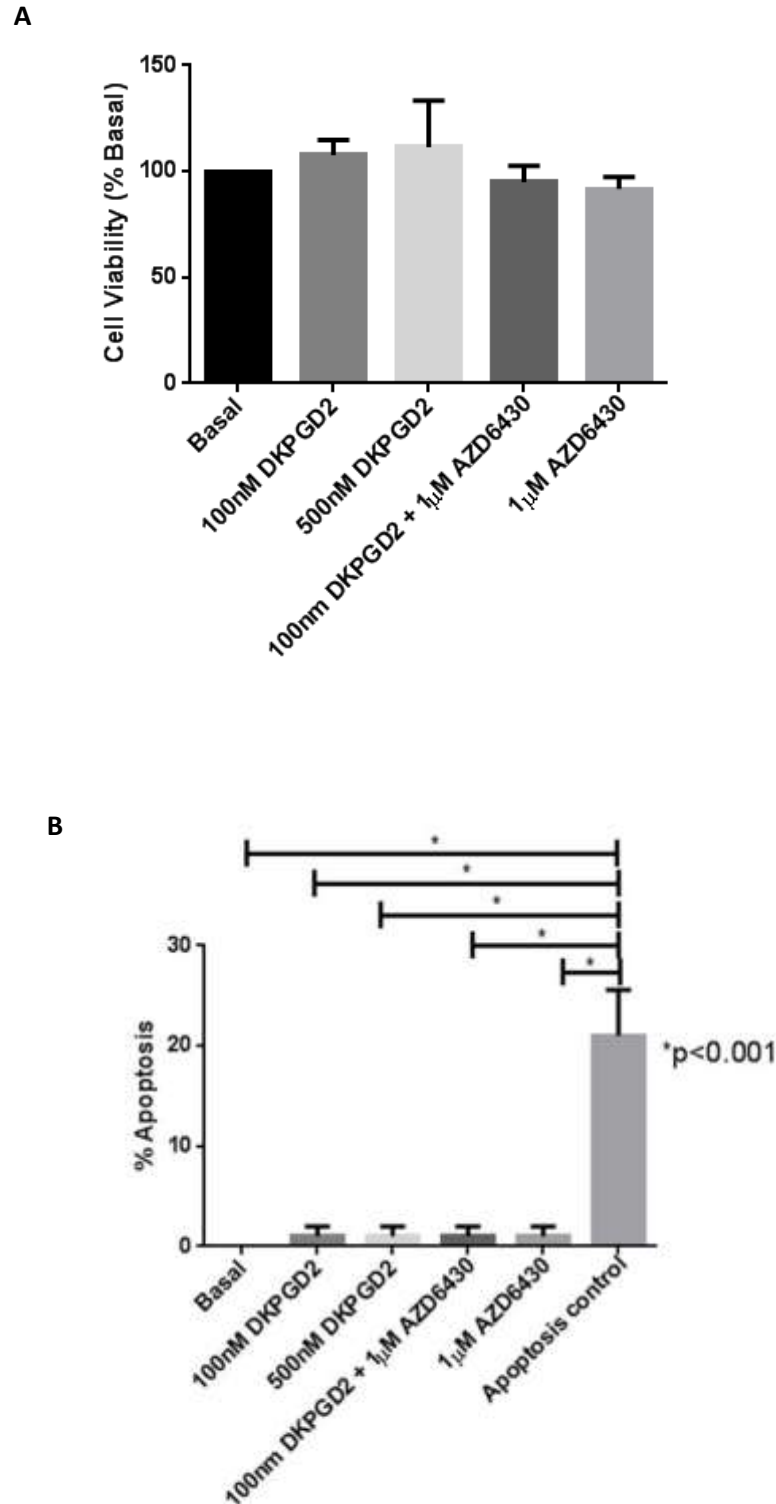
**Figure 4.2. CRTh2 mRNA expression on human bronchial epithelial cells.** A) Example of amplification plot for 18S housekeeping gene Taqman primers/probe. B) CRTh2 mRNA expression normalised to 18S housekeeping gene for epithelial cells. p values based on unpaired two-tailed t-tests.

#### ***4.4. Evaluating the effects of the CRTh2 selective agonist DK-PGD<sub>2</sub> and antagonist, AZD6430 on the viability of bronchial epithelial cells.***

In order to assess the effects of CRTh2 activation on bronchial epithelial cells drugs used for these studies were first tested for cellular toxicity and apoptotic effects on epithelial cells. DK-PGD<sub>2</sub> (104) was used as the selective agonist in these studies and AZD6430 as the selective antagonist.

To assess the toxicity of DK-PGD<sub>2</sub> and AZD6430 on bronchial epithelial cells an MTT assay was used. This assay utilises the MTT enzyme which is converted into formazan crystals by living cells, which determines mitochondrial activity. For most cell populations the total mitochondrial activity is related to the number of viable cells, so this assay can be used to establish the viability of cells in response to treatment. DK-PGD<sub>2</sub> treatment or AZD6430 treatment did not change cell viability at concentrations used for functional assays (**Figure 4.3A**). The effects of DK-PGD<sub>2</sub> and AZD6430 on apoptosis in the bronchial epithelial cells were also assessed using Annexin V-FITC binding in a flow cytometry assay. This assay utilises phosphatidyl-serine location to identify cells undergoing apoptosis (Annexin V-FITC binds to phosphatidyl-serine when it translocates to the external portion of the membrane when cells undergo apoptosis). DK-PGD<sub>2</sub> treatment or AZD6430 treatment did not affect levels of apoptosis in bronchial epithelial cells as no shift in the cell populations was observed when compared to untreated cells (**Figure 4.3B**). 1µM staurosporine was used as a positive control for apoptosis, and significant apoptosis was induced for this cell treatment, validating that the assay has worked (**Figure 4.3B**). This data suggested that DK-PGD<sub>2</sub> and AZD6430 would not affect

bronchial epithelial cell viability at relevant concentrations in the functional cellular assays described in the following sections.



**Figure 4.3. Effects of DK-PGD<sub>2</sub> and AZD6430 on cell toxicity and apoptosis levels in bronchial epithelial cells.** A) Cell viability as assessed by MTT assay for DK-PGD<sub>2</sub> and AZD6430 on bronchial epithelial cells. B) Cell apoptosis as assessed by Annexin V-FITC assay for DK-PGD<sub>2</sub> and AZD6430 on bronchial epithelial cells. p value based on one-way ANOVA. Overall p-value <0.001. Apoptosis control used 1µM staurosporine.

## ***4.5. Assessment of CRTh2 activation on functional responses in bronchial epithelial cells***

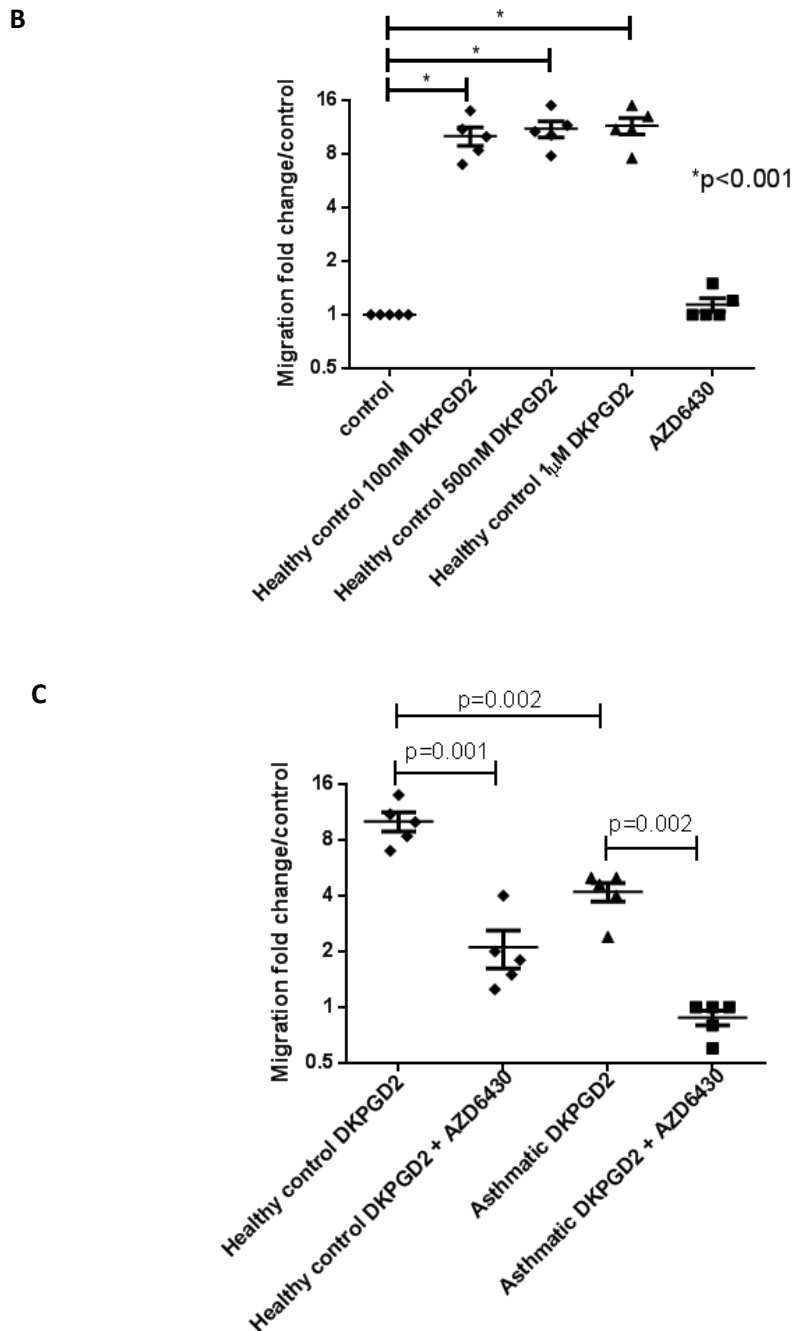
### ***4.5.1. Cell migration***

After establishing that CRTh2 was expressed at both the mRNA and protein level on bronchial epithelial cells functional assays were developed to assess the response of CRTh2 activation on these cells. The most well characterised functional response of CRTh2 activation on Th2 cells, eosinophils, basophils and innate-lymphoid type-2 cells (ILC2) is that of cell migration (104,169,170,208,228). The first functional effect of CRTh2 activation on bronchial epithelial cells that was investigated was therefore cell migration. This functional assay was also investigated because within the bronchial biopsies CRTh2 expression was observed on basal epithelial cells that had a flattened morphology in areas of epithelial loss (see result chapter 3.10). These cells may have been migrating to cover the denuded basement membrane and so it would be interesting to investigate if CRTh2 activation on epithelial cells *in vitro* causes cell migration. The cell migration assay was developed using a format that has not been used before for cultured bronchial epithelial cells (see methods) as many migration assays utilise non-adherent cells. The assay that was optimised for the bronchial epithelial cells is in a 96-well plate format, utilising a stopper which when applied to the centre of a well creates a migration zone. As this assay does not rely on migration induced via a scratch or wound, it is likely to minimise the proliferation of the cells but still instigate the activation of migratory features such as polarisation, protrusion, adhesion and translocation. Following application of the stopper to the centre of the well, cells are then seeded around the stopper and left to adhere to the well. The stopper is then

removed and agonist applied for 24 hours to allow migration. Cells are then fixed and stained with a fluorescence nuclear dye to allow for counting of the number of cells within the migration zone (**Figure 4.4Ai, ii, iii**). The fixation of cells and labelling with a nuclear dye step was developed separately from the manufacturer's instructions. The kit instructions suggested staining the cells with calcein, a fluorescent dye and then analysing the fluorescence levels with a plate reader. However, when this was tested, spurious results were obtained in the fluorescent readings, due to debris being present in some wells, therefore giving false-positive readings. During assay optimisation it was evaluated that the best way to assess changes in migration was to observe the cells using a microscope, and therefore the cell fixation and labelling with a fluorescence nuclear dye step was optimised. Within the assay control wells were used to identify non-specific migration. For vehicle-treatment (media + 1% DMSO) control wells, cell migration did occur compared to untreated-control wells. Therefore, for analysis of levels of migration, fold-change relative to vehicle-treated cells was assessed. Due to limited cell numbers a restricted dose-response to DK-PGD<sub>2</sub> was established for healthy control epithelial cells, of 100nM, 500nM and 1µM. AZD6430 alone was also tested in the assay. All concentrations of DK-PGD<sub>2</sub> promoted migration of epithelial cells but no significant differences were observed between the different concentrations (100nM DK-PGD<sub>2</sub> 10-fold[7-14], 500nM DK-PGD<sub>2</sub> 11-fold[7.8-15], 1µM DK-PGD<sub>2</sub> 11.5fold[7.6-1.5]. **Figure 4.4B**). This data suggests that maximal migratory activity is achieved at 100nM-1µM DK-PGD<sub>2</sub> concentrations. No significant migration was seen for AZD6430 alone compared to control (**Figure 4.4B**), helping to validate the selectivity of the AZD6430 compound. Due to no significant differences being

found between DK-PGD<sub>2</sub> concentrations, 100nM was used to further characterise the DK-PGD<sub>2</sub> migration response. DK-PGD<sub>2</sub> caused migration of healthy controls (10-fold [7-14]) and asthmatics (4-fold [2-5]), but was greater in the healthy controls than asthmatics (p=0.002) (**Figure 4.4C**). Migration was blocked by the CRTh2 antagonist AZD6430, in both healthy (10-fold [7-14] vs 2-fold [1-3], p=0.001) and asthmatic (4-fold [3-6] vs 0.8-fold [0.6-1], p=0.002) cells (**Figure 4.4C**). As AZD6430 blocked the migratory response this finding confirms that the migratory response to DK-PGD<sub>2</sub> was through the activation of CRTh2. The significant differences observed in the migratory response in the healthy control versus the asthmatic epithelial cells suggests there may be differences in the numbers of CRTh2 expressing cells between the healthy control and asthmatic cells. This observation is in keeping with previous results in this chapter which showed that healthy control cells contained a higher percentage of CRTh2 expressing cells when compared to asthmatics. A combination of both 10ng/ml TGFβ1 and 25ng/ml FGF was used as a positive control for the migration studies. TGFβ1 and FGF are growth factors which have been found to be important for migratory responses and cell migration in wound repair (394,415). Significant migration was observed in cells treated with both TGFβ1 and FGF (7-fold[3-12], p=0.018), a response that was not affected by AZD6430 (5-fold[1.7-15], p=0.102), helping again to confirm the selectivity of this compound for CRTh2.





**Figure 4.4. Effects of CRTh2 activation on bronchial epithelial cell migration.**

A) i) Hoescht+ epithelial cells within migration zone for vehicle control ii) Hoescht+ epithelial cells within migration zone for 100nM DK-PGD<sub>2</sub> treatment iii) Hoescht+ epithelial cells within migration zone for 100nM DK-PGD<sub>2</sub>+ AZD6430 treatment B) Dot plot of cell migration fold changes for 100nM, 500nM, 1 $\mu$ M DK-PGD<sub>2</sub> and AZD6430 alone. p value based on one-way ANOVA. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. C) Dot plot of cell migration fold change over vehicle control for both healthy control cells and asthmatic cells with 100nM DK-PGD<sub>2</sub> and 100nM DK-PGD<sub>2</sub> + 1 $\mu$ M AZD6430. p values based on paired two-tailed t-tests, healthy control vs asthmatic unpaired two-tailed t-test.



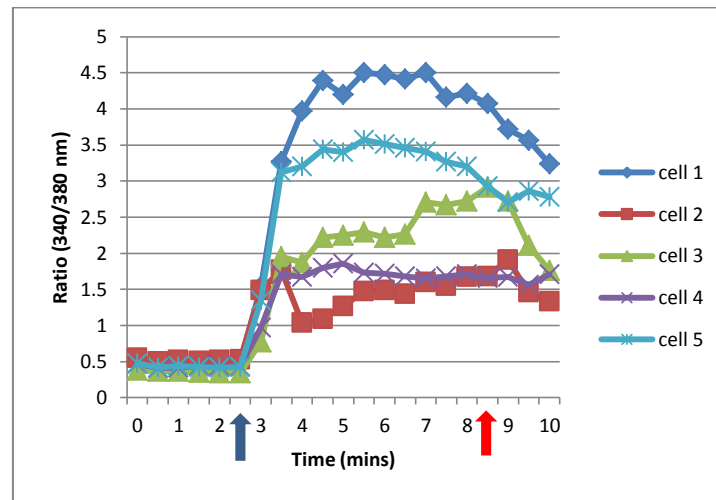
#### ***4.5.2. Calcium response***

CRTh2 has been found to signal via an increase in intracellular calcium for T-cells, basophils and osteoblasts (104,170,175). To establish if CRTh2 activation in bronchial epithelial cells elicits a calcium response, submerged healthy control epithelial cells were loaded with fura-2 and the 340nm/380nm ratio was assessed in response to DK-PGD<sub>2</sub> using a system similar to that previously used for primary osteoblasts (175). This technique was technically challenging as the epithelial cells did not adhere well to the glass coverslips used for the assay. Collagen coating of the cover slips did improve adherence but the cells were still only loosely adhered. The system used to assess the calcium response utilised a perfusion system which provided the advantage of bathing the cells with a uniform solution across the cells on the coverslip. Constant imaging (every 5 seconds) of the cells enabled any changes in the 340nm/380nm ratio to be observed immediately following agonist addition, an important technical aspect as the increase in intracellular calcium is rapid following agonist addition (104,175). Due to the technical difficulties of the assay and limited cell numbers, only a single dose of agonist was tested. Ideally a dose response to DK-PGD<sub>2</sub> would have been tested but within this assay format this was not possible. Cells were allowed to equilibrate for a few minutes before addition of agonist, agonist was added for a minimum of 5 minutes, then the agonist was removed whilst cells were still monitored. A profile of the 340/380nm ratio readings was plotted to monitor the calcium response during the recording period. Ionomycin was used as a positive control within the assay and was used to optimise conditions for the cells. Ionomycin creates pores within the cell membranes so calcium enters all cells, therefore changing the 340nm/380nm ratio. Background

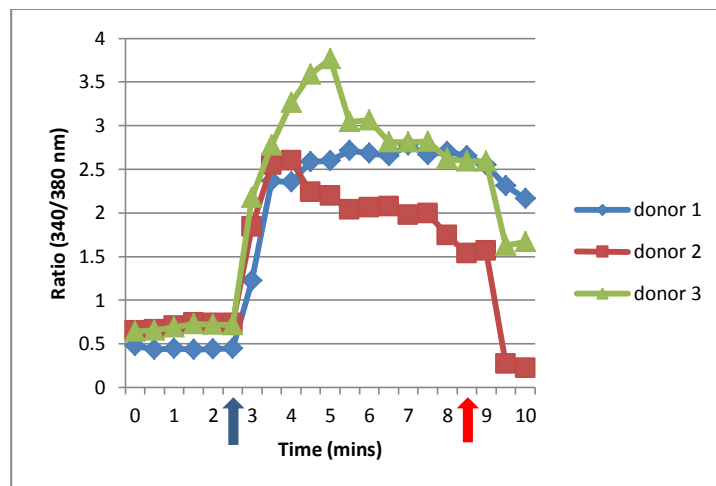
ratio levels for the bronchial epithelial cells were found to be in the range of 0.4-0.6. 1 $\mu$ M ionomycin elicited a calcium response in the bronchial epithelial cells (**Figure 4.5A**), validating that the methodology was working. There was a range of responses to ionomycin between the cells (**Figure 4.5A**), with the minimum peak 340/380nm ratio of 1.5 to a maximum of 4.5. The calcium response profile seen for ionomycin was consistent with what was expected as there was a peak following ionomycin addition which was maintained until it was removed (**Figure 4.5A**). This finding validated that the calcium response in the bronchial epithelial cells was as would be expected. An average response to ionomycin of 5-10 cells was assessed for 3 separate healthy control donors (**Figure 4.5B**). 1 $\mu$ M DK-PGD<sub>2</sub> also elicited a calcium response in the healthy control epithelial cells, not all cells responded, and the extent of the response varied between the cells (**Figure 4.5C**). These findings suggest that different levels of CRTh2 expression may be present on the cells, and that cells which did not respond do not express CRTh2. This would be in keeping with the expression analysis described previously in this chapter which demonstrated that not all bronchial epithelial cells grown in submerged conditions express CRTh2. The calcium response profile for DK-PGD<sub>2</sub> was different when compared to ionomycin, as there was an initial peak following agonist addition, which slowly decreased (**Figure 4.5C**). This finding is consistent with previous literature for CRTh2 on primary cells (103,175). The minimum 340nm/380nm peak was 0.65, with a maximum peak of 1.9. Similar to the ionomycin results an average response to DK-PGD<sub>2</sub> of 5-10 cells was assessed for 3 separate healthy control donors (**Figure 4.5D**). Addition of the CRTh2 antagonist 1 $\mu$ M AZD6430 with DK-PGD<sub>2</sub> reduced the peak of the 340nm/380nm ratio. **Figure 4.5E** shows the change

in peak 340nm/380nm ratio from the basal 340nm/380nm ratio, for 3 healthy control donors, with 1 $\mu$ M DK-PGD<sub>2</sub> alone treatment, 1 $\mu$ M DK-PGD<sub>2</sub> and 1 $\mu$ M AZD6430 and 1 $\mu$ M ionomycin. AZD6430 significantly reduced the change from basal to peak 340nm/380nm ratio (mean [SEM] DK-PGD<sub>2</sub> 2.7[0.04] versus DK-PGD<sub>2</sub> + AZD6430 1.3[0.1], p=0.005). These data suggest that CRTh2 activation on bronchial epithelial cells causes a calcium response.

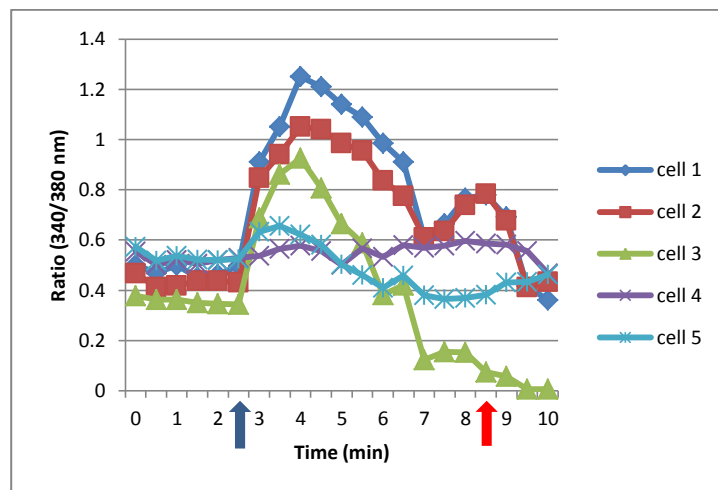
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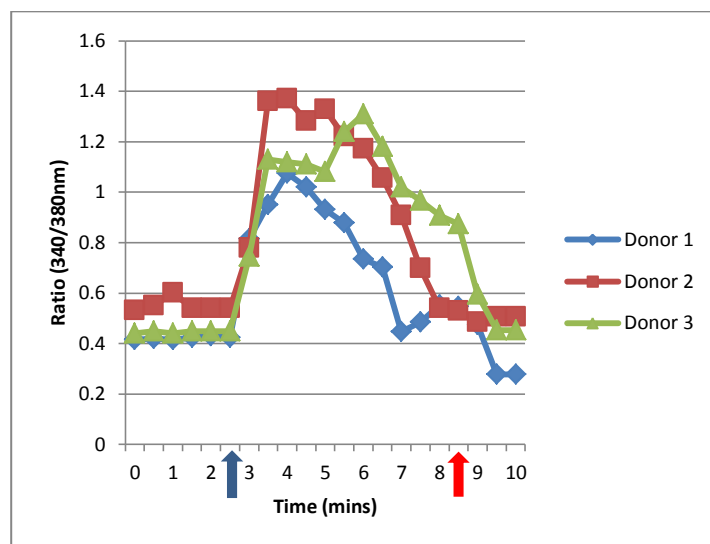
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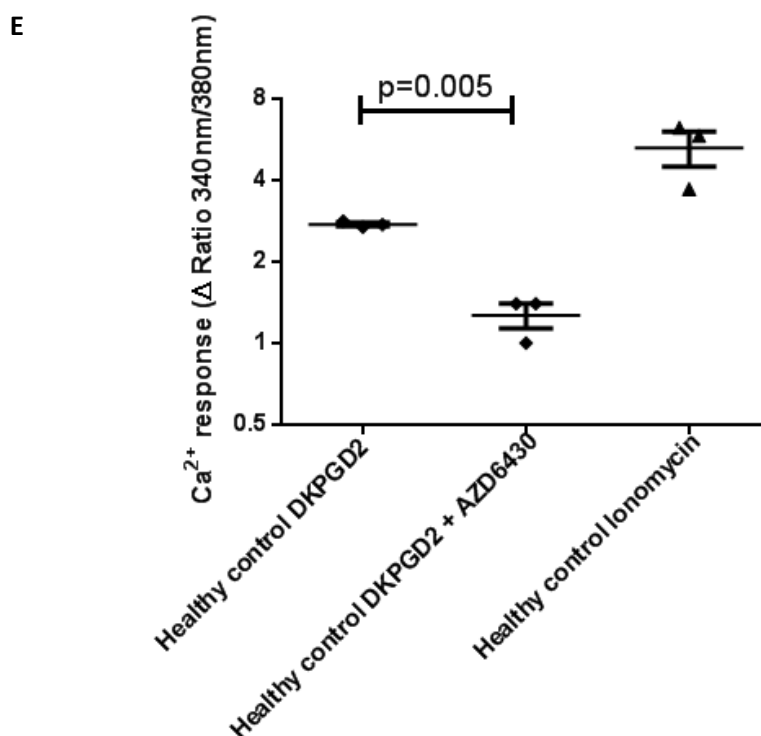


**C**



**D**





**Figure 4.5. Effects of CRTh2 activation on calcium responses in bronchial epithelial cells.** A) Calcium response curves (Fura-2 Ratio 340/380nm). Blue arrow indicates agonist addition; red arrow indicates removal of agonist A) 1 $\mu$ M ionomycin on human bronchial epithelial cells for 5 cells from a healthy control donor B) Average responses for three healthy control donors to 1 $\mu$ M ionomycin on submerged bronchial epithelial cells. C) 1 $\mu$ M DK-PGD<sub>2</sub> on human bronchial epithelial cells for 5 cells from a healthy control donor D) Average responses for three healthy control donors to 1 $\mu$ M DK-PGD<sub>2</sub> on submerged bronchial epithelial cells. E) Dot plot to show change in fura-2 ratio 340/380nm (peak/basal) for three healthy control donors to 1 $\mu$ M DK-PGD<sub>2</sub>, 1 $\mu$ M DK-PGD<sub>2</sub> + 1 $\mu$ M AZD6430 and 1 $\mu$ M ionomycin on submerged bronchial epithelial cells. p-value based on paired two-tailed t-test.

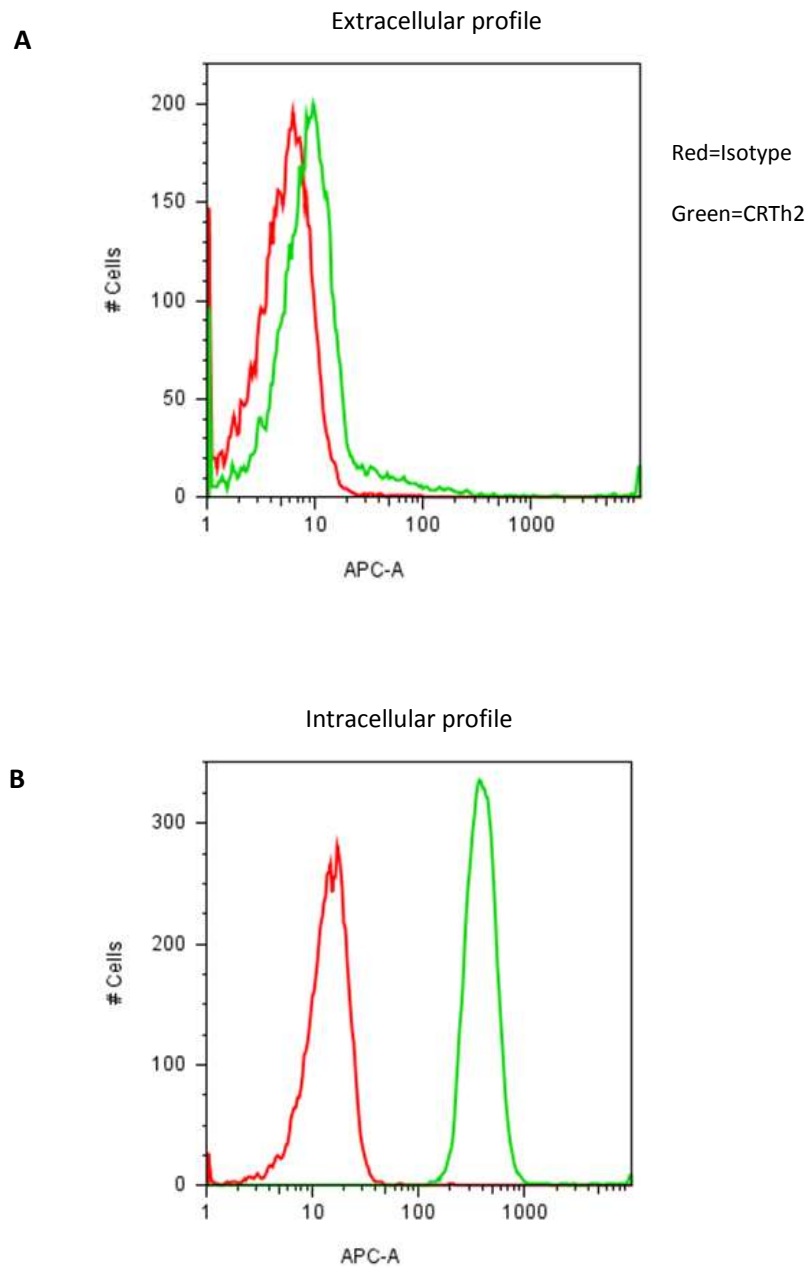
#### 4.6. Exploring CRTh2 protein expression on HMC-1 cells and isolated human lung mast cells

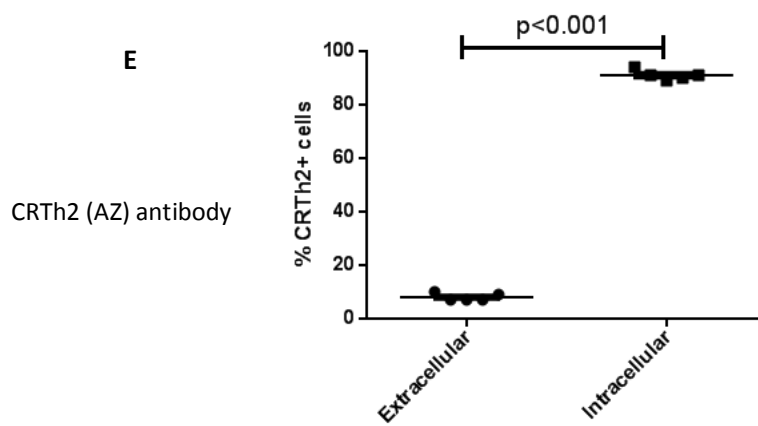
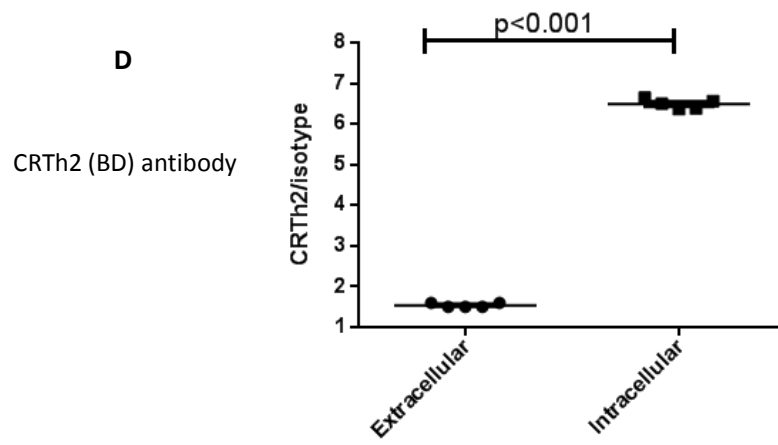
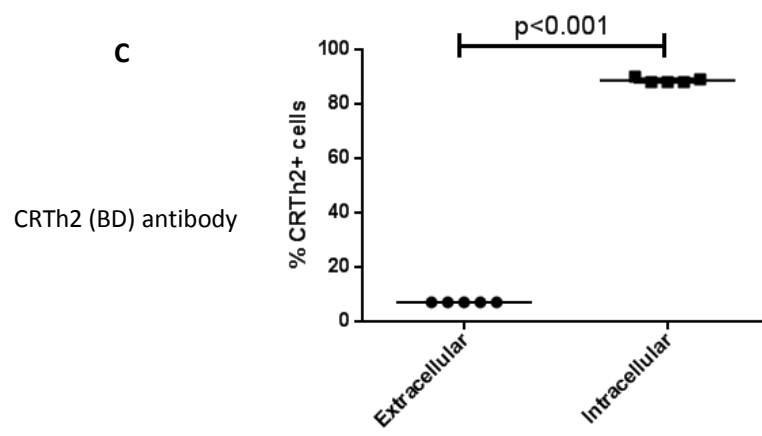
To confirm and extend upon the novel findings that CRTh2 was expressed on a sub-population of mast cells within the bronchial biopsies, CRTh2 expression was investigated on a mast cell line (HMC-1) and human lung mast cells. HMC-1 cells are a mast cell line and are considered to be of an immature mast cell

phenotype (384) as they lack native expression of the high affinity IgE receptor, FCεRI (385,386). However, as these cells are fast growing they are useful to work up methodologies for which human lung mast cells cannot be used due to lack of availability of cells. CRTh2 expression was explored using flow cytometry. For initial experiments using HMC-1 cells the flow cytometry expression profile for the cells obtained with the BD Biosciences antibody was compared to that of the CRTh2 AZ antibody. If similar expression profiles were obtained this data would help to further validate the CRTh2 AZ antibody.

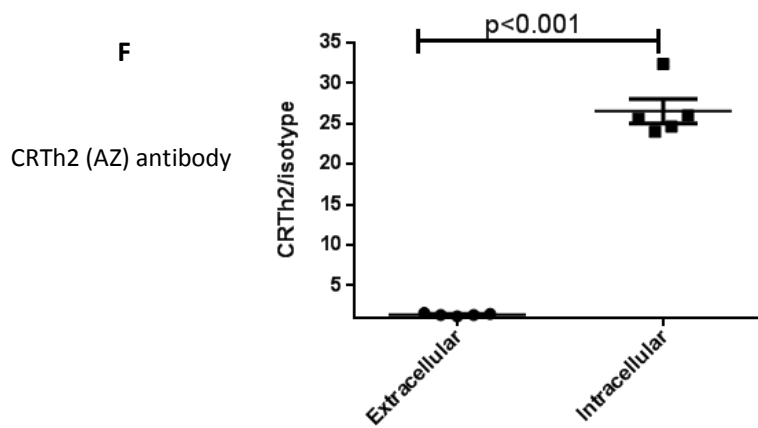
CRTh2 expression was found to be present on HMC-1 cells using both the BD Biosciences antibody and the CRTh2 AZ antibody. Similar percentages of positive cells were obtained for both antibodies helping to further validate the CRTh2 AZ antibody (**Figure 4.6C, E**). Extracellular expression demonstrated that 7[0]% of the HMC-1 cells expressed CRTh2. A significantly higher percentage of cells expressed CRTh2 when intracellular expression was investigated (mean [SEM] extracellular 7[0]% versus intracellular 89[0.4]%,  $p < 0.001$ . **Figure 4.6A-C**). Similarly, for both antibodies, the level of CRTh2 expression (geometric mean fluorescence units) was significantly higher for intracellular CRTh2 expression compared to extracellular expression, although the levels of fluorescence found for the CRTh2 AZ were higher than those for the BD Biosciences antibody (BD Biosciences antibody extracellular 1.5[1.4-1.6] versus intracellular 6.5[6.3-6.6],  $p < 0.001$ . AZ antibody extracellular 1.4[1.2-1.6] versus 26[22.6-30.6],  $p < 0.001$ . **Figure 4.6D, F**). The difference in the level of expression between the two antibodies is probably due to the BD Biosciences antibody being directly conjugated to APC-fluorescent dye, whereas a secondary APC-fluorescent antibody was applied

for the CRTh2 AZ antibody, contributing to elevated geometric fluorescent unit measurements. These data suggests that CRTh2 is expressed on HMC-1 cells, with more expression present within the intracellular compartment of the cells.





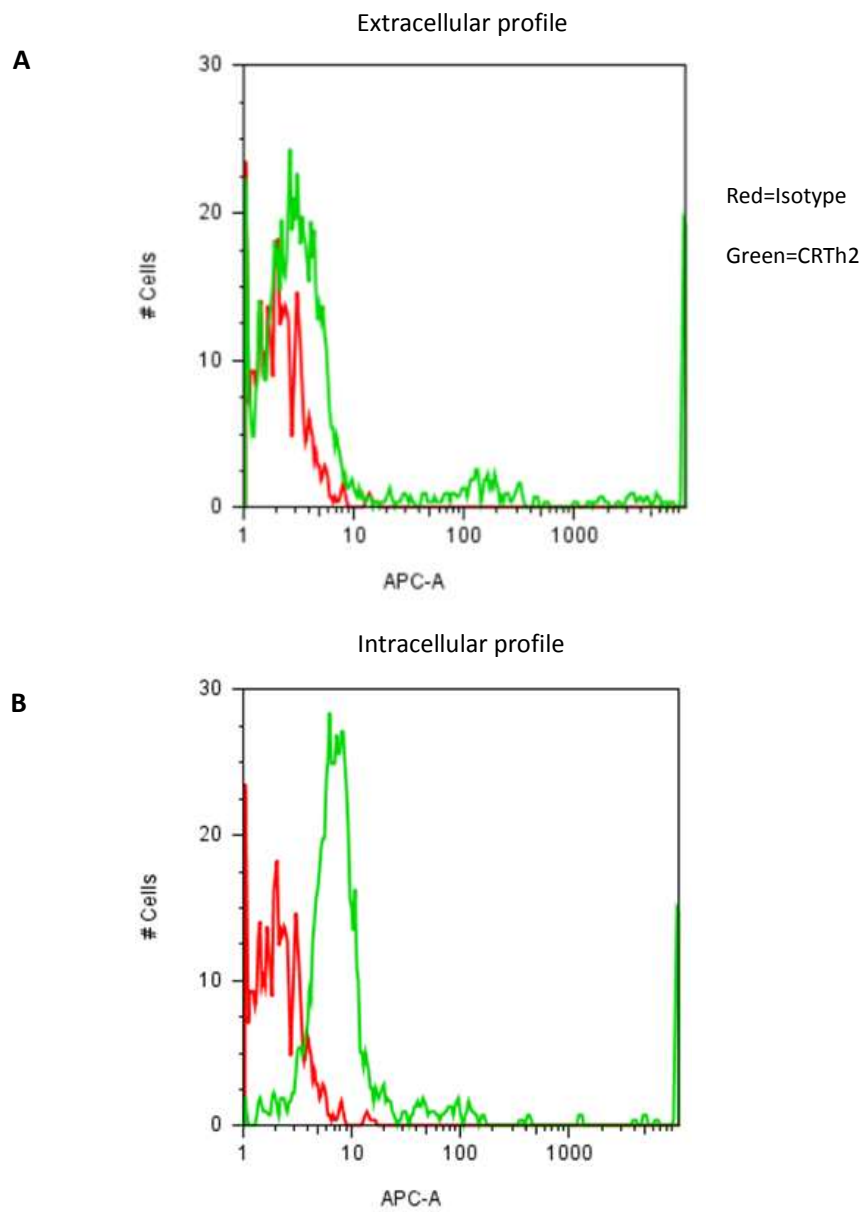


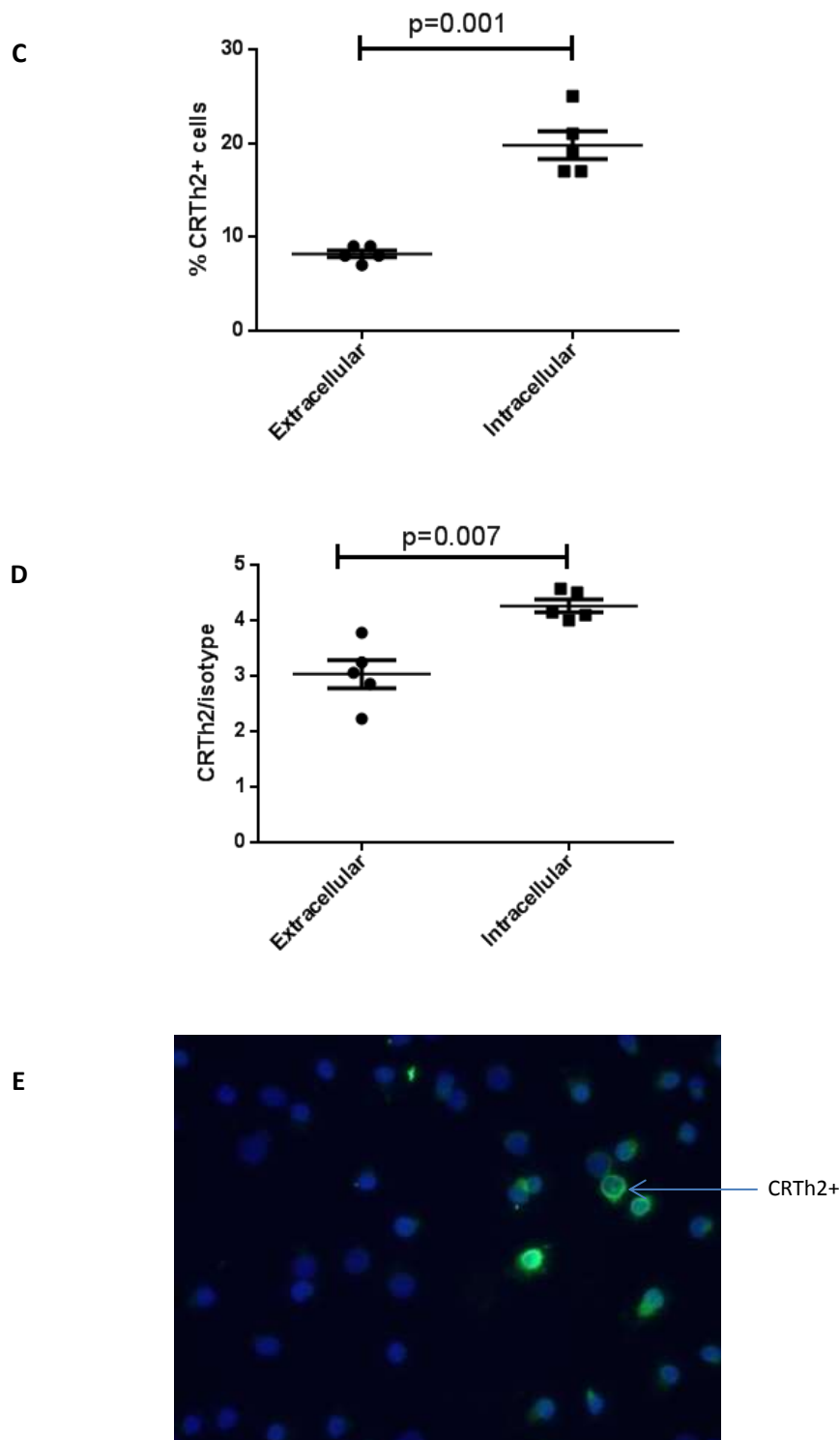


**Figure 4.6. CRTh2 expression on HMC-1 cells.** A) Extracellular CRTh2 expression profile for BD Biosciences antibody on HMC-1 cells. B) Intracellular CRTh2 expression for BD Biosciences antibody on HMC-1 cells. C) Dot-plot to show % CRTh2+ HMC-1 cells extracellular expression vs intracellular expression using BD Sciences antibody. p-value based on 2-tailed paired t-test. D) Dot-plot to show levels of CRTh2 expression (geometric mean fluorescent units) for HMC-1 extracellular expression vs intracellular expression using BD Sciences antibody. p-value based on 2-tailed paired t-test. E) Dot-plot to show % CRTh2+ HMC-1 cells extracellular expression vs intracellular expression using AZ antibody. p-value based on 2-tailed paired t-test. F) Dot-plot to show levels of CRTh2 expression (geometric mean fluorescent units) for HMC-1 extracellular expression vs intracellular expression using AZ antibody. p-value based on 2-tailed paired t-test.

CRTh2 protein expression was also identified on human lung mast cells using flow cytometry. The human lung mast cells are isolated from lung resection tissue. As with the HMC-1 cells, the percentage of CRTh2+ human lung mast cells was significantly higher for intracellular CRTh2 expression compared to extracellular expression (extracellular 8.2[0.8]% vs intracellular 19.8[3.3]%,  $p=0.001$ . **Figure 4.7A-C**). Similarly, the level of CRTh2 expression (geometric mean fluorescence units) was significantly higher for intracellular CRTh2 expression compared to extracellular expression (extracellular 3[2.3-3.8] vs intracellular 4.3[4-4.3],  $p=0.007$ . **Figure 4.7A, B, D**). In addition, CRTh2 mRNA

expression was found to be present for human lung mast cells (three donors combined, mean [SEM] 4.3[0.8] mRNA expression). Using an alternative technique to explore the protein expression of CRTh2, fluorescent cell staining on a human lung mast cell cytopsin demonstrated CRTh2 expression (green) on 5-10% of non-permeabilized cells within the cytopsin population (**Figure 4.7E**). Dual fluorescent staining for CRTh2 and mast cell chymase was also carried out on the cytopsin (data not shown). Positive chymase expression was observed on 2-5% of the cells within each cytopsin. There was co-localisation of 1-2% of the mast cells for CRTh2 and chymase for each cytopsin. CRTh2 expression was therefore present on chymase+ and chymase- mast cells. These data suggests that CRTh2 is expressed on a sub-population of lung mast cells, but not specific to chymase+ mast cells. This data is supportive of the findings on the bronchial biopsies demonstrated in chapter 3 (chapter 3.9.3). The HMC-1 and human lung mast cell data support and extend upon the observations within the bronchial biopsies that CRTh2 is expressed on a small percentage (5-10%) of mast cells.





**Figure 4.7. CRTh2 expression on isolated human lung mast cells (HLMC).** A) Extracellular CRTh2 expression profile for BD Biosciences antibody on HLMC. B) Intracellular CRTh2 expression for BD Biosciences antibody on HLMC. C) Dot-plot to show % CRTh2+ HLMC extracellular expression vs intracellular expression using BD Sciences antibody. p value based on paired two-tailed t-test D) Dot-plot to show levels of CRTh2 expression (geometric mean fluorescent units) for HLMC

extracellular expression vs intracellular expression using BD Sciences antibody. p value based on paired two-tailed t-test E) Fluorescent cell staining (green) on HLMC using CRTh2 AZ antibody.

#### 4.7. Chapter 4 Summary

The aim of this work was to explore the expression and function of CRTh2 on bronchial epithelial cells. CRTh2 was found to be expressed on isolated, cultured bronchial epithelial cells using flow cytometry, fluorescent cell staining and mRNA expression profiling techniques. These findings help to confirm and extend upon the novel observations found for the bronchial biopsies in chapter 3. Consistent with the findings for the bronchial biopsies the number of CRTh2 positive epithelial cells was reduced in cells from moderate-severe asthma, suggesting that the differentials in CRTh2 expression observed *in vivo* remain *in vitro*.

Tools to investigate the effects of activation of CRTh2 were evaluated. A CRTh2 selective agonist, DK-PGD<sub>2</sub>, and selective antagonist, AZD6430, were found to cause no increases in cellular toxicity or apoptosis in bronchial epithelial cells compared to controls when used at concentrations used within assays. These findings confirmed that these reagents were suitable for use to explore the activation effects of CRTh2 on bronchial epithelial cells. CRTh2 activation on bronchial epithelial cells caused cell migration and an increase in intracellular calcium. These findings demonstrate that activation of CRTh2 on bronchial epithelial cells causes two of the functional responses that have previously been described for this receptor on Th2 cells, eosinophils and basophils (104,169,170,228,416).

CRTh2 expression was also confirmed upon a sub-population of human lung mast cells, confirming and extending upon observations made within bronchial biopsies in chapter 3.

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## **Chapter 5**

### **Results**

# **Effects of CRTh2 activation on Air Liquid Interface (ALI) cultures**

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## 5.1. Chapter Overview

**The aims of this chapter were to investigate CRTh2 activation on epithelial cells grown at air liquid interface (ALI). This followed on from the novel observations of human CRTh2 expression on human bronchial epithelial cells within bronchial biopsies and submerged cultured cells.**

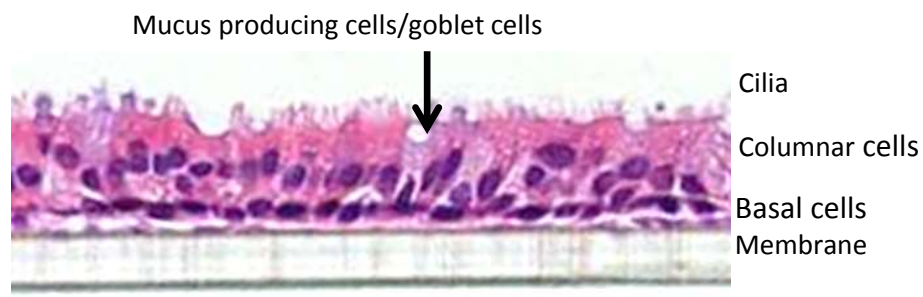
ALI cultures consist of differentiated epithelial cells, more similar to *in vivo* bronchial epithelium containing features such as mucus producing cells and ciliated cells, which are not present under submerged conditions. It was hoped that investigation into the effects of CRTh2 activation on epithelial cells in a differentiated format may indicate potential pro- or anti-remodelling processes. This information could then help to provide evidence of the effects CRTh2 activation *in vivo* and if CRTh2 activation may have a role in airway remodelling in asthma. This could then provide additional useful information for future CRTh2 antagonist studies in asthma.

A CRTh2 selective agonist and antagonist were used to treat ALI cultures. The effects of CRTh2 activation on the morphological characteristics of healthy control and asthmatic ALI cultures was investigated at both the protein and mRNA level. To investigate endogenous agonist effects, human lung mast cells were co-cultured with ALI cultures as mast cells have been reported to release the CRTh2 agonist prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) (163). To help elucidate if effects of CRTh2 activation were pro- or anti-remodelling comparisons were made for healthy control ALI cultures with asthmatic ALI cultures.



## 5.2. Description of Air Liquid Interface cultures (ALI)

Air liquid interface cultures (ALI) are bronchial epithelial cells that are grown at an air interface. This allows epithelial differentiation into basal and columnar cells, with the presence of mucus producing cells and ciliation (**Figure 5.1**). This system is therefore more similar to *in vivo* compared to epithelial cells grown under submerged conditions (417). Therefore the effects of CRTh2 activation on all of the components of the epithelium can be investigated using ALI cultures



**Figure 5.1. Haematoxylin & Eosin stained ALI culture from a healthy control donor.**

Different methods have been adopted to grow ALI cultures (418-420). The ALI cultures used for this study were from a commercial company called Epithelix™. These cultures are grown in an environment where the airflow across the ALI culture is carefully controlled using a system called VITROCELL™. This system more closely mimics an *in vivo* environment such as in human airways as compared to the more traditional approach of growing ALI cultures in a culture plate with the lid on within an incubator. The details of the Epithelix™ MucilAir cultures used for the current studies are shown in **table 5.1**. No significant differences were observed between the ALI cultures from donors with normal pathology compared to

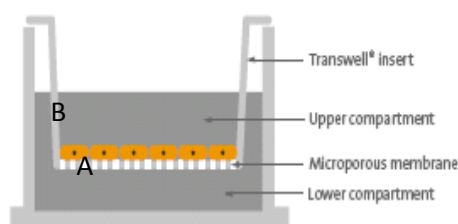
those from asthmatic donors for age (mean [SEM] 55[3] versus 46[7],  $p=0.240$ ), sex or ciliary beat frequency (CBF) (mean [SEM] 8[0.09] versus 8[0.08],  $p=0.402$ ).

**Table 5.1.** Epithelix MucilAir ALI culture details (taken from certificate of analysis)

Batch number	Age	Sex	Pathology	CBF (Hertz)
MD0442401	61	Male	Normal	8.5Hz
MD039301	69	Male	Normal	7.2Hz
MD005302	50	Female	Normal	7.2Hz
MD034300	47	Female	Normal	7.3Hz
MD0843300	26	Female	Normal	8.2Hz
MD1404502	54	Female	Normal	8.3Hz
MD1346701	62	Female	Normal	8.1Hz
MD1346600	65	Male	Normal	8.3Hz
MD020103	67	Female	Normal	7.9Hz
MD049901	63	Female	Normal	7.9Hz
MD020102	67	Female	Normal	7.9Hz
MD0440001	47	Male	Normal	7.8Hz
MD1256600	44	Male	Normal	7.9Hz
MD049901	63	Female	Normal	7.9Hz
MD0441701	30	Male	Normal	7.9Hz
MD0456200	63	Male	Normal	7.9Hz
MD0451501	46	Female	Asthmatic	7.9Hz
MD0441402	33	Male	Asthmatic	8.1Hz
MD0444700	68	Male	Asthmatic	8.2Hz
MD0445500	31	Female	Asthmatic	8.2Hz
MD1440601	54	Female	Asthmatic	7.8Hz

### 5.3. Air Liquid Interface cultures (ALI) experimental read-outs

The aim of this series of experiments was to investigate the effects of CRTh2 activation on ALI cultures. The ALI cultures are grown on small inserts. To maximise experimental read-outs following treatment each ALI membrane (**Figure 5.2 label A**) was carefully removed from its insert (**Figure 5.2 label B**) and cut. A portion of the membrane was processed for histology/immunohistochemical read-out and remaining membrane was processed for RNA/QT-PCR (see methods). A similar approach to processing ALI cultures has been used previously (351).



**Figure 5.2. Diagram of an ALI culture.** A=membrane with cells growing on it, B=insert which provide support for growing culture.

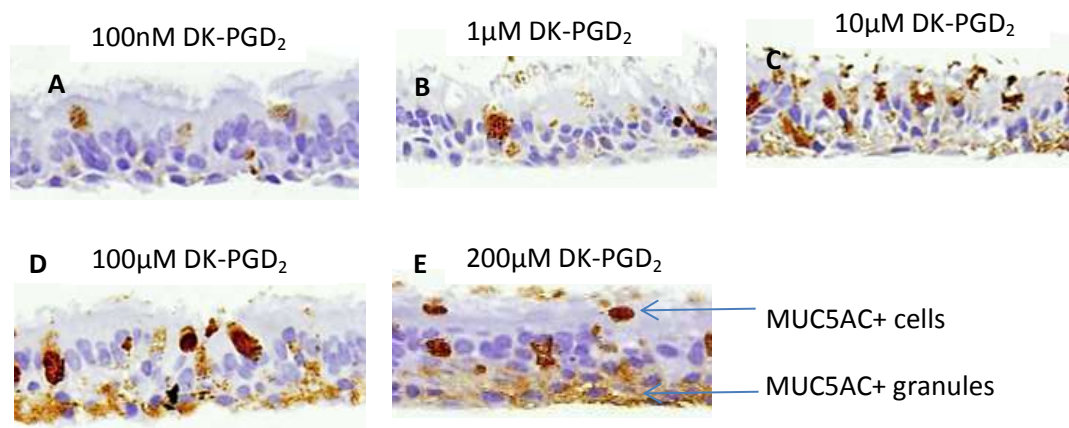
CRTh2 activation was carried out using the CRTh2 selective agonist DK-PGD<sub>2</sub> (104). To further validate that responses observed were through CRTh2 activation a CRTh2 selective antagonist AZD6430 was used.

### 5.4. Effects of CRTh2 activation on ALI culture morphology

To investigate the effects of CRTh2 activation in response to DK-PGD<sub>2</sub> treatment on the unique morphological components of ALI cultures, the differentiation status of the ALI culture was assessed, by investigating the goblet cells and the overall architecture of the ALI culture.

#### 5.4.1. Effects of CRTh2 activation on mucus producing cells

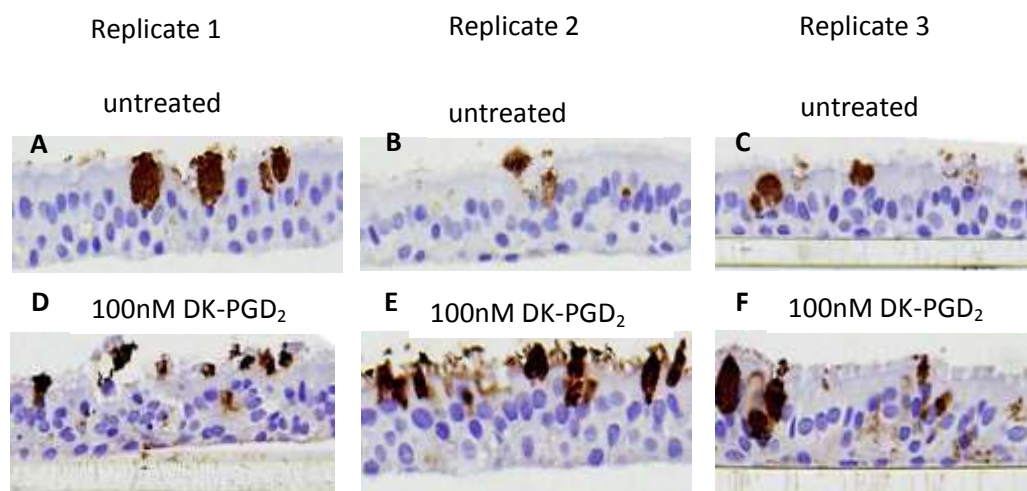
For healthy control ALI cultures a dose response to DK-PGD<sub>2</sub> was assessed for MUC5AC, the predominant mucin in goblet cells (62). For all doses investigated there was an increase in the number of MUC5AC+ cells compared to the untreated ALI culture, in addition MUC5AC+ granules were frequently seen for all the DK-PGD<sub>2</sub> treated cultures and were particularly clear at higher doses (**Figure 5.3**). These findings suggest that activation of CRTh2 influences the number of MUC5AC+ goblet cells, and MUC5AC+ granules which provide evidence of mucin synthesis (421).



**Figure 5.3. Dose response of DK-PGD<sub>2</sub> on MUC5AC+ staining for healthy control ALI cultures.** A) 100nM DK-PGD<sub>2</sub> B) 1µM DK-PGD<sub>2</sub> C) 10µM DK-PGD<sub>2</sub> D) 100µM DK-PGD<sub>2</sub> E) 200µM DK-PGD<sub>2</sub>.

As effects on the numbers of MUC5AC+ cells were observed at the lowest concentration of DK-PGD<sub>2</sub> of 100nM, this concentration was used for further studies as it was thought to be more physiologically relevant to levels of PGD<sub>2</sub> found released by mast cells (250). To minimise the costs of experiments the variability between ALI cultures in MUC5AC+ cells, receiving the same treatment was assessed for triplicate repeats. For the triplicate repeats there was low

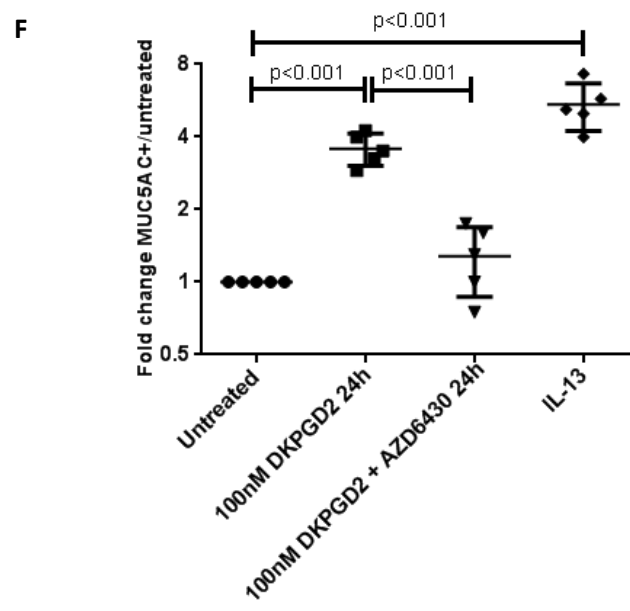
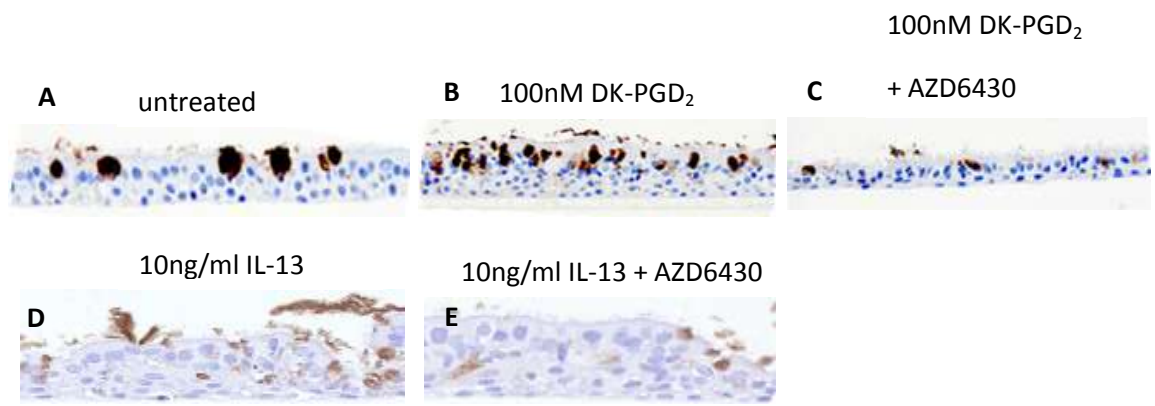
variability observed between ALI cultures receiving the same treatment (**Figure 5.4**), with the co-efficient of variation being 14%. This finding is in keeping with previous studies using Epithelix MucilAir™ cultures which demonstrated a low intra-experimental variability coefficient of variation of 15% (422). From these data it was decided to save costs duplicate ALI cultures would be sufficient to give representative results.

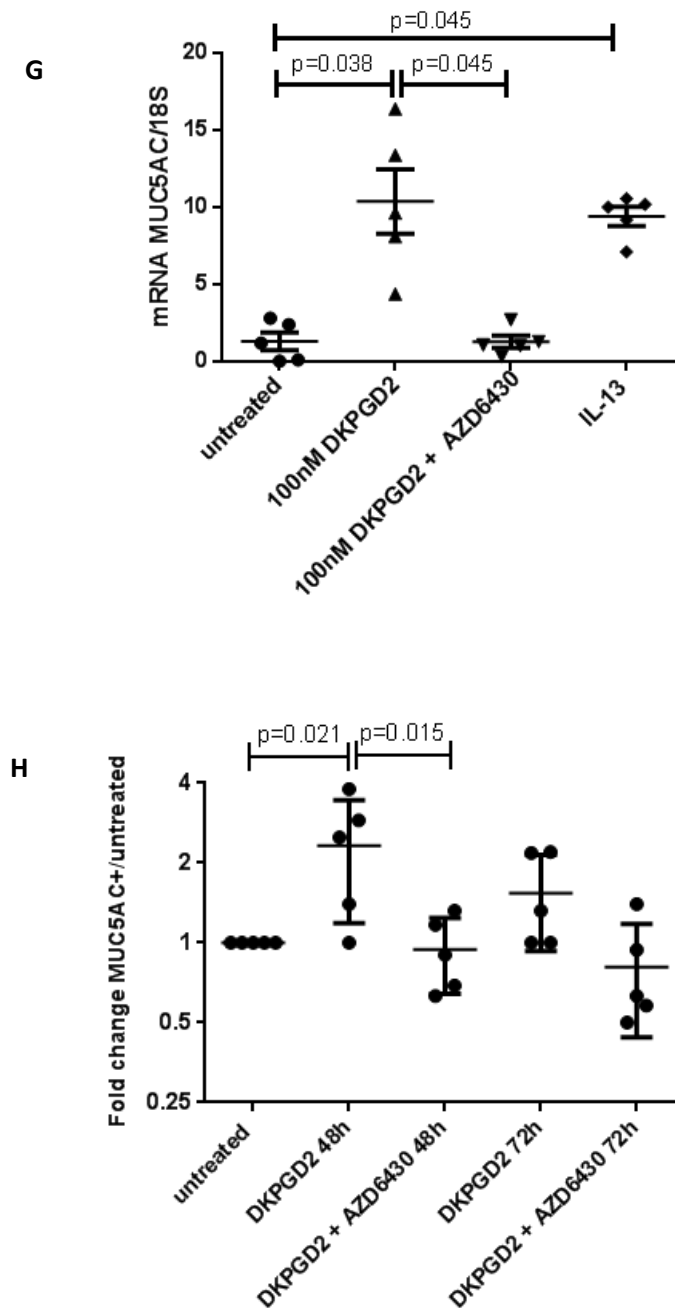


**Figure 5.4. Effects of treatment on triplicate repeats on MUC5AC+ staining for healthy control ALI cultures.** A) untreated B) untreated C) untreated D) 100nM DK-PGD<sub>2</sub> E) 100nM DK-PGD<sub>2</sub> F) 100nM DK-PGD<sub>2</sub>.

To confirm and extend upon observations for the effects of DK-PGD<sub>2</sub> treatment on MUC5AC+ cells, five separate donors of healthy ALI cultures were treated with vehicle control, DK-PGD<sub>2</sub> or DK-PGD<sub>2</sub> and AZD6430. Treatment for 24 hours with DK-PGD<sub>2</sub> produced an increase in the goblet cells present quantified by MUC5AC+ staining, which could be blocked with AZD6430 (**Figure 5.5A-C, F**). No differences were observed between untreated and vehicle control treated cultures, therefore all differences were assessed relative to untreated ALI cultures. A significant fold-increase in the number of MUC5AC+ cells was observed with

DK-PGD<sub>2</sub> treatment when compared to untreated ALI (4-fold [3-4] increase;  $p<0.001$ ), which decreased significantly in the presence of AZD6430 (**Figure 5.5A-C, F**). This finding validated that the effect on the number of MUC5AC<sup>+</sup> cells was through CRTh2 activation. IL-13, used as a positive control, caused a significant fold increase in the number of MUC5AC<sup>+</sup> cells compared to untreated (IL-13 5-fold [4-7] increase,  $p<0.001$ . **Figure 5.5D, F**). This finding validated the assay as increases in MUC5AC<sup>+</sup> cells in response to IL-13 within ALI cultures has been shown previously (358,423). AZD6430 did not affect IL-13 responses (5.6-fold [4-7] versus IL-13 alone 5-fold [4-7], **Figure 5.5D, E**) helping to give confidence in the selectivity of the compound to CRTh2. MUC5AC mRNA analysis showed similar results to the protein expression (**Figure 5.5G**), giving more confidence to findings. Taken together this information strongly suggests that CRTh2 activation can influence mucus producing cells within the epithelium, causing mucus cell hyperplasia. More chronic effects of DK-PGD<sub>2</sub> incubation were also assessed at 48h and 72h. An increase in MUC5AC<sup>+</sup> cells compared to untreated was maintained at 48h DK-PGD<sub>2</sub> (2-fold [1-4];  $p=0.021$ ), but this effect diminished at 72h (**Figure 5.5H**). This information suggests that increases in MUC5AC<sup>+</sup> cells within the epithelium are an acute response to CRTh2 activation.



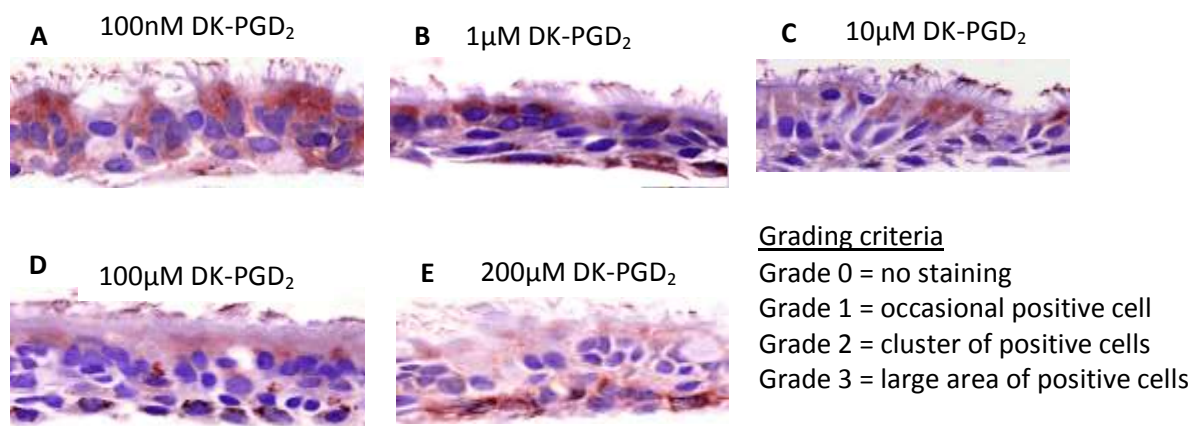


**Figure 5.5. Effects of CRTh2 activation on MUC5AC+ staining for healthy control ALI cultures.** Representative images of healthy control ALI cultures (A-E) of MUC5AC+ staining (brown) x200 magnification. A) untreated, B) 100nM DK-PGD<sub>2</sub> for 24h C) 100nM DK-PGD<sub>2</sub> + 1 $\mu$ M AZD6430 for 24h. D) 100ng/ml IL-13 for 24h. E) 100ng/ml IL-13 + 1 $\mu$ M AZD6430 for 24h F) Dot plot to show fold-change in MUC5AC+ cells per mm of culture over untreated for cultures with 24h treatment. p value based on one way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. G) Dot plot to show mRNA expression for MUC5AC normalised to 18S expression for cultures with 24h treatment. p value based on Kruskal Wallis. Overall p-value 0.003; p-values shown in figure based on Dunn's post hoc test. H) Dot plot to show quantitation of MUC5AC staining for cultures with 48h and 72h treatment. p value based on one way analysis of variance. Overall p-value 0.005; p-values shown in figure based on Tukey post hoc test.



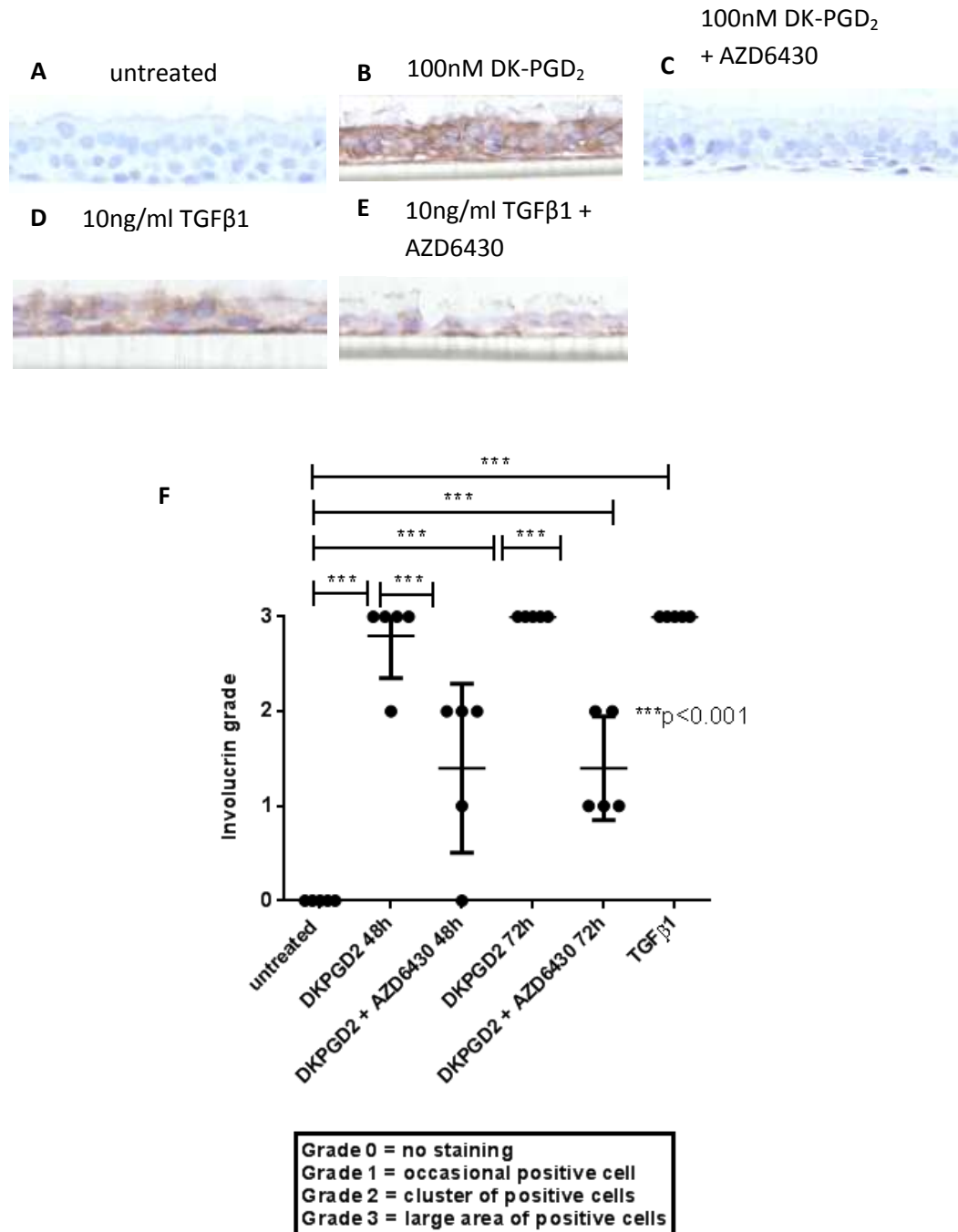
#### ***5.4.2. Effects of CRTh2 activation on squamous metaplasia***

The results from the current study suggest that CRTh2 activation causes mucous hyperplasia. Mucus cell hyperplasia is a recognised feature of airway remodelling in asthma (62-64). Epithelial metaplasia has also been identified as part of the remodelling response of the epithelium (68), therefore this response was investigated in ALI cultures in response to CRTh2 activation. Squamous metaplasia is the most common metaplasia associated with epithelial tissue (69). To investigate if CRTh2 activation also affected the differentiation status of the ALI cultures in terms of squamous metaplasia, a well validated squamous marker, involucrin (312) was used. Staining was graded according to the same criteria as used for the biopsies (see results 3.14). Involucrin staining was investigated in response to a dose-response range of DK-PGD<sub>2</sub> treatment. Involucrin staining was associated with all concentrations of DK-PGD<sub>2</sub> tested (**Figure 5.6**). In order to quantify the involucrin staining, grading criteria were assigned to the area of staining associated with the ALI cultures (**Figure 5.6**), ranging from grade 0 for no staining to grade 3 for a large area of staining. ALI cultures treated with DK-PGD<sub>2</sub> were associated with the highest involucrin grade of 3 (a large area of involucrin staining) being observed for all DK-PGD<sub>2</sub> concentrations. This information suggests that CRTh2 activation of ALI cultures induces squamous metaplasia, therefore influencing the differentiation status of the epithelium.



**Figure 5.6. Dose response of DK-PGD<sub>2</sub> on involucrin+ staining for healthy control ALI cultures.** A) 100nM DK-PGD<sub>2</sub> B) 1μM DK-PGD<sub>2</sub> C) 10μM DK-PGD<sub>2</sub> D) 100μM DK-PGD<sub>2</sub> E) 200μM DK-PGD<sub>2</sub>.

To further characterise and extend upon the findings that DK-PGD<sub>2</sub> induces squamous metaplasia involucrin staining was assessed for five healthy control cultures in response to 100nM DK-PGD<sub>2</sub> and AZD6430 was used as well to confirm that the response was through CRTh2 activation. A significant increase in involucrin staining was seen for the ALI cultures treated with DK-PGD<sub>2</sub> at 48h and 72h treatments (untreated grade 0[0-0], DK-PGD<sub>2</sub> 48h grade 3[2-3]  $p < 0.001$ , DK-PGD<sub>2</sub> 72h grade 3 [3-3]  $p < 0.001$ . **Figure 5.7A, B, F**). AZD6430 significantly decreased involucrin staining compared to DK-PGD<sub>2</sub> alone (**Figure 5.7B, C, F**). This finding indicates that induction of the squamous response is through CRTh2 activation. TGFβ<sub>1</sub> was used as a positive control, as this has previously been shown to induce squamous metaplasia in ALI cultures (325). TGFβ<sub>1</sub> caused a significant increase in involucrin staining (untreated grade 0[0-0] versus TGFβ<sub>1</sub> grade 3[3-3],  $p < 0.001$ ) (**Figure 5.7D, F**). This finding helped to validate the approach used to assess squamous metaplasia in ALI cultures. The effects of TGFβ<sub>1</sub> were unaffected by AZD6430 (TGFβ<sub>1</sub> grade 3[3-3] versus TGFβ<sub>1</sub> + AZD6430 grade 3[3-3] **Figure 5.7D, E**), helping to validate the selectivity of AZD6430 for CRTh2.



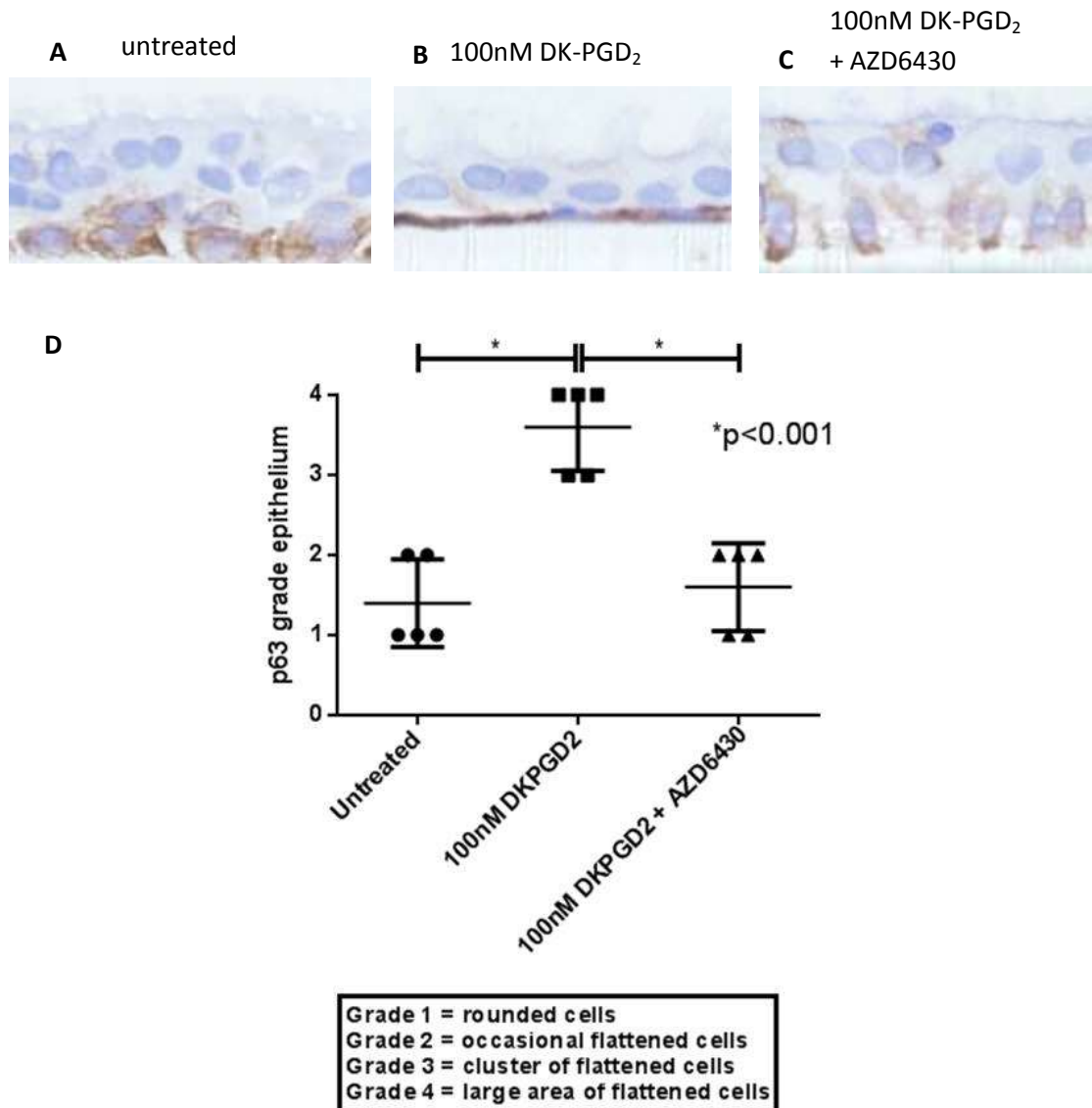
**Figure 5.7. Effects of CRTh2 activation on involucrin+ staining for healthy control ALI cultures.** Representative images of healthy control ALI cultures (A-E) of Involucrin+ staining (brown) x400 magnification. A) untreated, B) 100nM DK-PGD<sub>2</sub> for 48h C) 100nM DK-PGD<sub>2</sub> + 1μM AZD6430 for 48h. D) 10ng/ml TGFβ1 for 72h. E) 10ng/ml TGFβ1 + 1μM AZD6430 for 72h F) Representative image involucrin+ staining for an untreated asthmatic ALI culture. G) Dot blot to show quantitation of involucrin staining for cultures with 48 and 72 hour treatments. p value based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test.

To further investigate the differentiation status of ALI cultures upon CRTh2 activation a marker for basal epithelial cells investigated. Basal epithelial cells identified by p63 under normal differentiation conditions are confined to the basal layer of the epithelium and the cells have characteristic rounded basal cell morphology (400). It was of interest to address if these characteristics changed upon CRTh2 activation as basal cells are also considered to be the migratory cells of the epithelium (275,276) and CRTh2 activation was shown to promote epithelial migration in the current study (chapter 4).

#### ***5.4.3. Effects of CRTh2 activation on basal epithelial cells***

The effects of CRTh2 activation on the morphology of the basal epithelial cells of the ALI culture, identified with p63/high molecular weight cytokeratin (400) were investigated for 5 healthy control ALI culture donors. For the ALI cultures treated with 100nM DK-PGD<sub>2</sub> the basal epithelial cells were more flattened against the membrane, and adopted a more-spindle like morphology, when compared to the untreated ALI cultures and the DK-PGD<sub>2</sub> + AZD6430 treated cultures (**Figure 5.8A, B, C**). Grading criteria based on the morphology of the basal cells were devised to describe the morphology of the p63 cells, as shown in **figure 5.8D**. There was a significant change in morphology of the p63 cells upon DK-PGD<sub>2</sub> treatment. Upon DK-PGD<sub>2</sub> treatment the p63+ cells adopted a more flattened morphology compared to the untreated cultures where the p63+ cells had characteristic rounded basal cell morphology (median [IQR] untreated grade 1[1-2] versus DK-PGD<sub>2</sub> grade 4[3-4],  $p < 0.001$ . **Figure 5.8D**). AZD6430 treatment restored a rounded basal cell morphology for the majority of the p63+ cells (DK-PGD<sub>2</sub> grade 4[3-4] versus DK-PGD<sub>2</sub>+AZD6430 grade 2[1-2],  $p < 0.001$ . **Figure 5.8D**). These results suggest that

DK-PGD<sub>2</sub> treatment affects the morphology of basal epithelial cells. The flattened, spindle like cells could potentially indicate a change of the cells to a more mesenchymal phenotype (323), or migratory phenotype (424).



**Figure 5.8. Effects of CRTh2 activation on basal epithelial cells for healthy control ALI cultures.** Representative images of healthy control ALI cultures of p63/HMW cytochrome staining (brown) x400 magnification. A) untreated, B) 100nM DK-PGD<sub>2</sub> C) 100nM DK-PGD<sub>2</sub> + 1 $\mu$ M AZD6430 D) Dot blot to show grading of the morphology of the p63+ cells for healthy control ALI cultures. p value based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test.

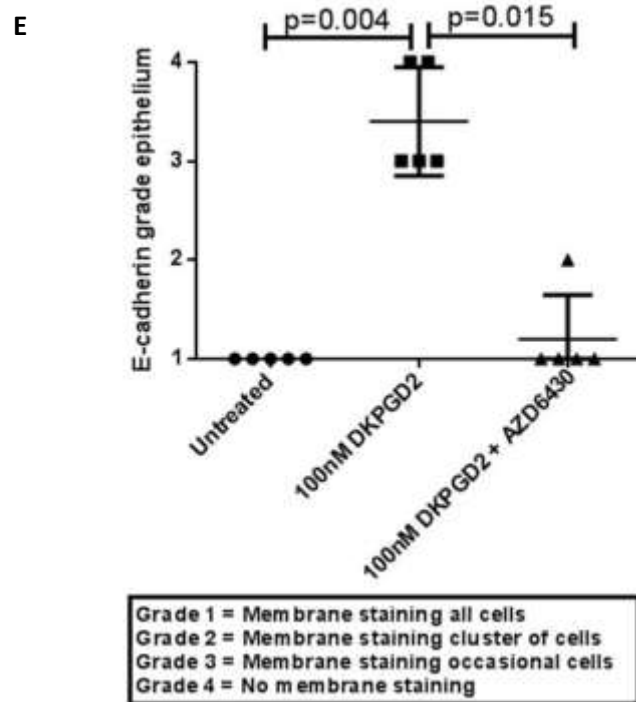
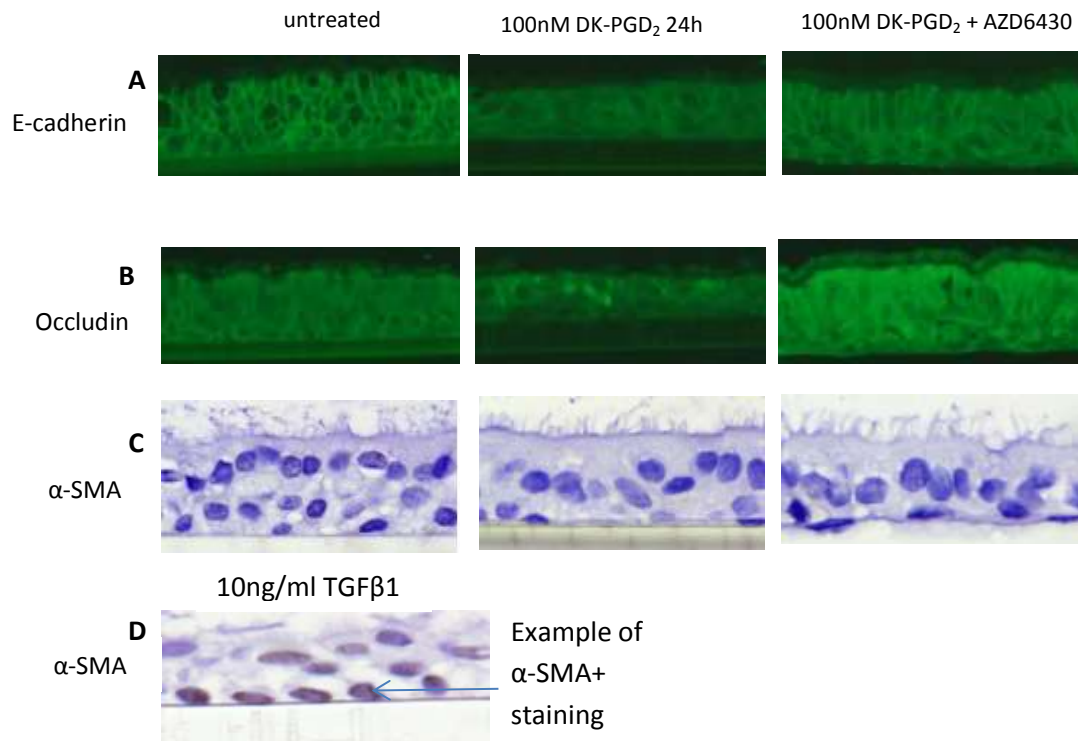
#### ***5.4.4. CRTh2 activation effects on E-cadherin, occludin and $\alpha$ -SMA staining***

The flattened morphology of the DK-PGD<sub>2</sub> treated basal epithelial cells could indicate a phenotype switch, or epithelial-mesenchyme transition (EMT), of the cells as has been observed previously for TGF $\beta$ 1 treated ALI cultures (323). An EMT transition in epithelial cells can be identified if cells lose their epithelial phenotype and gain a mesenchymal or fibroblast-like phenotype (317,425). An early molecular sign of EMT is a down-regulation of E-cadherin expression (317). A later stage of EMT is the cells gaining expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (322). Expression of E-cadherin can also help to indicate the integrity of the epithelium. In addition, E-cadherin has been shown to be essential for epithelial tissue integrity and morphogenesis through the initiation and formation of adherens junctions (277). Expression of the tight junction protein, occludin, can also help to indicate the integrity of the epithelium (277). Healthy control ALI cultures treated with DK-PGD<sub>2</sub> and DK-PGD<sub>2</sub> + AZD6430 were immunostained for E-cadherin,  $\alpha$ -SMA and occludin. Immunofluorescent staining was used for E-cadherin and occludin to be able to identify membrane associated staining more easily, and chromagen staining was used for  $\alpha$ -SMA. For untreated healthy control ALI cultures clear membrane E-cadherin expression was observed (**Figure 5.9A**). DK-PGD<sub>2</sub> treatment disrupted the staining, with a loss of membrane staining around individual epithelial cells (**Figure 5.9A**). Treatment with AZD6430 in addition to DK-PGD<sub>2</sub> reinstated membrane bound E-cadherin staining (**Figure 5.9A**). The location of the E-cadherin staining was assessed using the grading criteria shown in figure **5.9E**. The E-cadherin staining was significantly altered in the DK-PGD<sub>2</sub> treated cultures, with significantly fewer cells being associated with E-cadherin

expression around the membrane of individual cells (median [IQR] untreated grade 1[1-1] versus DK-PGD<sub>2</sub> grade 3[3-4],  $p=0.004$ . **Figure 5.9E**). With AZD6430 treatment membrane associated E-cadherin staining was restored (DK-PGD<sub>2</sub> grade 3[3-4] versus DK-PGD<sub>2</sub>+AZD6430 1[1-2],  $p=0.015$ . **Figure 5.9E**). These findings suggest that CRTh2 activation causes disruption to the integrity of the ALI culture junctions. The re-distribution of E-cadherin has been reported before during epithelial repair (426). The expression of occludin, a tight junction protein was also investigated on the ALI cultures. For untreated healthy control ALI cultures occludin expression was observed uniformly throughout the epithelium (**Figure 5.9B**). With DK-PGD<sub>2</sub> treatment expression appeared brighter along the apical surface of the epithelium with some bright spots of occludin staining (**Figure 5.9B**). With addition of AZD6430 and DK-PGD<sub>2</sub> treatment, brighter occludin staining was seen which was consistent across the epithelium (**Figure 5.9B**). This suggests that CRTh2 activation affects the distribution of occludin tight junctions, potentially creating a more permeable epithelium. In areas of squamous metaplasia it has been reported that tight junction protein re-distribute to the apical surface of the epithelium (427). This finding is consistent with the distribution of staining for occludin in DK-PGD<sub>2</sub> treated cultures and therefore suggests the development of squamous metaplasia in these cultures, in agreement with the findings for involucrin described previously. The healthy control ALI cultures or DK-PGD<sub>2</sub> treated cultures did not express  $\alpha$ -SMA protein (**Figure 5.9C**). However, ALI cultures treated with TGF $\beta$ 1 did express  $\alpha$ -SMA protein, as would be expected from previous literature (**Figure 5.9D**), therefore validating that the immunostaining had worked. The findings from this data would suggest that CRTh2 activation does not fully induce



epithelial-mesenchyme-transition in healthy control ALI cultures to produce cells of a fibroblast phenotype.

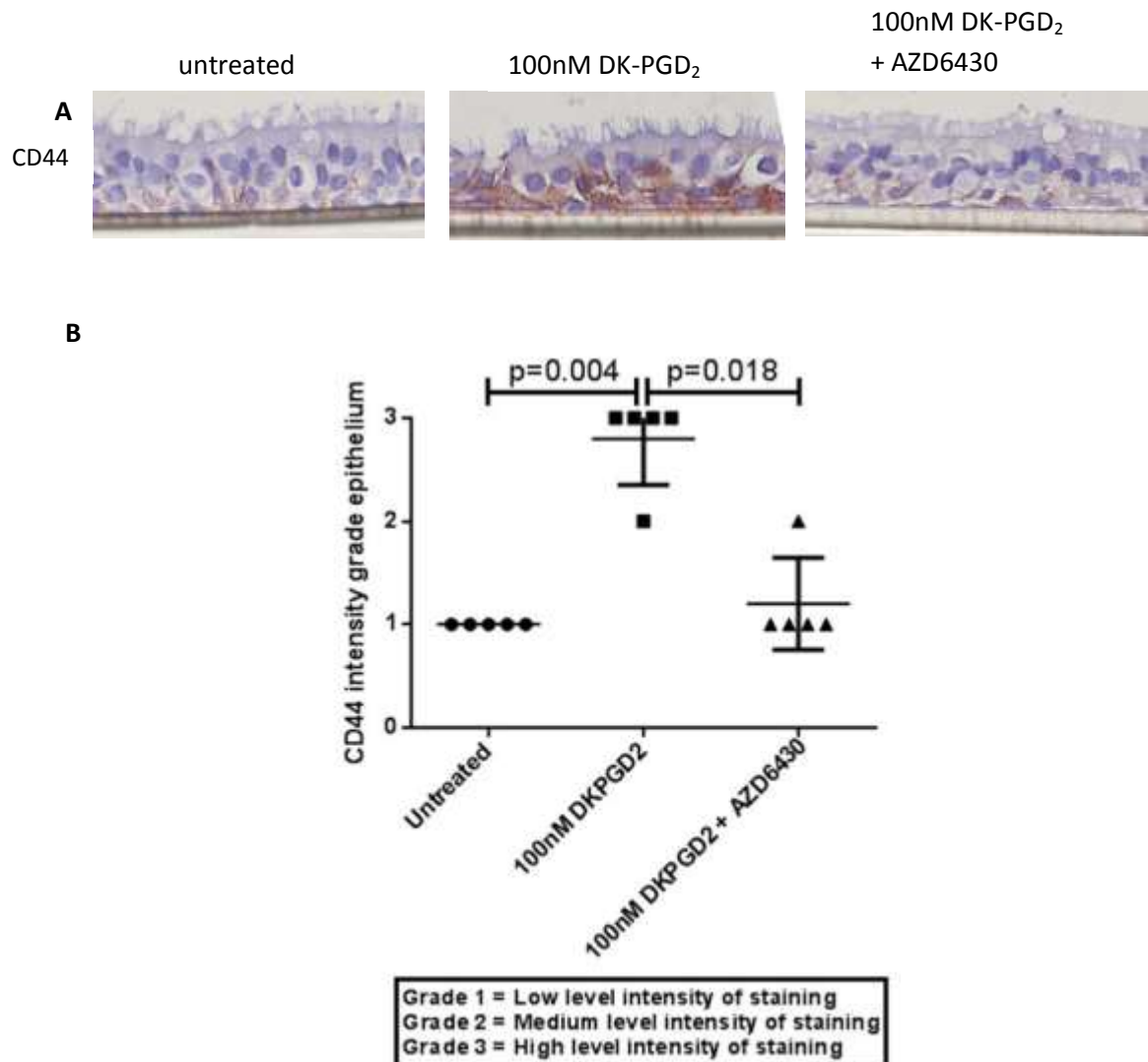


**Figure 5.9. Effects of CRTh2 activation on epithelial-mesenchyme transition and epithelial integrity for healthy control ALI cultures.** Representative images of healthy control ALI cultures of A) E-cadherin staining (green) x200 magnification for untreated, 100nM DK-PGD<sub>2</sub>, 100nM DK-PGD<sub>2</sub> + 1μM AZD6430. B) Occludin staining (green) x200 magnification for untreated, 100nM DK-PGD<sub>2</sub>, 100nM DK-PGD<sub>2</sub> + 1μM AZD6430. C) α-SMA staining (brown) x400 magnification for untreated, 100nM DK-PGD<sub>2</sub>, 100nM DK-PGD<sub>2</sub> + 1μM AZD6430. D) 10ng/ml TGFβ1. E) Dot blot to show grading of the distribution of E-cadherin staining for healthy control ALI cultures. p value based on Kruskal-Wallis. Overall p-value = 0.001; p-values shown in figure based on Dunn's post hoc test.

#### ***5.4.5. Activation of CRTh2 influences migratory features of the cells within ALI cultures***

CRTh2 activation on Th2 cells, basophils and eosinophils has been shown to activate cell migration (104,169,170,208,228). In addition, in the current study CRTh2 activation was shown to cause epithelial cell migration (see results chapter 4). The marker CD44 is a cell adhesion molecule, which is a receptor for hyaluronan and fibronectin. Both of these components form the provisional matrix that facilitate cell migration (424,428,429). CD44 has also been found to have increased expression in areas of repairing epithelium (298). Changes in CD44 expression can therefore indicate changes in the migratory activity of the epithelial cells within the ALI culture. For five healthy control donors ALI cultures treated with DK-PGD<sub>2</sub> and DK-PGD<sub>2</sub> + AZD6430 were assessed for CD44 immunostaining. The CD44 staining was observed predominantly on the basal cells of the ALI cultures (**Figure 5.10A**), consistent with previous literature (430). Treatment with DK-PGD<sub>2</sub> increased the intensity of staining within the ALI cultures (**Figure 5.10A**), and this was reduced by AZD6430 (**Figure 5.10A**). The intensity of the staining was assessed according to the intensity grading criteria described in figure 5.10B. The intensity of CD44 was significantly increased with DK-PGD<sub>2</sub>

staining (median [IQR] untreated 1[1-1] versus DK-PGD<sub>2</sub> 3[2-3],  $p=0.004$ . **Figure 5.10B**), and significantly reduced back to untreated levels with AZD6430 (DK-PGD<sub>2</sub> 3[2-3] versus DK-PGD<sub>2</sub>+ AZD6430 1[1-2],  $p=0.018$ . **Figure 5.10B**). These data suggest that CRTh2 activation up-regulates CD44 expression in ALI cultures, indicating potential effects on the migratory activity of the epithelial cells.



**Figure 5.10. Effects of CRTh2 activation on CD44 expression for healthy control ALI cultures.** Representative images of healthy control ALI cultures of CD44 staining (brown) x200 magnification. A) untreated, 100nM DK-PGD<sub>2</sub> for 24h, 100nM DK-PGD<sub>2</sub> + 1μM AZD6430 for 24h B) Dot blot to show quantitation of the intensity of CD44 staining for cultures with 24 hour treatments. p value based on Kruskal-Wallis. Overall p-value = 0.002; p-values shown in figure based on Dunn's post hoc test.

### ***5.5. CRTh2 activation with endogenous PGD<sub>2</sub> from human lung mast cells and activated human lung mast cell supernatants***

Human lung mast cells produce the CRTh2 agonist PGD<sub>2</sub> (163), with elevated levels released from activated mast cells (228). To investigate if endogenous levels of PGD<sub>2</sub> replicated the changes in the ALI cultures seen with DK-PGD<sub>2</sub>, healthy control ALI cultures were cultured with either human lung mast cells present in the basal compartment of the ALI, or with the supernatant of activated mast cells present in the basal compartment of the ALI. During optimisation of experiments, human lung mast cells were also incubated for 2 hours on the apical surface of the ALI culture to see if the mast cells would move between the epithelial cells.

#### ***5.5.1. Apically applied mast cells for 2 hours do not integrate within the epithelial cells of ALI cultures***

Mast cells were applied to healthy control ALI cultures to the upper, apical compartment in mast cell media for 2 hours. After 2 hours the mast cells were removed and placed into the basal compartment of the ALI cultures so as not to interfere with the air interface for a sustained period of time. ALI cultures were stained for mast cell tryptase to identify if any cells had moved between the epithelial cells. No tryptase<sup>+</sup> cells were identified within the epithelium of the ALI.

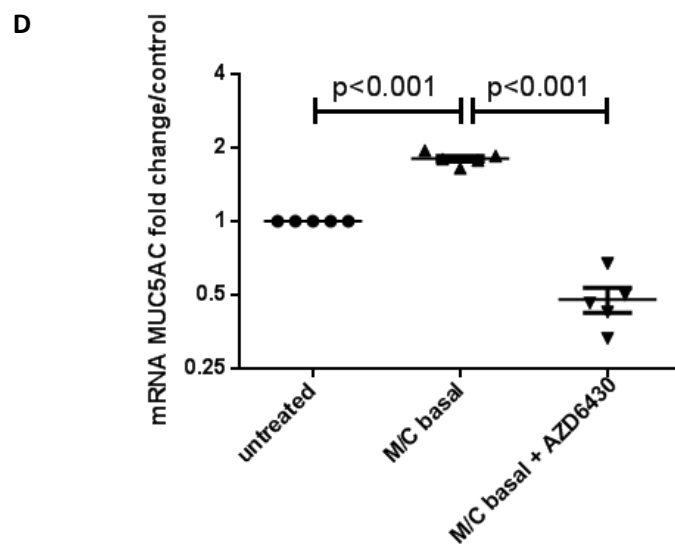
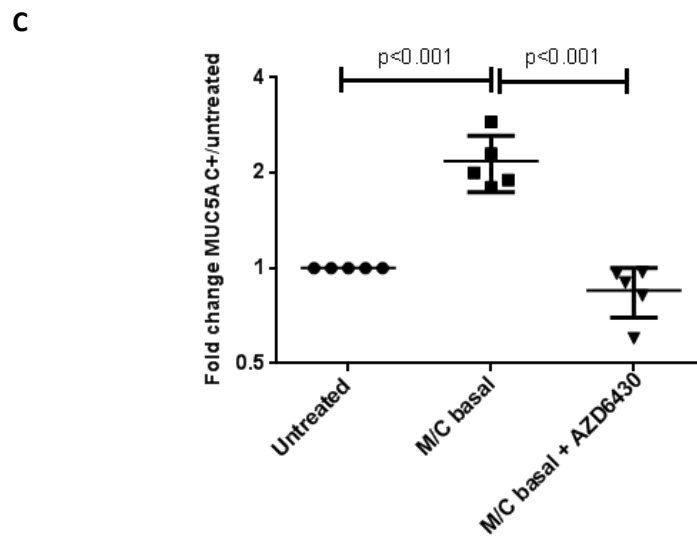
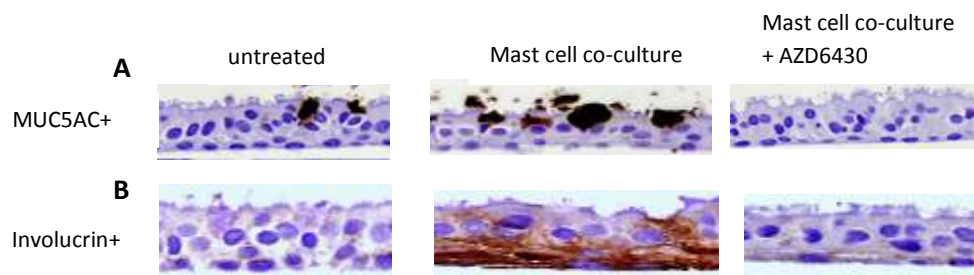
### ***5.5.2. Prostaglandin D<sub>2</sub> was present within the mast cell cultures and activated mast cell supernatants***

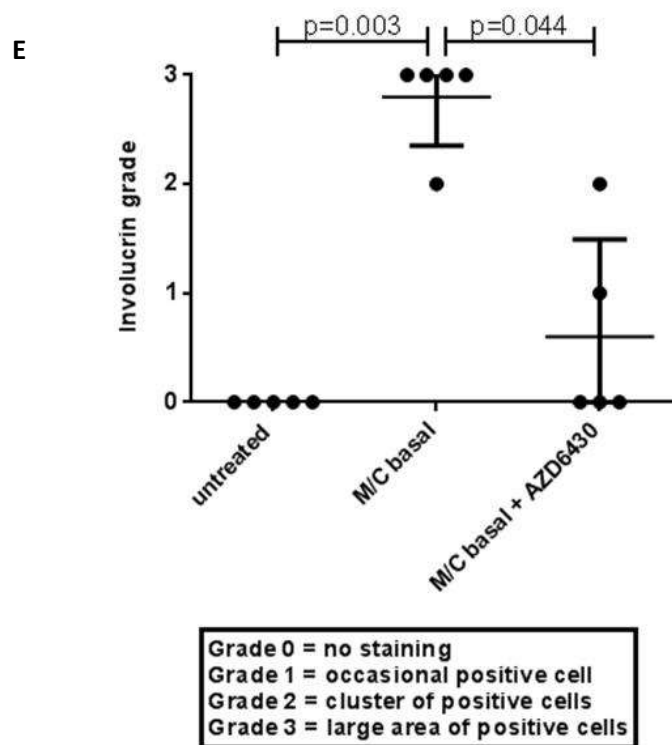
To establish if prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) was present in the mast cell cultures co-cultured with the ALI cultures, an ELISA for PGD<sub>2</sub> (see methods) was performed on methoxylamine hydrochloride (MOX-HCl)-treated samples. PGD<sub>2</sub> rapidly degenerates and so conversion into PGD<sub>2</sub>-MOX prevents further chemical degradation. For control supernatants (epithelium media, mast cell media alone) no PGD<sub>2</sub> could be detected. For supernatants containing un-activated mast cells PGD<sub>2</sub> levels were found to be 2[0.5]ng/ml, and for activated mast cell supernatants 33[4]ng/ml. These levels of PGD<sub>2</sub> are similar to previous studies (250,388). These findings validated that the reagents used for HLMC:ALI co-culture experiments.

### ***5.5.3 Co-culture of mast cells with healthy control ALI cultures mimics changes observed with DK-PGD<sub>2</sub> activation***

Mast cells together with mast cell media were added in a ratio of 1:1 with ALI media in the basal compartment of healthy control ALI cultures and cultured for 24 hours. Mast cell media and ALI culture media were used in 1:1 ratio so as not to affect the viability of either the mast cells or the epithelial cells. A significant increase in the number of MUC5AC<sup>+</sup> cells was observed for the mast cell:ALI co-cultures compared to the untreated ALI cultures (untreated 1[1-1]fold versus mast cell:ALI 2[2-3]fold,  $p < 0.001$ , **Figure 5.11A, C**). This increase was blocked with AZD6430 (mast cell:ALI 2[2-3]fold versus mast cell:ALI+AZD6430 0.8[0.6-1]fold,  $p < 0.001$ , **Figure 5.11A, C**). Although some fold change points for HLMC:ALI + AZD6430 were lower than untreated controls, no significant difference was

observed (**Figure 5.11C**). Observations that mucin secretion in cells is attenuated to a level below that seen in control cells has been made previously (431), and has been reported as a phenomenon that occurs often in cells exposed to strong inhibitors of various molecules involved in the secretory process (431,432). Similar results were also seen at the mRNA level (**Figure 5.11D**). These results show that PGD<sub>2</sub> can mimic the effects observed with the CRTh2 selective agonist DK-PGD<sub>2</sub>. Blocking the effects on MUC5AC+ cells with AZD6430 suggests the PGD<sub>2</sub> induced response is through CRTh2 activation. There was also a significant increase in involucrin staining for the mast cell:ALI co-cultures compared to untreated (median [SEM] untreated 0[0-0] versus mast cell:ALI 3[2-3], p=0.003, **Figure 5.11B, E**), which could be blocked with AZD6430 (mast cell:ALI 3[2-3] versus mast cell:ALI+AZD6430 0[0-2], p=0.044, **Figure 5.11B, E**). This data is similar to the observations made with DK-PGD<sub>2</sub> which suggests that induction of squamous metaplasia within the ALI cultures is due to CRTh2 activation. Taken together these findings provide evidence that mast cells co-cultured with ALI cultures cause CRTh2 activation, probably through PGD<sub>2</sub>, affecting the differentiation status of the ALI cultures.





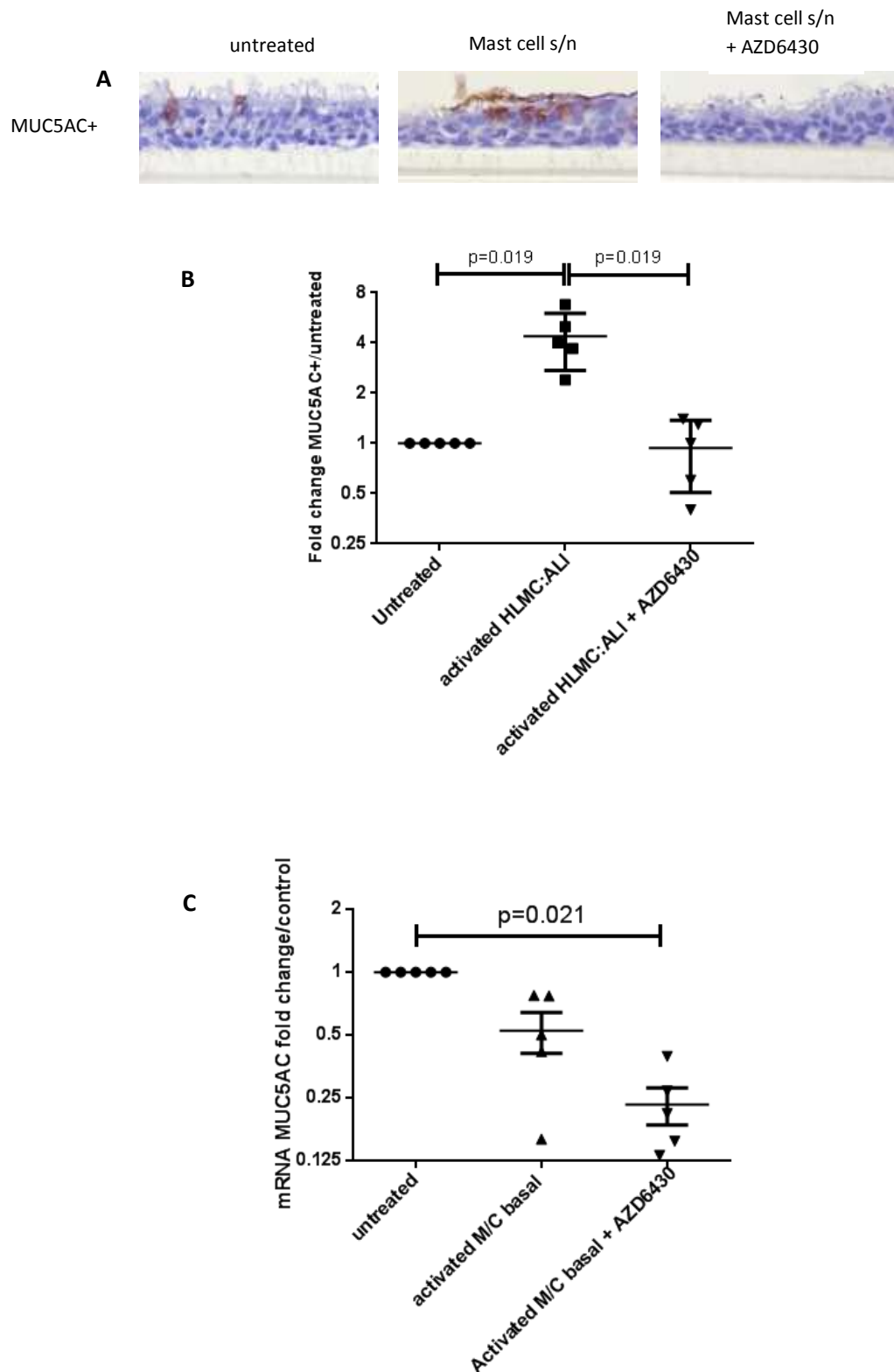
**Figure 5.11. Effects of mast cell co-culture on healthy control ALI cultures.** Representative images of healthy control ALI cultures of MUC5AC+ staining (brown) x400 magnification. A) untreated, mast cell co-culture 24h, mast cell co-culture + AZD6430 24h. Involucrin+ staining (brown) x400 magnification B) untreated, mast cell co-culture 24h, mast cell co-culture + AZD6430 24h. C) Dot plot to show fold-change in MUC5AC+ cells per mm of culture over untreated for cultures with 24h treatment. p value based on one way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. D) Dot plot to show mRNA expression for MUC5AC normalised to 18S expression for cultures with 24h treatment. p value based on one way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey's post hoc test. E) Dot blot to show quantitation of involucrin staining for cultures. p value based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test.

#### 5.5.4. Activation of CRTh2 through activated mast cell supernatant

Activated mast cells have been shown to release high levels of PGD<sub>2</sub> (228,388). To investigate if supernatants from activated mast cells were sufficient to



activate CRTh2 within the ALI culture and produce similar observations as with DK-PGD<sub>2</sub> healthy control ALI cultures were incubated with activated mast cell supernatant in a 1:1 ratio with ALI culture media. A significant increase in the number of MUC5AC<sup>+</sup> cells was observed at the protein level (untreated 1 [1-1]fold versus activated M/C supernatant 4[2.5-7]fold,  $p=0.019$ , **Figure 5.12A, B**), however, no significant increases in MUC5AC expression were observed at the mRNA level (**Figure 5.12C**). There was a decrease in MUC5AC mRNA expression for the activated mast cell supernatant (although this was not significant compared to untreated), and activated mast cell supernatant in addition with AZD6430, caused a further significant decrease in MUC5AC expression when compared to untreated cultures (untreated 1[1-1]fold versus activated M/C supernatant 0.2[0.1-0.4]fold,  $p=0.021$ , **Figure 5.12C**). The increase at the protein level could be blocked with AZD6430 (activated M/C supernatant 4[2.5-7]fold versus activated M/C supernatant+AZD6430 0.8[0.4-1.6]fold,  $p=0.0185$ , **Figure 5.12A, B**). No significant changes were observed for involucrin staining for the mast cell supernatant cultures compared to untreated. These findings suggest that activated mast cell supernatant, without mast cells present, does not cause the same activation of CRTh2 as DK-PGD<sub>2</sub> or mast cells. The finding that activated mast cell supernatants only affected the protein levels of MUC5AC<sup>+</sup> cells in a similar way to DK-PGD<sub>2</sub>, suggests that only the acute CRTh2 activation effects are observed. Activated mast cells release a wide range of mediators (241) and these have the potential to affect the effects of CRTh2 activation via PGD<sub>2</sub>.



**Figure 5.12. Effects of activated mast cell supernatant on healthy control ALI cultures.** Representative images of healthy control ALI cultures of MUC5AC+ staining (brown) x400 magnification. A) untreated, activated mast cell supernatant 24h, activated mast cell supernatant + AZD6430 24h. B) Dot plot to show fold-change in MUC5AC+ cells per mm of culture over untreated for cultures with 24h

treatment. p value based on Kruskal Wallis. Overall p-value = 0.002; p-values shown in figure based on Dunn's post hoc test. C) Dot plot to show fold-change in MUC5AC mRNA expression over untreated for cultures with 24h treatment. p value based on Kruskal Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test.

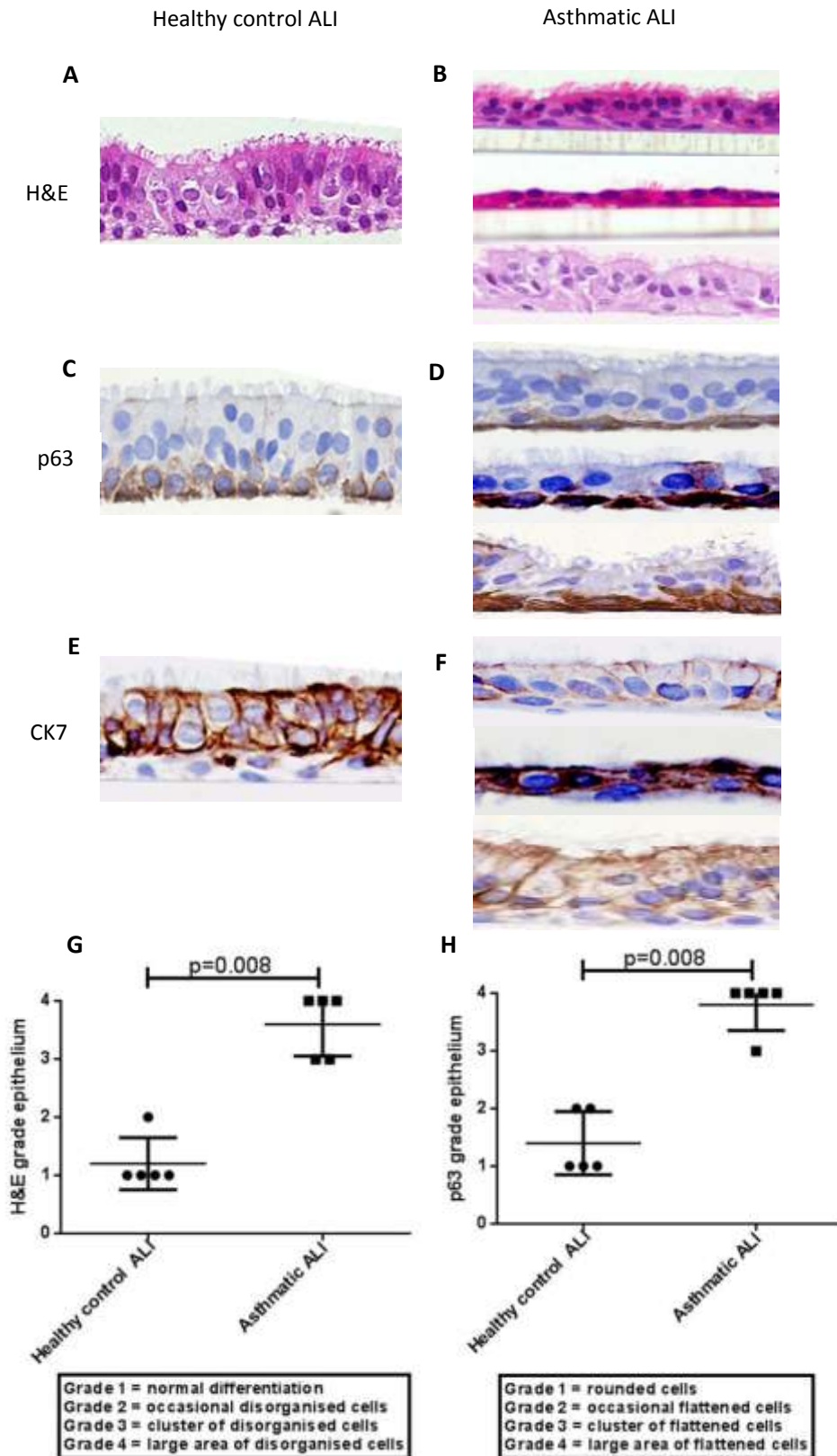
## ***5.6. Morphological analysis of ALI cultures derived from asthmatic patients***

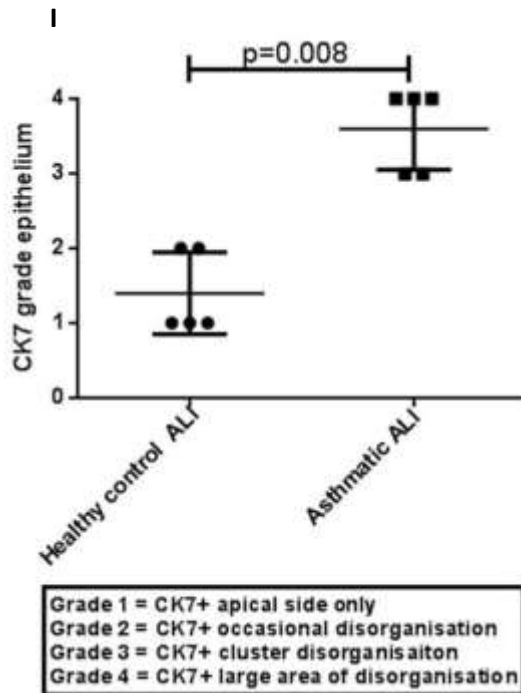
To identify if the changes observed for the healthy control ALI culture with CRTh2 activation were characteristic of pro- or anti-inflammatory changes in asthmatic epithelium, healthy control cultures were compared to asthmatic ALI cultures. The phenotypic markers investigated for the CRTh2 activation studies on the ALI cultures were applied to ALI cultures derived from asthmatic patients. Unfortunately the Epithelix™ asthmatic ALI cultures do not have associated clinical data so differentials in phenotype markers between asthma severities cannot be established.

### ***5.6.1. Asthmatic ALI cultures have altered histology compared to healthy control ALI cultures***

Initially the overall morphology of the ALI cultures from asthmatics were compared to the healthy control ALI cultures as assessed by H&E staining. The distribution of basal cells (p63+) and mature epithelial cells (cytokeratin 7+) were also compared between healthy control and asthma to identify any differences in the overall structure of the epithelium. The epithelial cells within the asthmatic ALI cultures have a flatter morphological appearance and the epithelial cells were more disrupted when compared to healthy control ALI cultures (**Figure 5.13A, B**). The H&E staining was graded according to the grading criteria shown in **figure 5.13G**.

The asthmatic ALI cultures had a significantly more disorganised structure as assessed by H&E staining when compared to the healthy control cultures (median [IQR] healthy control grade 1[1-2] versus asthmatic grade 4[3-4],  $p=0.008$ . **Figure 5.13G**). The asthmatic ALI cultures also contained significantly more flattened basal cells identified by p63/high molecular weight cytokeratin when compared to the more rounded, characteristic morphology of the healthy control ALI cultures (median [IQR] healthy control grade 1[1-2] versus asthmatic grade 4[3-4],  $p=0.008$ . **Figure 5.13C, D, H**). These observations are similar to those observed for DK-PGD<sub>2</sub> treated ALI cultures. For the phenotype marker cytokeratin 7, which identifies mature epithelial cells for the asthmatic ALI cultures the cytokeratin 7+ cells had a significantly more disorganised, less polarised appearance when compared to healthy control ALI cultures (median [IQR] healthy control grade 1[1-2] versus asthmatic grade 4[3-4],  $p=0.008$ . **Figure 5.13E, F, I**). These findings indicate that morphological differences are present between healthy control and asthmatic ALI cultures.





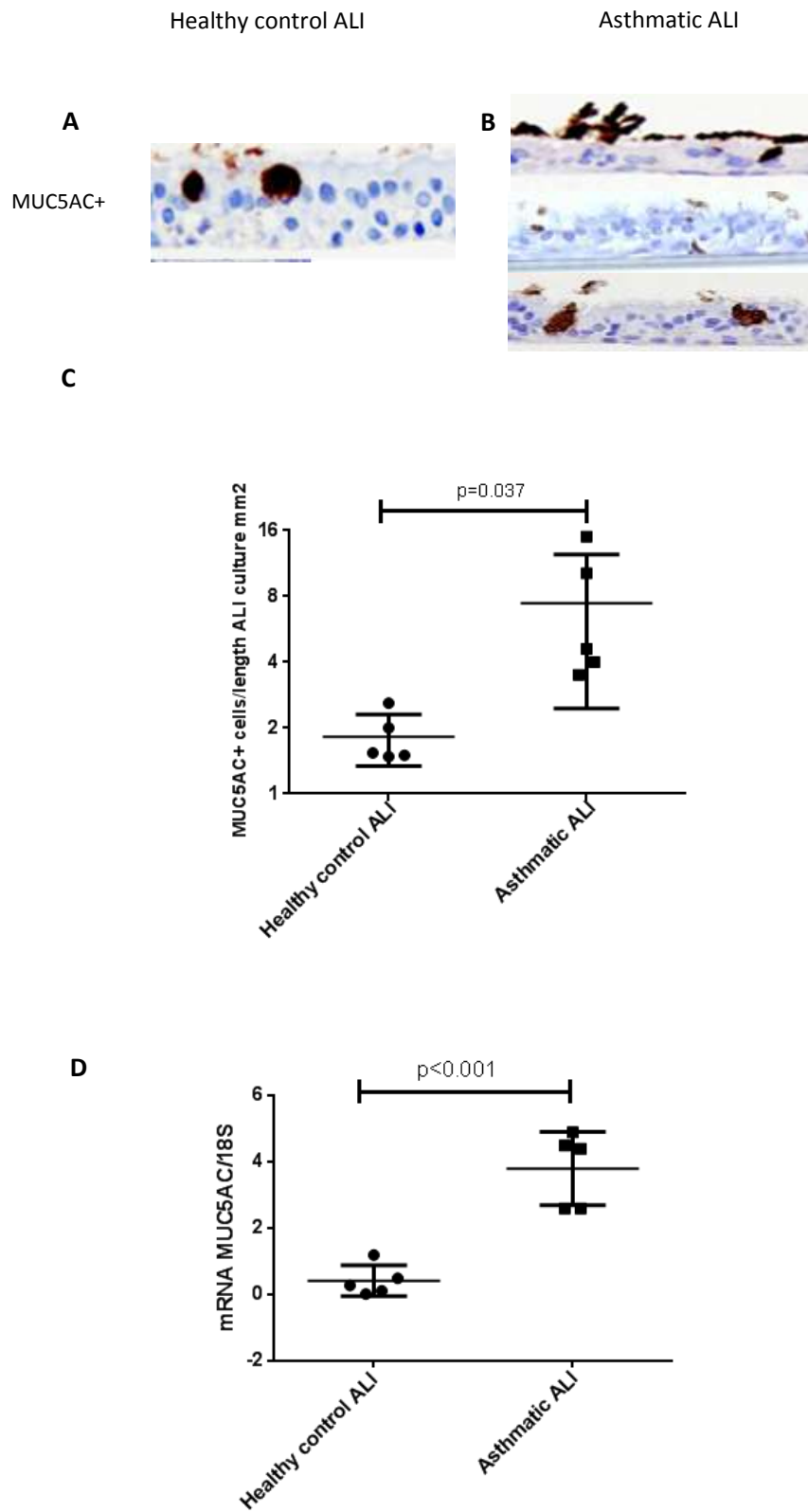
**Figure 5.13. Morphological analysis of ALI cultures derived from asthmatic subjects.** H&E staining for x400 magnification for A) untreated healthy control B) untreated asthmatic donor C) p63/HMW cytokeratin+ staining for untreated healthy control D) untreated asthmatic donor. E) Cytokeratin 7+ staining for untreated healthy control F) untreated asthmatic donor. G) Dot blot to show grading of H&E staining for cultures. p-value based on two-tailed Mann-Whitney test. H) Dot blot to show grading of p63+ cell morphology for cultures. p-value based on two-tailed Mann-Whitney test. I) Dot blot to show grading of CK7+ staining for cultures. p-value based on two-tailed Mann-Whitney test.

### 5.6.2. Asthmatic ALI cultures do not express $\alpha$ -SMA

As with the DK-PGD<sub>2</sub> treated cultures, the asthmatic ALI cultures contained basal cells of a flattened morphology. As assessed with the DK-PGD<sub>2</sub> treated cultures the asthmatic ALI cultures were immunostained for  $\alpha$ -SMA to identify if cells had undergone full EMT (323). The asthmatic ALI cultures did not express  $\alpha$ -SMA (data not shown), suggesting that full EMT had not occurred within the asthmatic ALI cultures used in the current study.

### ***5.6.3. Asthmatic ALI cultures are associated with higher levels of MUC5AC expression***

Asthmatic ALI cultures at both the protein level (mean [SEM] healthy control 1.8[0.2] MUC5AC+ cells per mm length versus asthmatic 7.5[2.20] MUC5AC+ cells per mm length,  $p=0.037$ . **Figure 5.14A-C**) and RNA level (healthy control 0.4[0.2] versus asthmatic 4[1],  $p<0.001$ . **Figure 5.14D**) were associated with significantly higher levels of MUC5AC+ expressions compared to healthy control ALI cultures. This data may be expected from the literature as MUC5AC has been shown to be increased in asthmatics compared to healthy controls (62), and suggests that these differences are maintained for ALI cultures grown *in vitro*.

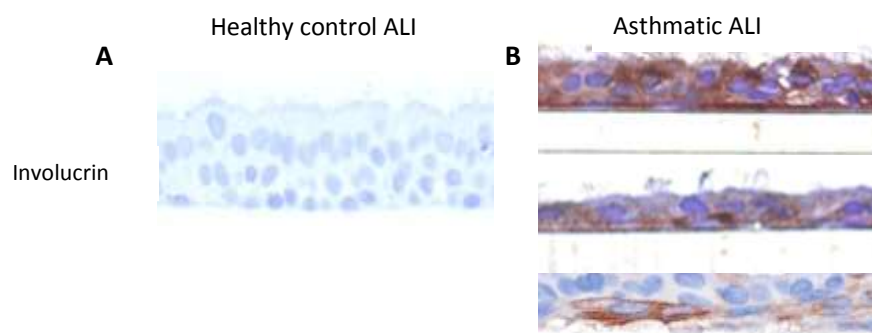


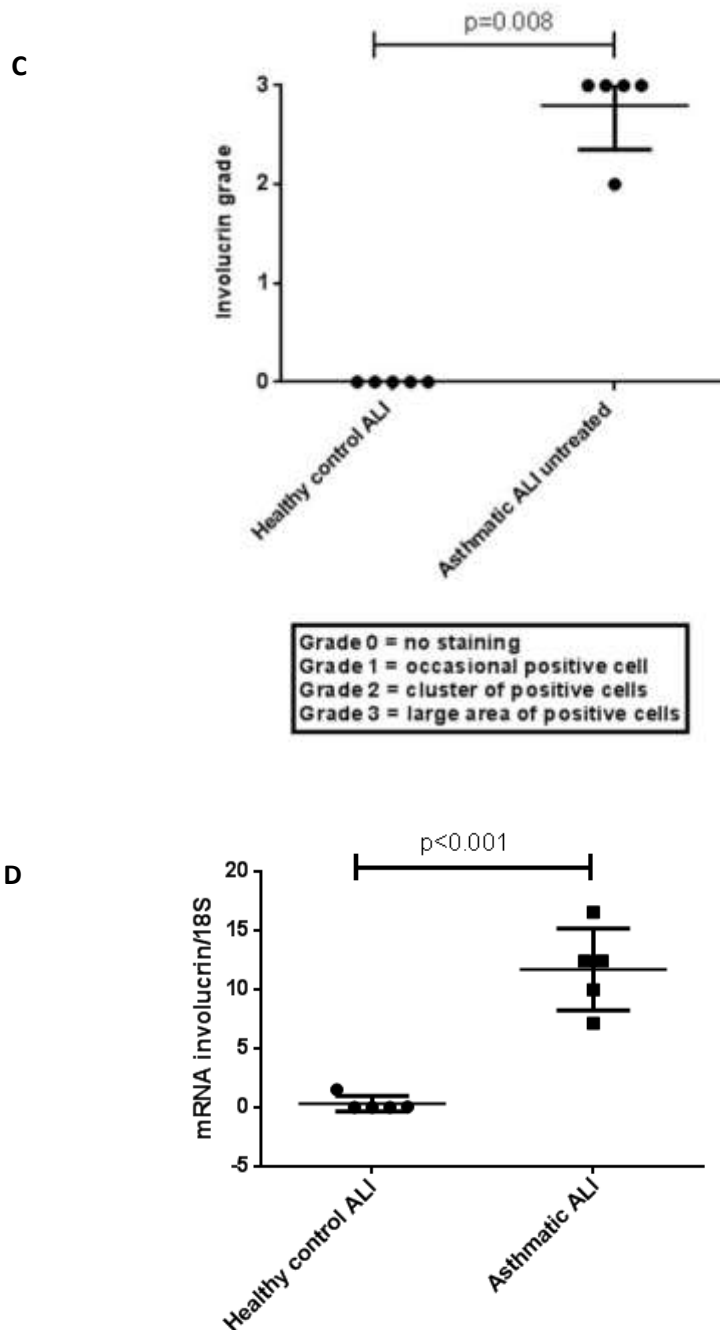


**Figure 5.14. MUC5AC+ expression in healthy control versus asthmatic ALI cultures.** MUC5AC+ staining for x400 magnification for A) untreated healthy control B) untreated asthmatic C) Dot-plot to show number of MUC5AC+ cells per mm length ALI for untreated healthy control ALI compared to asthmatic. p-value based on two-tailed unpaired t-test. D) Dot-plot to show mRNA expression for MUC5AC for healthy control compared to asthmatic ALI. p-value based on two-tailed unpaired t-test.

#### **5.6.4. Asthmatic ALI cultures are associated with higher levels of involucrin expression**

Asthmatic ALI cultures at both the protein (median [IQR] untreated grade 0[0-0] versus asthmatic grade 3[2-3],  $p=0.008$ . **Figure 5.15A-C**) and RNA levels (mean [SEM] healthy control 0.3[0.7] versus asthmatic 12[3],  $p<0.001$ . **Figure 5.15D**) were associated with higher levels of involucrin+ expression compared to healthy control ALI cultures. These data suggest that the novel observation made in this study that asthmatic biopsies are associated with an increased frequency of involucrin expression is maintained for asthmatic ALI cultures *in vitro*.

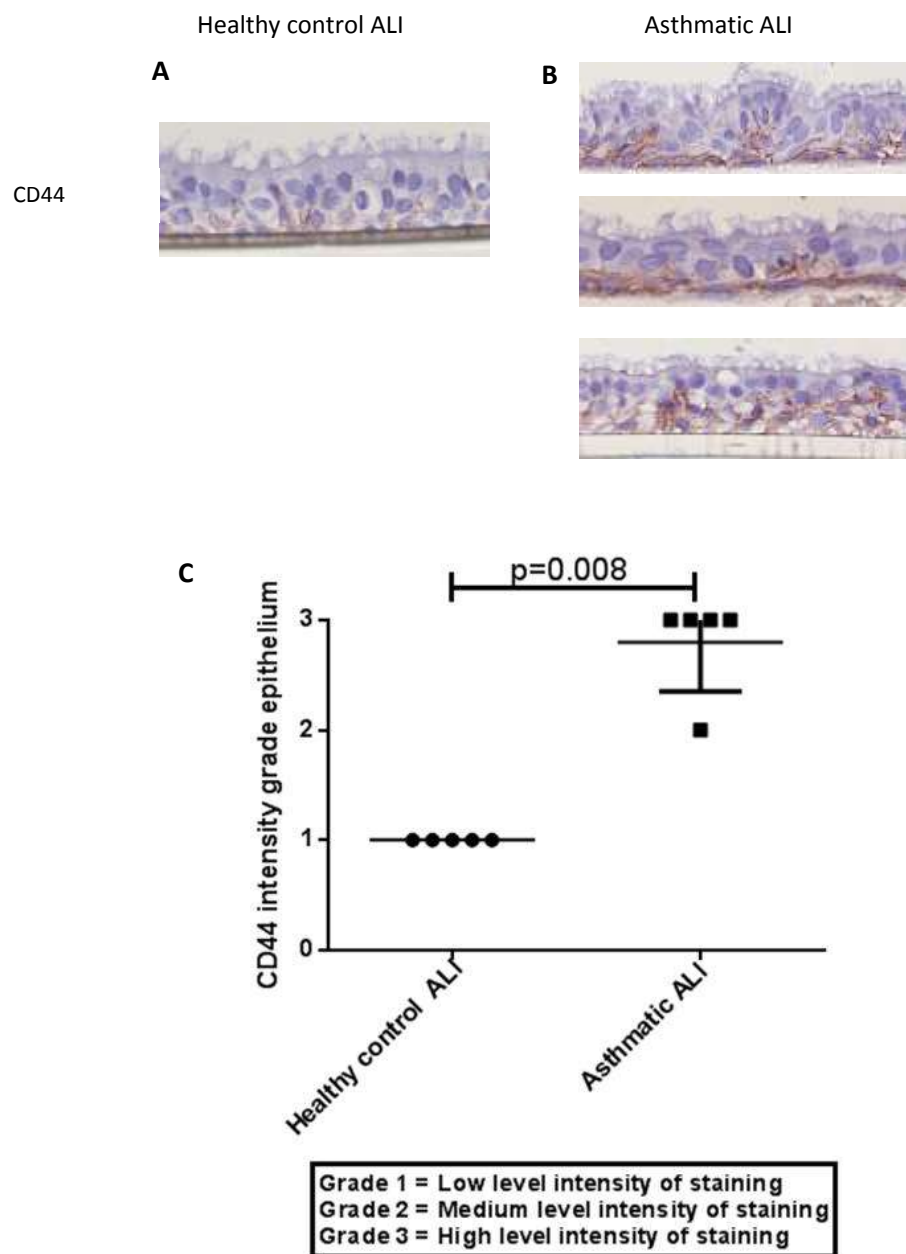




**Figure 5.15. Involucrin+ expression in healthy control versus asthmatic ALI cultures.** Involucrin+ staining for x400 magnification for A) untreated healthy control B) untreated asthmatic donor C) Dot blot to show quantitation of involucrin staining for healthy control compared to asthmatic cultures. p-value based on Mann Whitney test. D) Dot-plot to show mRNA expression for involucrin for healthy control compared to asthmatic ALI. p-value based on two-tailed unpaired t-test.

#### 5.6.4. Asthmatic ALI cultures are associated with higher levels of CD44 expression

Asthmatic ALI cultures were associated with higher levels of CD44 expression (median [IQR] healthy control 1[1-1] versus asthmatic grade 3[2-3],  $p<0.001$ . **Figure 5.16A-C**). These data suggests asthmatic cultures have evidence of increase cell migratory activity when compared to healthy control ALI cultures.

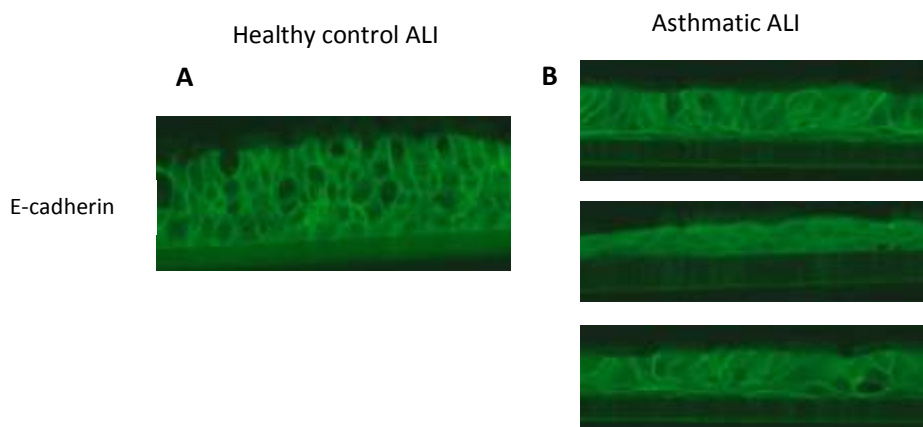


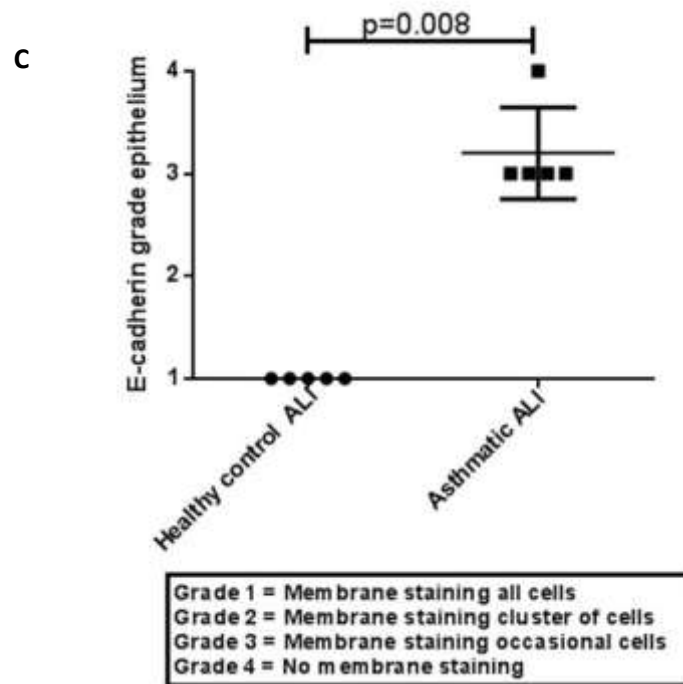
**Figure 5.16. CD44+ expression in healthy control versus asthmatic ALI cultures.** CD44+ staining for x200 magnification for A) untreated healthy control B) untreated asthmatic donor C) Dot blot to show quantitation of CD44 staining for healthy control compared to asthmatic cultures. p-value based on two-tailed unpaired t-test.

**5.6.6. Asthmatic ALI cultures have evidence of a disrupted structure as assessed by E-cadherin staining**

Asthmatic ALI cultures were associated with disrupted E-cadherin expression, as the staining was not as clearly defined around the membrane around the epithelial cells suggesting the epithelial barrier is disrupted in the asthmatic cultures (median [IQR] healthy control 1[1-1] versus asthmatic 4[3-4],  $p=0.008$ .

**Figure 5.17A, B, C).**





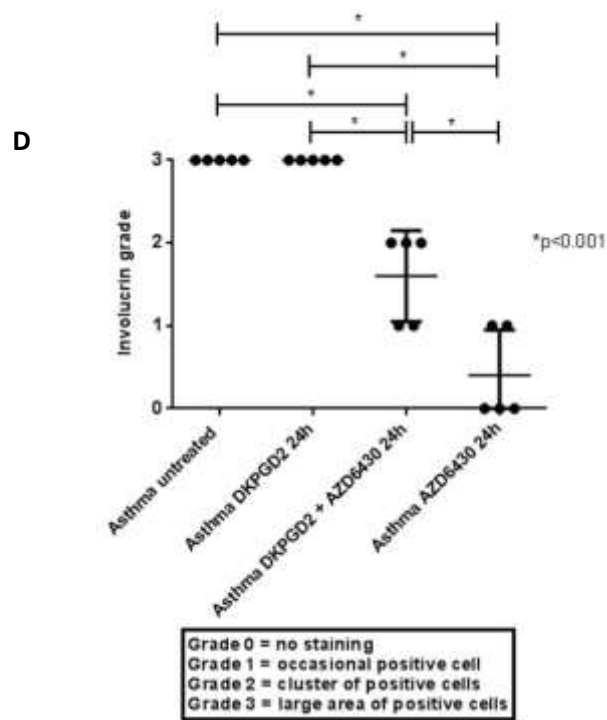
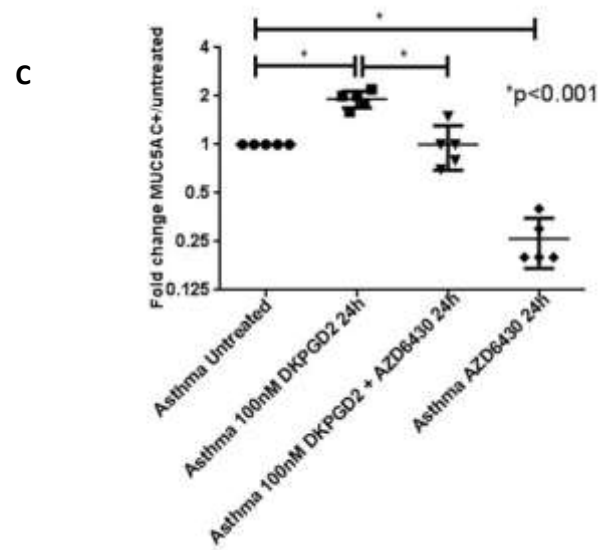
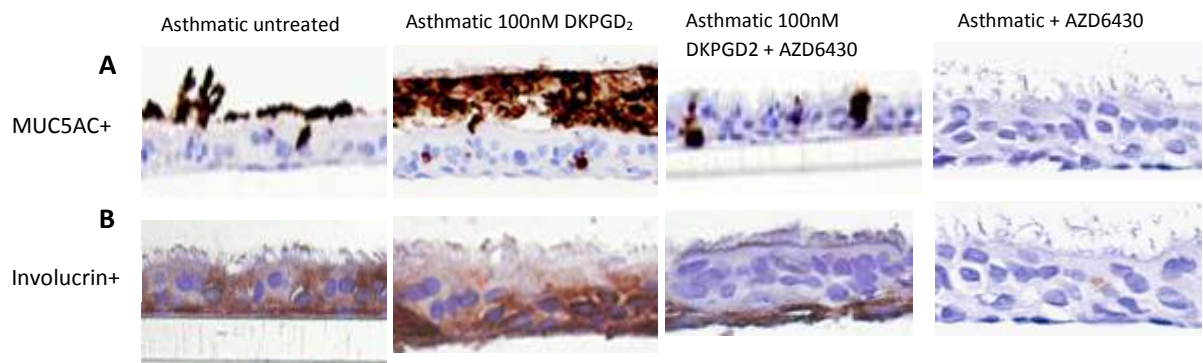
**Figure 5.17. E-cadherin+ expression in healthy control versus asthmatic ALI cultures.** E-cadherin+ staining for x200 magnification for A) untreated healthy control B) untreated asthmatic donor. C) Dot blot to show quantitation of E-cadherin staining for healthy control compared to asthmatic cultures. p-value based on Mann-Whitney test.

### 5.7. Effects of CRTh2 activation on asthmatic ALI cultures

To investigate the effects of CRTh2 activation on asthmatic ALI cultures that already have high basal levels of MUC5AC+ staining and involucrin staining, the same experiments as applied to healthy control ALI cultures were applied to asthmatic cultures. In addition, the effects of the CRTh2 antagonist alone, AZD6430, was investigated on the asthmatic ALI cultures, to establish if there were any effects of a CRTh2 antagonist on the basal levels of MUC5AC and involucrin expression. A significant increase in the number of MUC5AC+ cells was observed for the DK-PGD<sub>2</sub> treated asthmatic ALI cultures when compared to untreated (untreated 1[1-1]fold versus DK-PGD<sub>2</sub> 2[1.6-2.2]fold,  $p < 0.001$ . **Figure 5.18A, C**),

which was blocked with AZD6430 (DK-PGD<sub>2</sub> 2[1.3-2.8]fold versus DK-PGD<sub>2</sub> + AZD6430 1[0.4-2.6]fold,  $p < 0.001$ . **Figure 5.18A, C**). AZD6430 alone significantly decreased the number of MUC5AC<sup>+</sup> cells compared to untreated, DK-PGD<sub>2</sub> treated and DK-PGD<sub>2</sub> and AZD6430 (AZD6430 0.2[0.2-0.4]fold versus untreated 1[1-1]fold,  $p < 0.001$ ; versus DK-PGD<sub>2</sub> 2[1.3-2.8]fold,  $p < 0.001$ ; versus DK-PGD<sub>2</sub> + AZD6430 1[0.4-2.6]fold,  $p < 0.001$ . **Figure 5.18A, C**).

No increase in the area of involucrin staining was observed for the DK-PGD<sub>2</sub> treated asthmatic ALI cultures as the maximal response was already present in the untreated asthmatic cultures, so involucrin levels of expression were maintained with DK-PGD<sub>2</sub> (**Figure 5.18B, D**). The involucrin score was significantly reduced with DK-PGD<sub>2</sub> and AZD6430 when compared to untreated and DK-PGD<sub>2</sub> treated (median [IQR] DK-PGD<sub>2</sub>+AZD6430 2[1-2] versus untreated 3[3-3],  $p < 0.001$ ; DK-PGD<sub>2</sub> 3[3-3],  $p < 0.001$ . **Figure 5.18B, D**). AZD6430 alone significantly reduced the involucrin score when compared to untreated, DK-PGD<sub>2</sub> treated and DK-PGD<sub>2</sub>+AZD6430 treated cultures (AZD6430 0[0-1] versus untreated 3[3-3],  $p < 0.001$ ; DK-PGD<sub>2</sub> 3[3-3],  $p < 0.001$ ; DK-PGD<sub>2</sub>+AZD6430 2[1-2],  $p < 0.001$ . **Figure 5.18B, D**). These data suggests that CRTh2 activation on asthmatic ALI cultures still causes effects on mucus even with higher baseline levels of MUC5AC<sup>+</sup> cells. Interestingly, these data also show that AZD6430, a selective CRTh2 antagonist, reduced the baseline levels of mucus and involucrin expression. These data add further confidence that CRTh2 activation is important in mucus producing cell and squamous metaplasia effects in differentiated epithelium.



**Figure 5.18. Effects of CRTh2 activation on asthmatic ALI cultures.** MUC5AC+ staining x400 magnification for A) untreated asthmatic, asthmatic DK-PGD2 treated 24h, asthmatic DK-PGD2+AZD6430 24h. Involucrin+ staining x400 magnification for B) untreated asthmatic, asthmatic DK-PGD2 treated 24h, asthmatic DK-PGD2+AZD6430 24h. C) Dot-plot to show fold change of MUC5AC+ cells per mm2 over untreated for asthmatic ALI. p-value based on one way ANOVA, overall p-value  $p < 0.001$ , p-values shown in figure based on Tukey's post-hoc test. D) Dot blot to show quantitation of involucrin staining for cultures. p value based on Kruskal-Wallis. Overall p-value  $< 0.001$ ; p-values shown in figure based on Dunn's post hoc test.

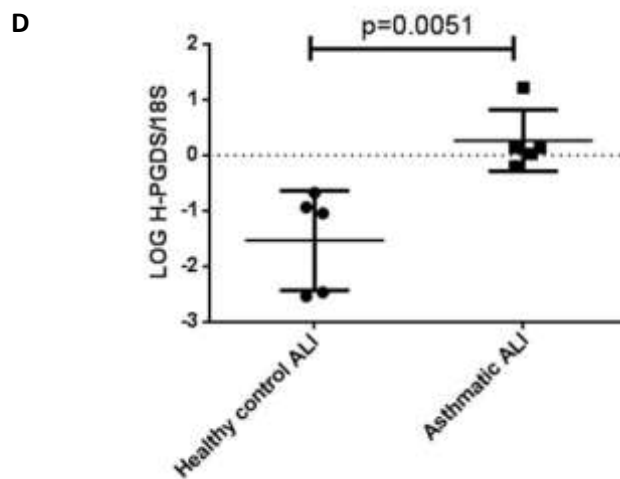
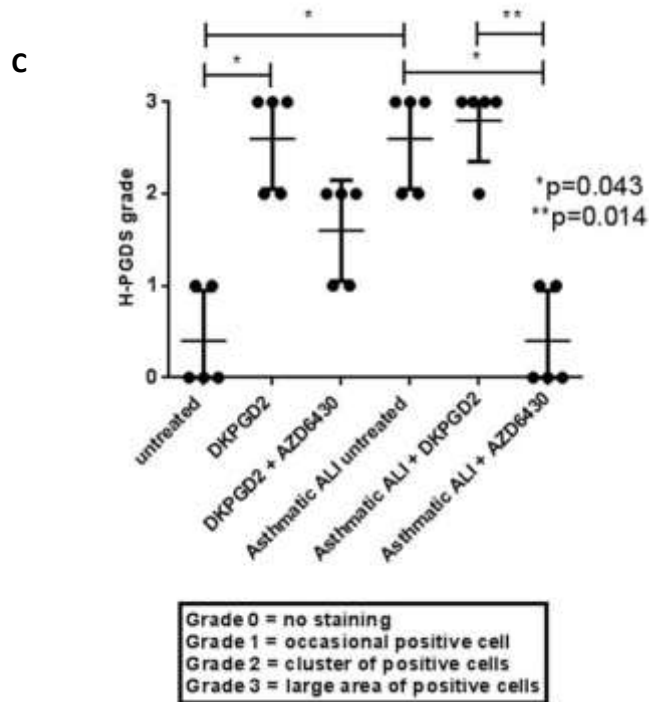
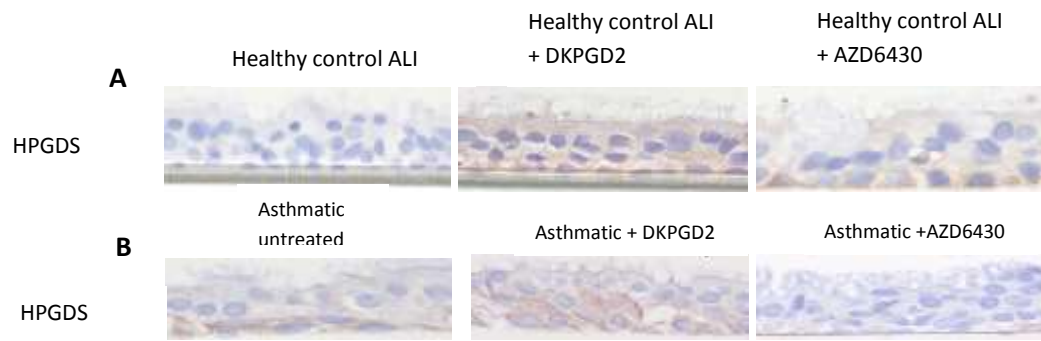
### **5.8. H-PGDS expression on ALI cultures**

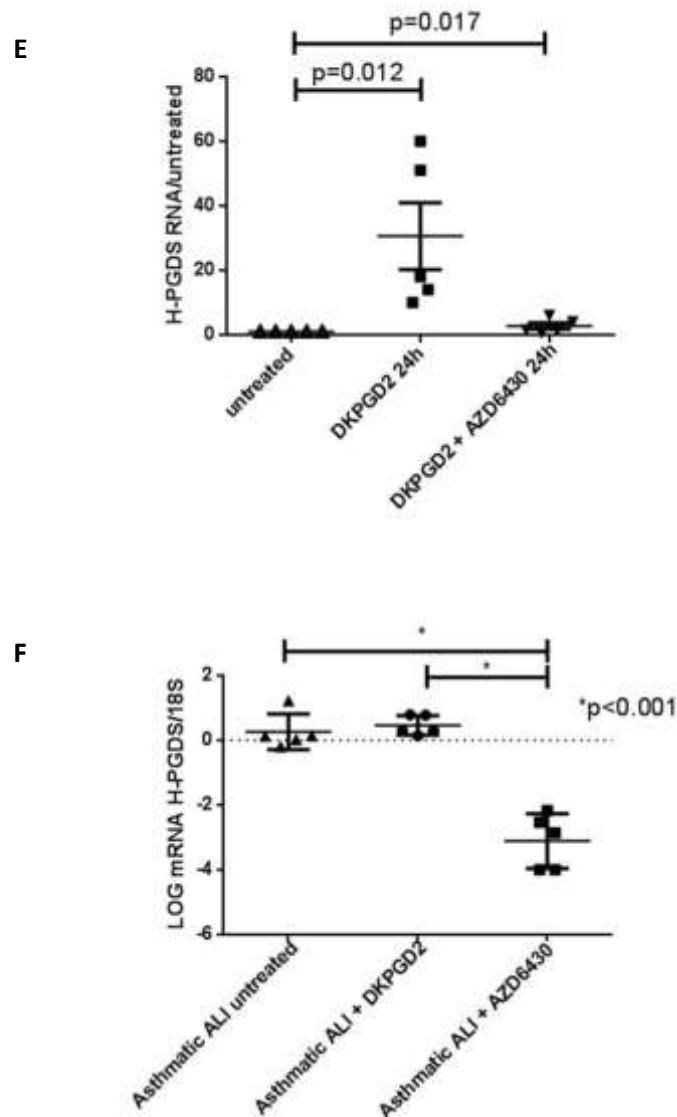
H-PGDS staining was observed within epithelial cells within bronchial biopsies (chapter 3.11). Immunostaining for H-PGDS was carried out on the ALI cultures to see if expression was present and whether its expression was affected by CRTh2 activation. The expression was quantified using the same criteria as used for the involucrin expression, grades range from grade 0 = no staining to grade 3 = large area of staining. Low levels or absence of expression was observed for the healthy control cultures (**Figure 5.19A, C**). There was a significant up-regulation of H-PGDS staining for the healthy control ALI cultures with DK-PGD<sub>2</sub> treatment (median [IQR] untreated 0[0-1] versus DK-PGD<sub>2</sub> 3[2-3],  $p = 0.043$ . **Figure 5.19A, C**), which was reduced with the addition of AZD6430, although not significantly (DK-PGD<sub>2</sub> 3[2-3] versus DK-PGD<sub>2</sub>+AZD6430 2[1-2],  $p = 0.06$ . **Figure 5.19A, C**). Interestingly, asthmatic untreated ALI cultures already had evidence of H-PGDS staining (**Figure 5.19B**). No significant increase in H-PGDS staining was observed for the asthmatic ALI cultures when incubated with DK-PGD<sub>2</sub> (**Figure 5.19B**). However, AZD6430 alone significantly reduced H-PGDS staining when it was incubated with the asthmatic cultures (asthmatic untreated 3[2-3] versus asthmatic untreated + AZD6430 0[0-1],  $p = 0.043$ . **Figure 5.19B, C**). Similarly to the protein



expression the mRNA expression for H-PGDS was up-regulated in the asthmatic ALI cultures when compared to the healthy control ALI cultures (mean [SEM] healthy control 0.09[0.04] versus asthmatic 4[3.1],  $p=0.0051$ , **Figure 5.19D**). In addition to the protein response for the healthy control cultures, DK-PGD<sub>2</sub> treatment significantly increased the mRNA expression of H-PGDS (**Figure 5.19E**). For the asthmatic ALI cultures DK-PGD<sub>2</sub> treatment did not induce H-PGDS mRNA levels, but AZD6430 alone did significantly reduce H-PGDS mRNA expression (**Figure 5.19F**). These data indicates that CRTh2 activation induces H-PGDS expression within healthy control ALI cultures and that asthmatic ALI cultures without DK-PGD<sub>2</sub> treatment already have elevated H-PGDS expression. Elevated H-PGDS levels were present in the DK-PGD<sub>2</sub> treated cultures and asthmatic cultures that were found to have increased numbers of MUC5AC<sup>+</sup> cells and involucrin expression. The level of H-PGDS expression correlated with MUC5AC<sup>+</sup> cell numbers ( $r_s=0.805$ ,  $p<0.001$ ), and involucrin score ( $r_s=0.932$ ,  $p<0.001$ ), demonstrating that elevated H-PGDS levels are present in ALI cultures with increased MUC5AC<sup>+</sup> cell numbers and involucrin staining. The presence of H-PGDS staining on the asthmatic ALI cultures provide more evidence that CRTh2 is activated in asthma.

To establish if the ALI cultures produced PGD<sub>2</sub>, apical washes taken during experiments were measured using an ELISA for PGD<sub>2</sub>. PGD<sub>2</sub> levels were found to be below detection limits of the ELISA. However, as the apical washes were not taken specifically to measure PGD<sub>2</sub> levels, MOX reactions which prevent the degradation of PGD<sub>2</sub>, were not carried out so apical washes may not be of sufficient quality to detect PGD<sub>2</sub>.





**Figure 5.19. H-PGDS staining for ALI cultures.** H-PGDS+ staining x400 magnification for A) untreated healthy control, DK-PGD2 treated 24h, DK-PGD2+AZD6430 24h. B) untreated asthmatic, untreated asthmatic DK-PGD2 treated 24h, asthmatic+AZD6430 24h. C) Dot blot to show quantitation of H-PGDS staining for cultures. p value based on Kruskal-Wallis. Overall p-value <0.001; p-values shown in figure based on Dunn's post hoc test. D) Dot-plot to show H-PGDS mRNA expression for healthy control compared to asthmatic ALI cultures. P-value based on two-tailed unpaired t-test. E) Dot-plot to show mRNA expression for H-PGDS on treated ALI cultures. p value based on one way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test. F) Dot-plot to show mRNA expression for H-PGDS on asthmatic ALI cultures. p value based on one way analysis of variance. Overall p-value <0.001; p-values shown in figure based on Tukey post hoc test.

***5.9. mRNA expression profiling of the effects of CRTh2 activation of healthy control ALI cultures for key genes identified as important in the regulation of mucus.***

Given the effects of CRTh2 activation on MUC5AC and involucrin expression, genes that have been reported to be involved in the development of mucus production and squamous metaplasia were investigated to establish if CRTh2 activation affected these pathways. Quantitative RT-PCR using Taqman probes was carried out for a number of different genes of interest.

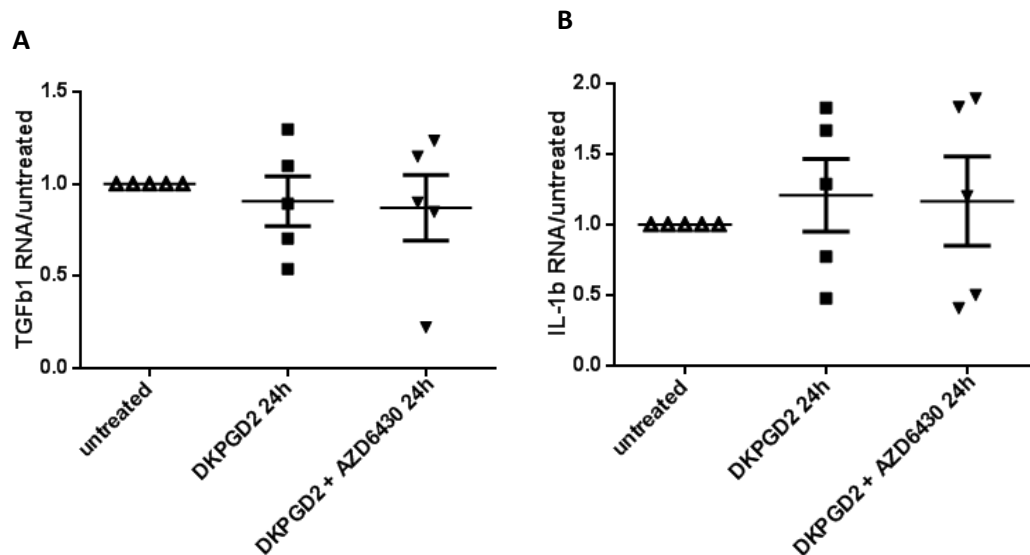
IL-13 has been shown to up-regulate MUC5AC (358), so this gene was profiled on untreated and treated ALI cultures. However, no mRNA expression was observed for untreated, DK-PGD<sub>2</sub> treated healthy control ALI cultures.

Another gene that has been implicated in both mucus cell hyperplasia and squamous metaplasia is TGFβ1 (433), and more specifically, the development of squamous metaplasia in ALI cultures (325). TGFβ1 mRNA expression was detected in the ALI cultures but no significant changes were observed with DK-PGD<sub>2</sub> or AZD6430 treatment (**Figure 5.20A**). However, as TGFβ1 is in an inactive form within cells, to fully establish if TGFβ1 could be affected by DK-PGD<sub>2</sub> within the ALI cultures an alternative assay such as an ELISA of apical washes from the ALI cultures, or a reporter gene assay would be useful.

IL-1β has been shown to up-regulate MUC5AC (352), and be involved in the development of squamous metaplasia (312). In addition, a CRTh2 antagonist has been shown to down-regulate IL-1β in an OVA-induced allergic cutaneous model (138). IL-1β mRNA expression was detected in the ALI cultures but no significant

changes were observed with DK-PGD<sub>2</sub> or AZD6430 treated cultures (**Figure 5.20B**).

These data suggest that selected key genes reported as being involved in the promotion of mucus cell hyperplasia are not up-regulated upon CRTh2 activation. A global gene array experiment would be useful to identify other genes that are regulated by CRTh2 activation in epithelial cells in a differentiated format.



**Figure 5.20. mRNA expression profiles for TGFβ1, IL-1β and PCNA on healthy control ALI cultures.** A) Dot-plot to show TGFβ1 mRNA expression for untreated , DK-PGD<sub>2</sub> treated and DK-PGD<sub>2</sub>+AZD6430 treated healthy control ALI cultures. B) Dot-plot to show IL-1β mRNA expression for untreated , DK-PGD<sub>2</sub> treated and DK-PGD<sub>2</sub>+AZD6430 treated healthy control ALI cultures.

## 5.10. Chapter 5 Summary

The aim of this work was to investigate the effects of CRTh2 activation in epithelial cells in a fully differentiated format that contains goblet cells and ciliated cells, therefore being more similar to *in vivo*. ALI cultures are currently considered to be the gold standard *in vitro* model for airway epithelial biology research (434), and genome-wide expression profiling of epithelial cells have reported that ALI cultures most closely resemble *in vivo* (417). It was hoped that this information could indicate whether CRTh2 activation would promote pro- or anti-remodelling processes. Using the CRTh2 selective agonist, DK-PGD<sub>2</sub> (104), and the CRTh2 selective antagonist AZD6430 ALI cultures were treated for 24, 48 or 72 hours. Using these reagents on well differentiated ALI cultures from a commercial source, the effects of CRTh2 activation were robustly demonstrated to up-regulate goblet cell numbers (MUC5AC) and squamous metaplasia (involucrin) features within healthy control ALI cultures. Mucus and involucrin read-outs were validated using IL-13 (423) and TGFβ1 (325), respectively. CRTh2 activation also caused changes to the morphology of some of the epithelial cells, in particular basal epithelial cells. Despite affecting the differentiation status of the ALI cultures and morphology of some of the epithelial cells, CRTh2 activation was found not to fully induce epithelial-mesenchyme transition within the ALI cultures. The changes to the differentiation status of healthy control ALI cultures in response to a CRTh2 selective agonist, and endogenous PGD<sub>2</sub> from mast cells, and those responses being blocked with a CRTh2 selective antagonist, provides strong evidence that CRTh2 activation has an impact on the differentiation status of bronchial epithelial cells in a differentiated format.

#### **5.10.1. CRTh2 activation increases the number of MUC5AC+ cells in ALI cultures**

The effects of CRTh2 activation on mucus producing cells as identified in the current study is novel data for human epithelial cells. However, in previous allergic mouse models a CRTh2 antagonist has been shown to decrease mucus metaplasia (134,367,435). The data from the current study therefore extends upon this animal data and shows similar effects on human epithelial cells. Mucus is required within the airways to trap pathogens and inhaled particles for clearance via the muco-ciliary escalator (83,436). The balance between clearance and production of mucus depends on optimum mucus quantities; however, in asthma, goblet cell hyperplasia (63,83,348) disrupts this balance contributing to the pathogenesis of asthma, demonstrating the importance in maintaining a balance of mucus production and clearance. Therefore, the abnormal activation of CRTh2 on epithelial cells has the potential to change mucus levels within the epithelium. Given the reported high levels of PGD<sub>2</sub> that have been reported in asthma (116), excessive activation of CRTh2 may contribute to increased mucus levels in asthma. Interestingly, asthmatic ALI cultures were found to be associated with higher levels of mucus producing cells and a CRTh2 antagonist was shown to significantly reduce the number of mucus producing cells. These data highlight that CRTh2 may already be activated within asthmatic epithelial cells, and a CRTh2 antagonist may be beneficial in asthma in reducing mucus levels.

### **5.10.2. CRTh2 activation induces squamous metaplasia in ALI cultures**

As well as affecting mucus metaplasia within the ALI cultures, CRTh2 activation also induced squamous metaplasia. In addition, areas of squamous metaplasia were associated with asthmatic ALI cultures. Two potential mechanisms have been described for the development of squamous metaplasia. One mechanism is through hyper-proliferation of epithelial cells, then when cell division ceases, the cells differentiate towards a squamous phenotype and form a cross linked cornified envelope (69). However, no evidence was found in this study that CRTh2 activation causes an increase in epithelial cell proliferation. Another mechanism hypothesised for the development of squamous metaplasia is that squamous metaplasia is a response to cell injury and is part of a repair mechanism. In a naphthalene induced epithelial injury mouse model, a squamous metaplastic response was described which was thought to be the epithelial cells trying to form a protective barrier (437). The hypothesis from this mouse model study was that neighbouring epithelial cells to the site of injury, infiltrated the injured area, differentiated and ultimately repaired the ciliated cells. The development of involucrin positive cells, indicating cells of a squamous phenotype, upon activation of CRTh2 may link with the hypothesis of the Park model, in that CRTh2 activation causes epithelial cell migration, which if cell injury is absent will be an abnormal response, with the cells ultimately developing squamous metaplasia. This hypothesis would also be supported by the up-regulation of CD44 expression, a migratory linked protein marker, upon CRTh2 activation and within asthmatic ALI cultures. An epithelial barrier containing squamous metaplastic cells for a sustained length of time could be detrimental to its required functions of protecting the underlying tissue. Studies have shown squamous ALI



cultures are more susceptible to viral infection (438), and that these cultures release more cytokines than non-squamous cultures, such as IL-1 $\beta$ , which in turn has been shown to influence remodelling events in underlying tissue (312). Therefore activation of CRTh2 in asthma may contribute to remodelling changes within the airways.

### **5.10.3. CRTh2 activation causes disruption to the tight junction proteins in ALI cultures**

Evidence that the changes to the ALI cultures with CRTh2 activation caused a disruption in the integrity of the epithelium was shown by investigating the expression of E-Cadherin and occludin. These are tight junction proteins which are involved in maintaining the integrity of the epithelium (277). Knockdown of E-cadherin has been shown to decrease the resistance of epithelial monolayers (281) even in the absence of differences in trans-epithelial resistance measurements (TER), therefore showing that E-cadherin is more sensitive to address changes in epithelial resistance compared to TER measurements. Activation of CRTh2 with DK-PGD<sub>2</sub> redistributed the expression of E-cadherin from being associated with membrane to being associated with occasional cells only. Expression was restored when AZD6430 was present. These findings indicate that CRTh2 activation may affect the integrity of the epithelium potentially increasing accessibility of allergens and viruses into the airway tissue (439-441), through increased permeability of the epithelial barrier. Indeed, epithelial disruption in tight junctions has been observed in asthma (319) and excessive mucosal permeability has been found in atopic individuals (442). In addition, in animal models of asthma, decreases in E-cadherin mediated cell-cell contacts have been observed during the asthmatic response

leading to an increase in epithelial permeability and AHR (443). For the tight junction protein, occludin, DK-PGD<sub>2</sub> treatment caused a re-distribution of the protein from uniformly expressed across the epithelial layers to bright staining being confined to the apical surface. This finding is consistent with the expression of tight junction proteins in areas of squamous metaplasia (427), adding to the evidence that CRTh2 activation induces a squamous metaplastic response.

#### **5.10.4. CRTh2 activation changes ALI cultures to be more ‘asthmatic’-like. CRTh2 activation responses in epithelial cells are pro-remodelling.**

To address if the changes observed for the ALI cultures upon CRTh2 activation changed the ALI culture to be more ‘asthmatic-like’, the morphology was investigated for ALI cultures derived from asthmatic subjects. Previous studies have confirmed that ALI cultures derived from asthmatic patients maintain the morphological imbalances initially observed in endobronchial biopsy (286). Significant differences were present for the asthmatic ALI cultures when compared to healthy control ALI cultures. Asthmatic cultures had a less organised structure compared to healthy control ALI cultures, with cells that had a more disorganised structure and mixtures of immature and mature cells throughout the epithelium, rather than the ordered immature cells at the bottom and mature cells at the top of the culture as seen for the healthy control cultures. Asthmatic ALI cultures had higher MUC5AC expression, which would be expected from current literature (286). Asthmatic ALI cultures also expressed high levels of involucrin; this finding has not been previously reported, and could suggest that asthmatic epithelial cells are inherently different to healthy control epithelial cells, and that this altered phenotype is maintained even in culture. These findings also indicate that CRTh2 activation

induces pro-remodelling responses in ALI cultures, as the DK-PGD<sub>2</sub> treated ALI cultures contained more of the features as identified with the asthmatic ALI cultures than with the untreated healthy control cultures.

CRTh2 activation of asthmatic ALI cultures was assessed to see if significant effects on mucus and squamous metaplasia could be observed with higher base-line levels of these markers. Significant increases in MUC5AC expression were observed for the asthmatic cultures upon CRTh2 activation, which could be blocked with AZD6430. However, the fold increase in MUC5AC positive cells in response to CRTh2 activation was not as high as observed with the healthy control cells (asthma 2-fold, healthy control 4-fold). In addition, AZD6430 treatment alone significantly reduced the number of MUC5AC positive cells even for the untreated asthmatic cultures, suggesting that CRTh2 may already be activated in asthmatic cultures. The levels of squamous metaplasia of the asthmatic ALI cultures were not increased with DK-PGD<sub>2</sub> treatment, however, the asthmatic ALI cultures already had maximum grading of staining so there was no scope for increase. However, AZD6430 did significantly decrease the area of involucrin staining of the untreated and DK-PGD<sub>2</sub> treated cultures. Again this finding suggests that CRTh2 may already be activated in asthmatic cultures.

#### **5.10.5. CRTh2 activation induces the expression of H-PGDS within epithelial cells. Epithelial cells from asthmatics express H-PGDS.**

To try to address if the asthmatic ALI cultures contained evidence of elevated ligand levels capable of activating CRTh2 in the absence of exogenously added ligand, H-PGDS levels within the ALI cultures were assessed. Healthy

control ALI cultures contained little if any H-PGDS expression, however DK-PGD<sub>2</sub> elevated the H-PGDS levels at both the mRNA and protein level, suggesting that that addition of endogenous ligand caused an up-regulation of the enzyme involved in producing PGD<sub>2</sub>. When AZD6430 was added the levels of H-PGDS was reduced in comparison to DK-PGD<sub>2</sub> treatment alone, but only significantly at the mRNA level. Interestingly, the untreated asthmatic ALI cultures expressed H-PGDS even in the absence of endogenously added DK-PGD<sub>2</sub> at both the mRNA and protein level. This data could suggest that asthmatic cultures are more activated and therefore more ready to produce endogenous PGD<sub>2</sub>, or, it could be that previous exposure to elevated PGD<sub>2</sub> levels, from mast cells and T-cells within the airway milieu, have up-regulated the H-PGDS levels and this has been maintained in culture. Irrespective of the mechanism, this data provides evidence to suggest that CRTh2 activation may be present in untreated asthmatic ALI cultures. In turn, this activation may lead to some of the aberrant changes that have been identified within this study for the asthmatic ALI cultures, such as elevated MUC5AC levels and the presence of squamous metaplasia.

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# **Chapter 6**

## **Discussion**

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## **6.1. Summary of main novel findings**

- Using a well validated antibody, CRTh2 was found to be expressed on bronchial biopsies from healthy control and asthmatic subjects.
- CRTh2 expression on inflammatory cells within the sub-mucosa of healthy control and asthmatic bronchial biopsies was identified on a sub-population of T-cells, eosinophils and mast cells. No CRTh2 expression was found on neutrophils within the sub-mucosa of the biopsies. The number of CRTh2 positive T-cells was significantly increased in the mild, moderate and severe asthmatic biopsies compared to the healthy control biopsies. The number of CRTh2 eosinophils was found to be significantly increased in moderate asthmatics biopsies compared to healthy control biopsies.
- CRTh2 expression was identified on bronchial epithelial cells within the healthy control and asthmatic bronchial biopsies. CRTh2 expression was present on basal epithelial cells and columnar epithelial cells but was not expressed on goblet cells. The number of CRTh2 positive epithelial cells was significantly reduced in the moderate-severe asthmatic biopsies compared to the healthy control biopsies.
- Areas of squamous metaplasia were significantly elevated in the moderate-severe asthmatic biopsies compared to the healthy control biopsies as assessed by histology of epithelial cells and with the squamous phenotype marker involucrin.
- CRTh2 expression was not present on epithelial cells in areas of squamous metaplasia and CRTh2 expression negatively correlated with a squamous metaplastic histology and the presence of involucrin staining.

- CRTh2 staining was absent in airway smooth muscle bundle tissue.
- H-PGDS, an enzyme involved in the synthesis of PGD<sub>2</sub>, was present on a sub-population of inflammatory cells within the sub-mucosa and within the epithelium, and the numbers of these cells were significantly increased in asthmatic biopsies compared to healthy control biopsies.
- Granular staining for H-PGDS was present within epithelial cells themselves within asthmatic biopsies.
- CRTh2 was expressed on a sub-population of epithelial cells from bronchial brushings of healthy control and asthmatic patients, demonstrating that CRTh2 expression is maintained by epithelial cells in culture in both submerged and ALI culture conditions.
- The number of CRTh2 positive bronchial epithelial cells in culture was significantly less in cells from moderate-severe asthmatics compared to healthy controls.
- CRTh2 was expressed on a sub-population of human lung mast cells in culture.
- Activation of CRTh2 on bronchial epithelial cells in culture elicited a calcium response and induced cell migration. The amount of migration induced for asthmatic bronchial epithelial cells was significantly lower than for healthy control epithelial cells.
- Activation of CRTh2 on bronchial epithelial cells in an ALI format induced goblet cell hyperplasia and squamous metaplasia.
- Activation of CRTh2 within ALI cultures via mast cells induced similar responses to those observed with a CRTh2 selective agonist.

- Activation of CRTh2 induced a number of similar phenotypes to those of asthmatic ALI cultures.
- Activation of CRTh2 induced expression of H-PGDS within ALI cultures.
- ALI cultures from asthmatic patients had significantly higher expression levels of MUC5AC, involucrin and H-PGDS compared to ALI cultures from healthy controls.
- A CRTh2 antagonist reduced the number of mucus producing cells and levels of squamous metaplasia in ALI cultures from asthmatic patients.

## **6.2. Summary of other findings**

- Phenotyping of T-cells, eosinophils and mast cells within the sub-mucosa across healthy control and asthmatic biopsies identified that T-cells were significantly elevated within moderate-severe asthmatic biopsies compared to healthy control biopsies.
- Numbers of epithelial cell markers for basal epithelial cells (p63) and mature epithelial cells (cytokeratin 7) were not significantly altered in asthmatic biopsies compared to healthy control biopsies. The area of goblet cells identified by MUC5AC, was significantly increased in the asthmatic biopsies compared to the healthy control biopsies.



### **6.3. CRTh2 is expressed on inflammatory cells within the submucosa and numbers of positive cells are increased in asthma – importance of findings**

Within the current study CRTh2 expression was observed on a sub-population of T-cells, eosinophils and mast cells within the sub-mucosa of the bronchial biopsies from healthy control and asthmatic subjects. Previous reports have demonstrated CRTh2 expression on peripheral blood derived T-cells and eosinophils (103,366), and T-cells, eosinophils and mast cells within nasal tissue (254). However, this study is the first to describe CRTh2 positive T-cells, eosinophils and mast cells within bronchial biopsies in patients with asthma.

Previous studies have identified an increase in the number of CRTh2 positive T-cells in nasal tissue of allergic subjects (254) and in the BAL of asthmatics (116,143). The current study is the first to identify a significant increase in the number of CRTh2 positive T-cells within asthmatic subjects compared to healthy control, with the majority of the T-cells being a CD4 positive phenotype. Innate lymphoid type 2 (ILC2) cells are also likely to contribute to the overall T-cell population as they express the CD3 marker and have been shown to express CRTh2 (227,228). Currently no selective ILC2 markers have been found so it is not possible to co-localise CRTh2 with ILC2 cells. These findings are significant as activation of CRTh2 on peripheral blood T-cells has been shown to cause the release of IL-4, IL-5 and IL-13 and a delay in T-cell apoptosis (105,106). This delay in apoptosis in response to CRTh2 activation may account for the increased T-cells being found within the sub-mucosa. The role of CRTh2 in driving allergic inflammation is supported by observations in asthma and allergic mouse models where CRTh2 antagonists reduced overall inflammation and the levels of a number

of cytokines including IL-4, IL-13 and IL-1 $\beta$  (134,138). It therefore seems likely that activation of CRTh2 positive T-cells within the sub-mucosa of the airways plays a role in the pathogenesis of asthma.

The observation that CRTh2 positive eosinophils were also found to be significantly elevated in moderate asthmatics may in part explain the clinical efficacy of CRTh2 antagonists in reducing sputum eosinophil numbers in allergen challenged steroid-naïve asthmatic subjects (123) and in allergic rhinitis patients (127). Pre-clinical evidence in allergic animal models also showed that CRTh2 antagonists significantly decreased eosinophilia (135,136), suggesting that CRTh2 activation of eosinophils could play a significant role in asthma and allergy via the recruitment of eosinophils. Indeed, *in vivo* findings are supported by a number of *in vitro* studies demonstrating that CRTh2 on activation on eosinophils elicits chemotaxis which offers an explanation for the increased number of eosinophils observed in the moderate asthmatic airways (126,169,173,208,210).

CRTh2 expression has been demonstrated on mast cells within human nasal tissue (254) and on murine bone marrow-derived mast cells (BMMC) (247). The current study is the first however, to demonstrate CRTh2 expression on human lung mast cells both within bronchial biopsies and on cells isolated from lung tissue. Mast cells have previously been linked to CRTh2 because they are the major producer of the CRTh2 ligand, PGD<sub>2</sub> (163,228). The levels of PGD<sub>2</sub> have been found to be elevated in asthmatic patients (110-112,116,444). The current study demonstrates that CRTh2 positive mast cells are also present within the sub-mucosa of asthmatic airways, providing evidence for a potential ligand-receptor dual role on mast cells contributing to pathogenesis in asthma. Balzar et al (2011) reported that

mast cells within the sub-mucosa of severe asthmatic patients were predominantly of a MC<sub>TC</sub> phenotype (112). In the current study, CRTh2 expression was found on a sub-set of mast cells within the sub-mucosa of both healthy control and asthmatic bronchial tissue and further studies need to address if CRTh2 expression is associated with a particular mast cell phenotype.

The findings from the current study show that the numbers of CRTh2 positive inflammatory cells (mostly T-cells) are significantly increased in the bronchial sub-mucosa of asthmatic patients. These observations are of clinical relevance as it demonstrates that CRTh2 antagonists have the potential to influence inflammatory cell accumulation within asthmatic airways of severe asthmatic patients. In addition, this infiltration of CRTh2 positive cells may also aid in patient selection for CRTh2 antagonist therapies.

#### **6.4. CRTh2 is expressed on bronchial epithelial cells and its activation has functional consequences – importance of findings**

CRTh2 has previously been described on normal human bronchial epithelial cells (255) and within COPD lung tissue (128), but limited information is available about the expression of CRTh2 on bronchial epithelial cells of asthmatic bronchial biopsies, and cultured epithelial cells from asthmatic bronchial brushings. This study is the first to demonstrate that in both biopsies and cultured bronchial epithelial cells the number of CRTh2 positive cells in asthmatic patients is significantly lower than for healthy controls. CRTh2 was found to have intracellular and extracellular expression on epithelial cells, similar to previous studies for this receptor (254,255). The expression of CRTh2 on bronchial epithelial cells suggests

that CRTh2 activation on these cells could have an impact in the pathogenesis of asthma, which most likely is mediated through cell surface receptors. However, intracellular receptor activation may also occur as has been reported for other GPCRs (445), and its functional importance requires further study.

The expression of CRTh2 on bronchial epithelial cells directed further investigation into the functional role of CRTh2 activation on cultured bronchial epithelial cells from healthy control and asthmatic patients. CRTh2 activation by PGD<sub>2</sub> has been shown to cause cell migration in Th2 cells, basophils and eosinophils (104,178). In the current study CRTh2 activation with the CRTh2 selective agonist, DK-PGD<sub>2</sub> (168,169) also caused cell migration of both asthmatic and healthy bronchial epithelial cells, an effect that was blocked with a CRTh2 selective antagonist. However, although the antagonist was highly selective it has been shown to have low affinity for other receptors and enzymes such as the TP receptor, thus off target effects cannot be fully excluded and these may need to be addressed in future studies. Migration was more pronounced in the healthy control cells when compared to the asthmatic cells, possibly due to the difference in cell surface receptor expression. However, a previous study demonstrated that the time to repair mechanical wounds was significantly decreased in cells from children with asthma when compared to healthy controls (306) suggesting the possibility that asthmatic cells may have abnormal migratory ability. A limitation of this study was that a dose-response curve was not fully explored for the migration response due in part, to limitations of cell number. However, increasing concentrations of DK-PGD<sub>2</sub> up to 1µM, revealed that maximal migratory responses were obtained. Future migration studies comparing different concentrations of DK-PGD<sub>2</sub> with other CRTh2 agonists

such as 15(R)-15-methyl-PGD<sub>2</sub> may help in uncovering differences between asthmatic and healthy states.

The functional role of CRTh2 on bronchial epithelial cells was also shown using a fluorescence based assay where activation with DK-PGD<sub>2</sub> caused an increase in intracellular calcium in healthy control bronchial epithelial cells. This functional response has also been identified for T-cells and basophils (103,170). Considering the differences observed in CRTh2 expression between healthy control and asthmatic epithelial cells a comparison of this response with asthmatic epithelial cells would be of interest as it may reveal differences in CRTh2 signalling in disease.

These findings demonstrate for the first time the existence of functional CRTh2 on bronchial epithelial cells.

#### **6.5. Areas of bronchial epithelial squamous metaplasia are more frequent in moderate-severe asthmatic patients – importance of findings**

Areas of squamous metaplasia were found to be more frequent in moderate and severe asthmatic biopsies. This information is of clinical interest as squamous metaplasia has been found to correlate with severity of airway obstruction (446). Also, *in vitro* experiments have demonstrated that epithelium of a squamous phenotype is more susceptible to viral infection due to a more disrupted barrier (447). The mechanisms driving bronchial epithelial squamous metaplasia are still unknown. In this study squamous metaplasia was increased in the moderate-severe asthmatic cohort irrespective of smoking status between the groups. This indicates that contrary to previous suggestions that squamous metaplasia is related to smoking status (448), other factors can contribute to the induction of this epithelial

phenotype. Indeed, a number of studies have hypothesised that squamous metaplasia is a response to injury (68,310,423), and so data from the current study may indicate that in moderate-severe asthmatic patients there is an increased frequency of active repair processes. Evidence from the current study that suggests that repair processes are active within asthmatic epithelium, are the re-organisation of the tight-junctional proteins, which indicates loosening of epithelial-epithelial contacts, a reported step within epithelial repair processes (426,449). These findings would fit with the suggestion that the epithelium in asthma is locked in a process of aberrant repair (287,295,297,314). Studies that help with the understanding of the alterations of the epithelium in asthma are needed as it may provide additional target features to consider in the development of new asthma therapies targeting pro-remodelling features.

#### **6.6. CRTh2 activation induces pro-remodelling responses – importance of findings**

The existence of functional CRTh2 on epithelium is supported by the present study and preclinical mouse studies where CRTh2 antagonists have been found to reduce mucus cell metaplasia and epithelial cell hyperplasia in response to either cigarette smoke (367) or allergen stimulation (435). In addition, in an allergen-sensitised skin model a CRTh2 antagonist down-regulated gene expression of a family of epithelial differentiation genes (138). Using the ALI culture system that closely mimics the *in vivo* environment (285) the current study provided additional evidence for a role of CRTh2 in driving phenotype changes of epithelial cells by showing that DK-PGD<sub>2</sub> treatment and mast cells co-cultured with ALI cultures induced not only increased goblet cell number when exposed acutely, but also

increased the area of involucrin expression in the epithelium with more chronic treatment. In addition, activation of CRTh2 disrupted the tight junction proteins within the ALI cultures. These aspects of epithelial change were also identified within ALI cultures derived from asthmatic cultures, demonstrating that CRTh2 activation promotes an ‘asthmatic-like’ phenotype. In addition, the effects of CRTh2 activation on ALI cultures derived from asthmatic patients indicated that a significant increase in the number of mucus producing cells could still be achieved despite the higher basal levels of these cells that were associated with the asthmatic cultures. Interestingly, a CRTh2 antagonist incubated alone with the asthmatic ALI culture significantly reduced the number of mucus producing cells. In addition, a CRTh2 antagonist reduced the area of squamous metaplasia within the asthmatic ALI culture. These findings are of interest as they show that a CRTh2 antagonist could influence pro-remodelling features within asthmatic airways. More importantly it suggests that these ALI cultures from asthmatic patients may produce the CRTh2 ligand, PGD<sub>2</sub> under basal conditions thus explaining the therapeutic benefit of the CRTh2 antagonist. This hypothesis is interesting and will be addressed in future studies. This information could also influence the design of future CRTh2 antagonist studies, which could incorporate end-points such as mucus production.

#### **6.7. H-PGDS expression is elevated in asthmatic biopsies – importance of findings**

Findings from the current study provided novel evidence that cells that express H-PGDS, a terminal enzyme involved in the production of the CRTh2 agonist PGD<sub>2</sub>, are found within the sub-mucosa of human bronchial biopsies. In

addition, H-PGDS positive cells infiltrating into the epithelium were observed for asthmatic biopsies. A sub-set analysis of the inflammatory phenotype of cells expressing H-PGDS revealed that a sub-set of mast cells and T-cells expressed H-PGDS, however, additional H-PGDS positive cells that were not mast cells or T-cells were also present. Previous studies have described the expression of H-PGDS predominantly in mast cells (163), with T-cells and eosinophils also being found to express H-PGDS (147,161). Additional cells that have been found to express H-PGDS are basophils (162), activated macrophages (450) and endothelial cells (451,452). Therefore one can speculate that the H-PGDS positive cells that did not co-localise with mast cells and T-cells markers in the current study may likely be one of these inflammatory cell types.

In addition to H-PGDS being expressed on inflammatory cells, granular staining was found within bronchial epithelial cells, which was more evident for the asthmatic biopsies. Interestingly, identification of newly synthesised  $\text{PGD}_2$ , using an EicosaCell assay (453) within eosinophils, reported a similar distribution of staining as was seen within the asthmatic bronchial epithelial cells (147), with a punctate cytoplasmic pattern proximate to but separate from the nucleus. H-PGDS expression has been described on epithelial cells from the retina, gut, skin and nasal polyps (165,405,454) but this is the first study to describe H-PGDS expression on bronchial epithelial cells within biopsies. Investigation of prostaglandin synthases (H-PGDS and L-PGDS) expression for the growth of ALI cultures from rat tracheal bronchial epithelial cells demonstrated that a co-ordinated expression of both synthases was required for the differentiation of the ALI culture (455,456). In addition, airway epithelial cells have been shown to express other proteins involved



in arachidonic acid metabolism (455-457). The presence of more evident epithelial H-PGDS staining for the asthmatic biopsies is of interest. A previous study investigating wound healing in the skin identified that H-PGDS expression was elevated in the later stages of wound healing (165), which could further link with the hypothesis that asthmatic epithelium is associated with activated repair processes. Expression of H-PGDS within bronchial epithelial cells might suggest that they are capable of producing PGD<sub>2</sub>. Indeed in a previous study, a significant correlation was found between epithelial cell number and PGD<sub>2</sub> levels recovered in lavage fluid after dry air challenge in dogs (166), a study supporting the idea that epithelial cells can produce PGD<sub>2</sub> and may be an important source of PGD<sub>2</sub> within asthmatic airways. The fact that CRTh2 expression and function have also been found on bronchial epithelial cells suggests that there is the potential role for an autocrine action of PGD<sub>2</sub> in CRTh2 function on epithelial cells. Indeed, CRTh2 expressing eosinophils and T-cells have been found to produce PGD<sub>2</sub> (105,147) and for these cells an autocrine loop for CRTh2 activation has been proposed. A limitation of the current study was that production of PGD<sub>2</sub> from epithelial cells could not be determined. Cell types have been found to express H-PGDS but not produce PGD<sub>2</sub> (458), so it is therefore important to ascertain if bronchial epithelial cells can release PGD<sub>2</sub>. In the current study, PGD<sub>2</sub> levels were assessed for apical washes from ALI cultures, but PGD<sub>2</sub> levels were too low for detection via ELISA. However, optimisation of the method of collecting the apical washes is still needed, as the method used for the current study was a single 30 seconds apical wash, in order to prevent damage to the ALI culture and preserve the histology of the culture as this was the primary read-out. In addition chemical reactions were not performed on the

apical washes to prevent the degradation of PGD<sub>2</sub>. Further studies are therefore needed to optimise collection of both basal and apical washes from ALI cultures to validate if bronchial epithelial cells produce PGD<sub>2</sub>, and whether this is different in health or disease.

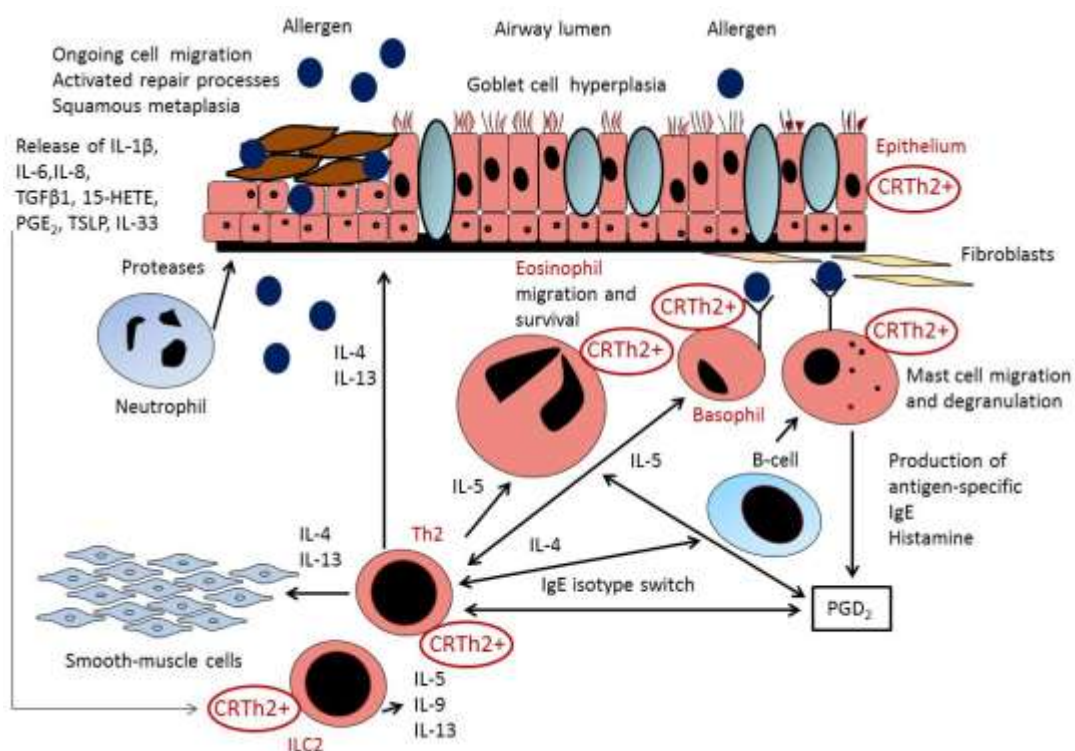
For healthy control ALI cultures, CRTh2 activation via DK-PGD<sub>2</sub> induced H-PGDS expression, which was blocked with a CRTh2 antagonist. As CRTh2 activation induced mucus hyperplasia, and squamous metaplasia this finding could suggest that this phenotype is linked with H-PGDS expression. Indeed, a significant correlation was observed for H-PGDS expression with the number of mucus producing cells and squamous metaplasia grade for the ALI cultures. This hypothesis would also be supported with the finding that ALI cultures derived from asthmatic patients that were associated with elevated mucus cell numbers and areas of squamous metaplasia, also expressed H-PGDS. These findings are intriguing and suggest that CRTh2 could be continuously activated within asthmatic ALI cultures through an autocrine loop, leading to elevated mucus levels and areas of squamous metaplasia. This hypothesis would be further supported by the observations described above that a CRTh2 antagonist significantly reduced the number of mucus-producing cells and areas of squamous metaplasia within asthmatic ALI cultures. These findings are important as they provide evidence that in addition to PGD<sub>2</sub> release from inflammatory cells within the airways there could be an additional source of PGD<sub>2</sub>. PGD<sub>2</sub> released from both inflammatory and epithelial cells could act in an autocrine manner to further drive accumulation of inflammatory cells into the asthmatic airways and also induce pro-remodelling changes within the epithelium.

## 6.8. Conclusions

This study has contributed to a better understanding of the expression and function of CRTh2 within asthmatic airways;

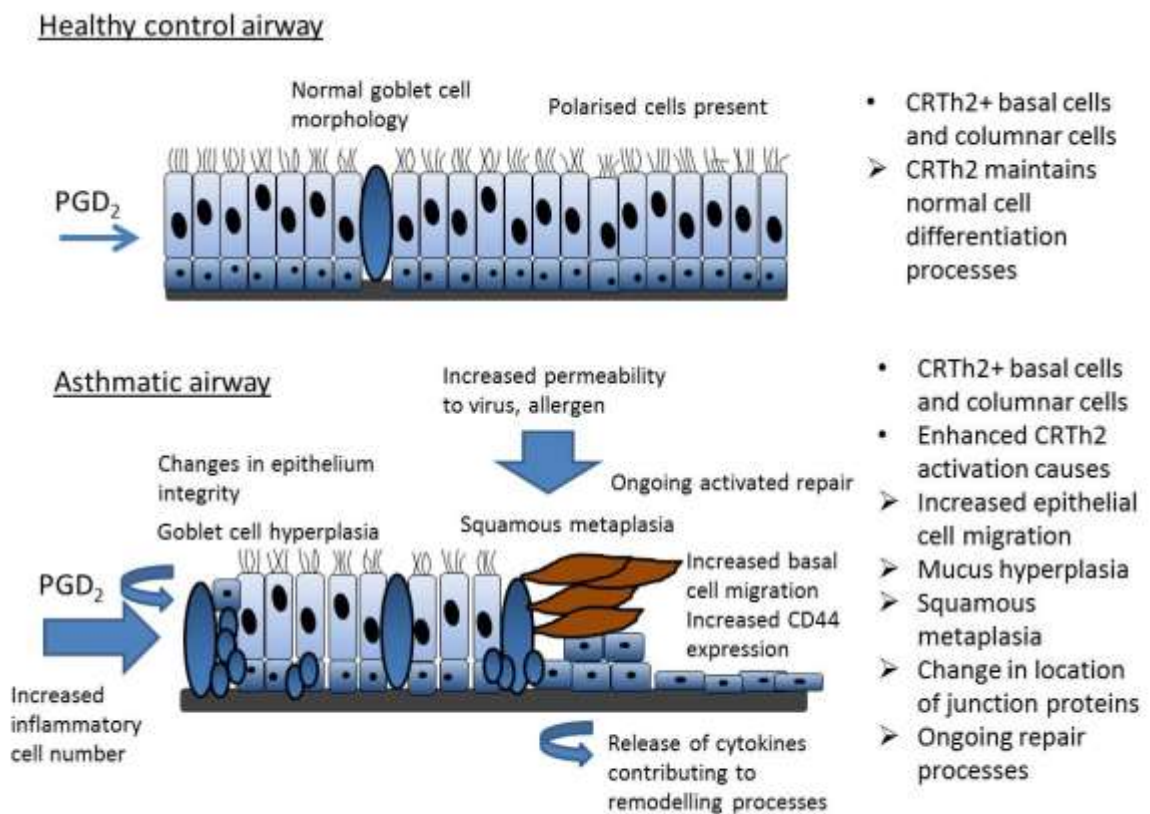
1. CRTh2 is present on a number of effector cells within the airways of asthmatics including inflammatory cells and structural cells. These cells are highlighted in red in **Figure 6.1**, which demonstrates CRTh2 expression on inflammatory cells and structural cells of asthmatic airways. If this figure is compared to that of **Figure 1.4**, the novel findings of the current study can be observed.
2. There is a differential in expression of CRTh2 in health and asthma.
3. The number of CRTh2 inflammatory cells is significantly increased in the airways of severe asthmatic patients.
4. CRTh2 is expressed on a sub-set of T-cells, eosinophils and mast cells within the airways.
5. The largest contribution of CRTh2 positive inflammatory cells within the sub-mucosa of severe asthmatic airways is the T-cell.
6. CRTh2 is expressed on bronchial epithelial cells within the airways in health and disease.
7. The number of CRTh2 positive bronchial epithelial cells is significantly reduced within moderate-severe asthmatic biopsies, which has been validated both *in vivo* and *in vitro*.

8. CRTh2 expression on bronchial epithelial cells is linked to the phenotype of cell, with epithelial cells of a squamous metaplastic phenotype losing the expression of CRTh2.
9. CRTh2 activation on bronchial epithelial cells induces pro-remodelling responses, changing the overall structure of the epithelial barrier to be more asthmatic-like. These conclusions have been validated using a CRTh2 selective agonist and antagonist and with endogenous agonist released from mast cells.
10. H-PGDS, an enzyme involved in the production of PGD<sub>2</sub> is elevated within asthmatic epithelium, suggesting a potential autocrine mechanism for CRTh2 activation on bronchial epithelial cells.



**Figure 6.1.** Summary of findings for the expression of CRTh2 on effector cells within asthmatic airways.

The findings from this study suggest that CRTh2 may have a homeostatic role on bronchial epithelial cells, maintaining the overall differentiation status and turn-over of the epithelial barrier. CRTh2 is expressed on both the basal epithelial cells and the columnar epithelial cells and potentially on migrating epithelial cells moving along the basement membrane. These cell types are essential for epithelial repair and maintenance (288) and a proposed mechanism for repair is that migratory epithelial cells infiltrate into areas of damage and then differentiate into epithelial cells of a squamous or goblet cell phenotype (275,288,399,437). The expression of CRTh2 on basal and columnar epithelial cells and the findings that CRTh2 activation can cause many of the repair step processes described above could indicate that this receptor plays a key role in the maintenance and repair of the epithelial barrier. Within the asthmatic airways, where there are elevated levels of PGD<sub>2</sub> originating from inflammatory cells (110,111,116), and from the present evidence also potentially from bronchial epithelial cells, it is likely that CRTh2 activation accelerates these functional responses on epithelial cells, causing an aberrant mucosal barrier phenotype. CRTh2 positive basal epithelial cells with increased migratory activity even in the absence of epithelial damage could lead to an increased frequency of mucus cell hyperplasia and squamous metaplasia. The findings from the current study that CRTh2 activation induces epithelial cell migration, elevates expression of the migratory protein CD44, induces goblet cell hyperplasia and squamous metaplasia would all support these conclusions. In addition, PGD<sub>2</sub> may be released from epithelial cells in asthma, providing an autocrine loop for CRTh2 activation, potentiating the pro-remodelling changes. These findings are summarised in **figure 6.2**.



**Figure 6.2.** Summary of findings for the role of CRTh2 activation on epithelial remodelling.

### Clinical implications of CRTh2-driven changes in epithelial cell function

These epithelial remodelling changes in asthma may significantly impact the pathogenesis of the disease. It has been reported that areas of squamous metaplasia have increased susceptibility to viral infection (447) and that viral infections are important triggers of acute exacerbations in asthma (93). In addition, respiratory viruses have been found to interfere with the structural integrity of the tight junctions and cause increased permeability of the epithelium (440,441), with the disruption lasting post infection (439). An epithelial barrier with areas of squamous metaplasia and disruption of tight junctions could therefore increase the risk of exacerbations. In addition, acute degranulation of hyperplastic goblet cells is thought to contribute to asthma exacerbations in mild and moderate asthma (62), and mucus

hypersecretion is thought to contribute to morbidity and mortality during exacerbations (83). An epithelial barrier with elevated numbers of mucus-producing cells may therefore increase the risk of more severe exacerbations. Furthermore, remodelled epithelial cells have been shown to secrete elevated levels of pro-inflammatory cytokines. An example, of this is that areas of squamous metaplasia have been shown to release elevated levels of IL-1 $\beta$ , which then contributes to remodelling of underlying fibroblasts (312). Therefore the epithelial barrier is likely to play a central role in the airway remodelling process in asthma.

To date, CRTh2 antagonists in asthma have had limited efficacy (119,120), however, the effects on remodelling end-points such as mucus production have not yet been explored. The data from the current study provides evidence that CRTh2 antagonists may be more effective in moderate-severe asthmatics as there are elevated CRTh2 positive inflammatory cells within the airway tissue and increased frequency of areas of squamous metaplasia and goblet cell hyperplasia within the epithelium for this sub-set of patients. As severe asthma is associated with the presence of frequent and/or severe exacerbations, low baseline lung function, reliance on high doses of inhaled and/or oral corticosteroids and nearly daily symptoms (25,459,460) further investigation of CRTh2 antagonist treatment for this group is important.

In conclusion, in addition to the CRTh2 driven inflammatory response in asthma, activation of this receptor on bronchial epithelial cells may contribute to airway remodelling processes within asthmatic airways. A CRTh2 antagonist may therefore be useful in dampening down CRTh2 activation on epithelial cells, restoring normal epithelial differentiation processes, as well as reducing numbers of

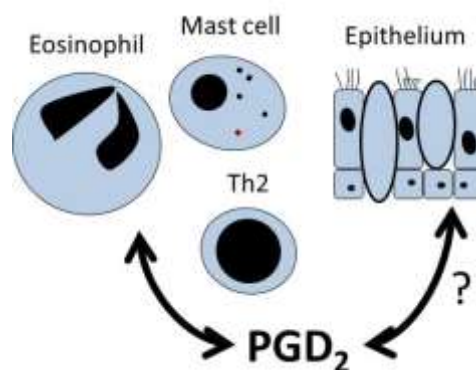
inflammatory cells. This information is important as it may influence the design of future CRTh2 antagonist clinical studies in asthma.



## 6.9. Further work

A number of further questions have arisen from the findings of the current study;

- 1) The finding that H-PGDS is expressed within bronchial epithelial cells and is up-regulated in asthmatic bronchial epithelial cells requires further study. It would be of interest to establish if bronchial epithelial cells can produce measureable quantities of  $\text{PGD}_2$ . These experiments are technically challenging due to the instability of  $\text{PGD}_2$ . However, optimisation of ALI culture assays specifically to measure released levels of  $\text{PGD}_2$  could improve the prospects of collecting supernatants with intact  $\text{PGD}_2$ , if it is present. In addition, the EicosaCell assay (453) can be used to identify newly synthesised  $\text{PGD}_2$  *in-situ* within a cell. It would be of interest to try to optimise this assay for bronchial epithelial cells and compare expression for healthy control bronchial epithelial cells compared to asthmatic bronchial epithelial cells. As discussed, previous studies have demonstrated that CRTh2 expressing cells can also produce  $\text{PGD}_2$  (161,163), and as summarised in **Figure 6.3** the current study provides evidence that epithelial cells express H-PGDS and therefore could potentially secrete  $\text{PGD}_2$ .



**Figure 6.3.** CRTh2 expressing cells that are also capable of producing  $\text{PGD}_2$

- 2) The finding that CRTh2 activation influences the differentiation status of the epithelium requires further study. It would be of interest to grow an ALI culture from transfer into ALI cultures conditions in the presence of the selective CRTh2 agonist, DK-PGD<sub>2</sub>. The findings from the current study would suggest that a differentiated epithelium similar to that found for an ALI culture from an asthmatic patient, with increased MUC5AC positive cells and areas of squamous metaplasia would be achieved.
- 3) It would be of interest to establish if the areas of squamous metaplasia that are present in asthmatic ALI cultures and following CRTh2 activation, secrete elevated levels of cytokines that could potentiate remodelling processes in other cell types. Experiments to optimise the collection of apical and basal supernatants from ALI cultures and the use of a multiplex cytokine ELISA system would aid in the investigation into this question.
- 4) It would be interesting to investigate the effects of CRTh2 activation on the calcium response within bronchial epithelial cells from asthmatic patients. This could not be completed for the current study due to availability of cells.
- 5) Limited gene expression changes were investigated in the current study for the effects of CRTh2 activation on bronchial epithelial cells. It would be of interest to carry out global gene array experiments to establish if there are any identifiable gene pathways leading to the elevated mucus producing cells and squamous metaplasia within the ALI cultures.
- 6) To establish the importance of the findings from the current study for asthmatic patients, a clinical study including end-points that interrogate

effects on bronchial epithelial cells such as mucus production and presence of squamous metaplasia, using methodologies such as endoscopic bronchoscopy, would be invaluable.

## 7.1. References

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