

INVITED REVIEW SERIES: CUTTING EDGE TECHNOLOGIES
SERIES EDITOR: DARRYL KNIGHT

Bronchial brushings for investigating airway inflammation and remodelling

KEVIN LOOI,¹ ERIKA N. SUTANTO,^{2,4} BALARKA BANERJEE,^{1,3} LUKE GARRATT,¹ KAK-MING LING,²
CLARA J. FOO,¹ STEPHEN M. STICK^{1,2,4*} AND ANTHONY KICIC^{1,2,4*}

¹*School of Paediatrics and Child Health, ²Telethon Institute for Child Health Research, Centre for Health Research and ³School of Medicine and Dentistry, The University of Western Australia, Nedlands, and ⁴Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, Western Australia, Australia*

ABSTRACT

Asthma is the commonest medical cause for hospital admission for children in Australia, affects more than 300 million people worldwide, and is incurable, severe in large number and refractory to treatment in many. However, there have been no new significant treatments despite intense research and billions of dollars. The advancement in our understanding in this disease has been limited due to its heterogeneity, genetic complexity and has severely been hampered particularly in children by the difficulty in obtaining relevant target organ tissue. This review attempts to provide an overview of the currently used and recently developed/adapted techniques used to obtain lung tissue with specific reference to the airway epithelium.

Key words: airway, airway inflammation, cytology brushing, epithelium, functional study.

INTRODUCTION

Asthma is a chronic inflammatory airway disease affecting as many as 300 million people worldwide and it is expected that this number will rise by a further 100 million by 2025.¹ In Europe alone, the social and economic burden of asthma has been estimated at close to €18 billion per annum² and in Australia this is estimated at 600 million dollars/year.³ The prevalence of asthma in Australia is relatively high by international standards, with up to 12% of adults (one in 10) and up to 16% (one in eight) of children of all ages and ethnicities being affected.⁴ Despite its high prevalence this disease remains incurable with no significant new treatments in over 30 years of intense investigation. The advancement in our understanding in this disease has been limited due to its heterogeneity and genetic complexity and thus the simple definition as 'a disorder of the conducting airways, which over-spontaneously contracts' remains vague and inaccurate. The definition proposed by the Global Strategy for Asthma Management and Prevention Report states that asthma can be defined by its pathologic and functional consequence which is, 'Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or early morning. These episodes are usually associated with widespread, but variable, airflow obstruction, within the lungs, that is often reversible either spontaneously, or with treatment.'¹ Similarly, although succinct, this definition still remains less than satisfactory and asthma continues to evade specific definition due to its complex gene and environment interactions. With no single factor accountable for the pathogenesis of asthma, respiratory clinicians have struggled to rely on inflammatory profiles and symptoms to classify asthma and administer the

The Authors: Kevin Looi is a PhD student at the University of Western Australia investigating epithelial barrier disruption post injury. Erika Sutanto is a Senior Researcher with expertise and interest in airway epithelial responses to injury in children with cystic fibrosis. Balarka Banerjee is a PhD student at the University of Western Australia investigating the processes leading to lung transplant rejection. Luke Garratt is a PhD student at the University of Western Australia investigating epithelial wound repair in children with cystic fibrosis. Kak-Ming Ling is a research scientist investigating aspects of dysregulated epithelial repair in children with asthma. Clara Foo is a PhD student at the University of Western Australia investigating innate immune responses of the airway epithelium post injury in children with cystic fibrosis. Stephen Stick is an NHMRC Practitioner Fellow and respiratory physician with a long-standing interest in paediatric airway disease. Anthony Kicic is a Senior Research Officer with expertise in functional airway epithelial studies.

Correspondence: Anthony Kicic, Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, WA 6001, Australia. Email: anthonyk@ichr.uwa.edu.au

*Authors contributed equally to this manuscript.

Received 1 April 2011; invited to revise 3 May 2011; revised 5 May 2011; accepted 21 May 2011.

appropriate treatment regimens. Momentum is now gathering to order this complex heterogeneity into particular 'asthma endotypes' with the aim of identifying definable subpopulations of asthma with discrete pathogenic pathways.⁴ The interactions between immunity, inflammation and remodelling have been central to a plethora of asthma research over the years; however, attempts at understanding asthma, particularly in children, have been severely hampered by the difficulty in obtaining relevant target organ tissue. This review attempts to provide an overview of the currently used and recently developed/adapted techniques used to obtain lung tissue with specific reference to the airway epithelium. This structure is emerging as an integral player as it is the interface between the environment and the submucosa and represents the first line of defence against inhaled exogenous agents that trigger asthma. In healthy tissue, damage to the epithelium caused by these agents trigger a cascade of events which leads to quick and efficient repair.^{5,6} However, in diseased tissue, failure to repair and restore full functional or structural capacity typically results in tissue remodelling, inflammation and fibrosis. This review begins by outlining the various direct sampling techniques presently utilized in the assessment of human airway inflammation and remodelling followed by a focused review on the bronchial cytology brush sampling method, with specific emphasis on the potential for subsequent functional investigative assessments. In order to provide a succinct overview we have chosen to focus on those key articles that have made significant contributions to this field.

ASSESSING AIRWAY INFLAMMATION AND REMODELLING

Airway inflammation in asthma often involves all structures of the bronchi, with inflammation and the remodelling processes occurring in both large and small airways.⁷ Progression of the inflammatory process to the smaller airways only occurs as the severity of the disease increases and becomes chronic in nature but otherwise, remains largely restricted to the larger airways.⁸ The exact location of airway inflammation remains controversial; however, it is accepted that inflammation can be exhibited in both large and small airways. In humans, markers of airway inflammation and remodelling can be sampled directly via whole lung sections, fibre-optic bronchoscopy, BAL, bronchial biopsies and cytology bronchial brushings. Sampling directly from the respiratory airways has major advantages as will be discussed; however, their invasive and labour intensive natures are often the greatest limitation to their routine employment.

Whole lung tissues

Human airway epithelial cells obtained from lungs not suitable for transplantation or from cadaver

sources can and has also been used to assess airway inflammation and remodelling. Hackett and co-workers utilized de-identified human lungs that were considered unsuitable for transplantation for the establishment of epithelial cells cultures and subsequent various downstream investigative assessments.^{9,10} In brief, isolation of the trachea and bronchi to the third generation involves their direct dissection and rinsing to remove blood and mucus. The epithelium on intact segments of trachea and bronchi are then dissociated via protease digestion. Afterwards, the trachea/bronchi tube is rinsed and dissociated cell clumps are strained through a nylon mesh and the protease neutralized. The cell suspension is then plated onto tissue culture flasks and incubated to deplete adherent cells. Approximately 20×10^6 cells in total are recovered from both the trachea and bronchial tissues. The obtained airway epithelial cells can either be grown to confluence for monolayer assessments or subsequently allowed to differentiate into air-liquid interface cultures. These specifically derived cells and resulting cultures have been successfully utilized to identify and characterize an epithelial subpopulation, termed side population (SP) cells.¹⁰ Further analysis revealed that although these CD45⁻ SP cells displayed progenitor cell function they were over expressed (4%) in asthmatic airways when compared with the healthy epithelial population (0.12%), indicating a possibly abnormality in the wound repair capacity of these cells.¹⁰ Furthermore, airway epithelial cells derived from the same methodology have been used to assess transforming growth factor $\beta 1$ (TGF- $\beta 1$)-induced epithelial to mesenchymal transition (EMT) in asthmatic epithelium.⁹ Here, direct evidence was provided that this phenomenon occurs in asthmatic cells via a SMAD3-dependent process and that in air-liquid interface cultures this is restricted to the basal cells, which collectively supported the hypothesis of an altered repair phenotype. Despite this sampling methodology's high recovery rate and the ability to perform epithelial specific analysis, its feasibility for large-scale routine research remains undetermined due to the irregular frequency at which non-transplanted lungs become available.

Bronchoscopy and associated methodologies

Since bronchoscopes became widely available, bronchoscopy and its associated sampling techniques, such as BAL, biopsies and cytology brushings, have enabled investigators to gain a better insight into the pathogenesis of various respiratory diseases.^{11,12} Although considered safe in experienced hands, bronchoscopy is still an invasive procedure involving the insertion of a bronchoscope down the airway of an anesthetized patient and concurrently performing bronchial biopsies, BAL or cytology bronchial brushings. Even though there is a cumulative record of safety in performing these techniques,¹³⁻¹⁶ concerns such as cough, wheeze or pneumothorax that may develop during or after their use^{17,18} certainly warrant constant monitoring and reporting of the use of such techniques.

BAL

BAL is generally performed to attain a representative sample of the distal airways and alveolar surfaces.¹⁹ In general, this involves the introduction of varying quantity (usually between 1 and 5 mL/kg) of sterile room-temperature saline aliquots through a flexible bronchoscope into the segmental or sub-segmental bronchus for a short duration, followed by removal into a sterile collection trap. The suctioned fluid is then termed BAL fluid (BALF) and cells from the fluid are harvested by low speed centrifugation and subsequently used for total cell counts or cytopsin preparations. The term BAL is not synonymous with bronchial lavage as the latter refers to washing of the larger airways either via a bronchoscope or directly through an endotracheal tube (ETT) in a ventilated patient. Information gained from the BALF will depend upon the status of the lavaged area and can often include but is not limited to cellular content including mast cells, neutrophils and eosinophils,^{20,21} amount of non-cellular compounds associated with inflammation such as cytokines,^{22,23} host defences comprising of alveolar macrophages and immune cells²⁴ as well as markers for lung damage like surfactant proteins A and D.²⁵ Despite being able to provide a vast array of information, the very nature of instilling saline to sample the bronchus presents an inevitable dilution effect the added saline has on the subsequently suctioned BALF. Although evidence has suggested the use of albumin as a suitable denominator for BALF,²⁶ presently, there remains no valid way to account for this dilution effect. Until fully capable of accounting for this dilution effect, the use of BAL would be limited to being a complementary technique to bronchoscopy in the diagnosis and research of airway-related diseases.

Biopsies

A vast majority of the human airway epithelium obtained during biopsies would contain areas of viable cells that can be used for the assessment of the integrity and structure of the airway epithelium.²⁷ Biopsies are performed by the insertion of cupped biopsy forceps in the closed position down the suction channel of a bronchoscope. Bronchial biopsy specimens are selected from areas previously untouched by the bronchoscope within the right upper and middle lobe bronchi. The forceps are then extended and under direct vision, opened and clamped around the area to be sampled. With a gentle tug, the forceps are retracted and removed from the bronchoscope. The biopsied samples are then sectioned and fixed and can then be examined using immunohistochemical analyses, together with a panel of monoclonal antibodies, to identify and quantify cellular or inflammation markers such as cytokeratins, eosinophils, neutrophils and other various inflammatory cells respectively.^{28–30} Biopsy-derived samples have also been utilized for cell culture establishment involved in studies of cell injury, proliferation, differentiation and repair in

response to injurious stimuli in the form of diesel exhaust particles.^{31,32} Bronchial biopsies are an ideal diagnostic tool for the observation of pathological changes of the respiratory airways; however, it remains typically unsuitable for routine human bronchial epithelial cell culture establishment due to its highly invasive nature, its requirement for competency in performing the procedure and the labour intensive nature in processing biopsied tissue for culture establishment and purification.

Cytology bronchial brushings

Cytology bronchial brushings are simple, relatively easy to perform and less invasive compared with either BAL or biopsies. The process of cytology bronchial brushing provides the opportunity to obtain airway epithelial cells of high viability and number to be analysed by various subsequent methodologies, including immunocytochemistry,³³ confocal microscopy,³⁴ electron microscopy³⁵ and flow cytometry.³⁶ However, this process has progressed beyond its initial purpose of cellular analysis to include establishment of primary human bronchial epithelial cell cultures from adult and paediatric patients.^{36–38}

The method of cytology bronchial brushing and sampling epithelial cells has been routinely used in the past for the diagnosis of tuberculosis,^{39,40} but has recently included other respiratory diseases, such as asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disorder.^{41–47} A majority of these studies^{37,43,44,46,47} have performed bronchial cytology brushings from adult patients however, Doherty and co-workers successfully executed non-bronchoscopic bronchial cytology brushings in children by the insertion of a sheathed bronchial cytology brush through an ETT. Here, sampling occurred via the gentle wedging of a sheathed cytology brush in a peripheral bronchus. Once wedged, the sheathed cytology brush was retracted 2–3 cm before the brush tip was advance 2–3 cm from the protective sheath. Gentle brush sampling was performed and upon completion, the brush tip was then retracted back into its protective sheath and completely removed from the ETT. The brush tip was then detached into collection media and the process repeated. Collected samples were then characterized and used to successfully establish primary bronchial epithelial cell cultures.³⁸ Since this initial study showcases the establishment of cultures from paediatric patients, we have established a similar blind, non-bronchoscopic technique with modifications for obtaining bronchial epithelial cells.⁴² Briefly, once each child is anaesthetized, using sevoflurane and propofol, and intubated in theatre an unsheathed soft nylon bronchial cytology brush is advanced through the ETT until the tip hits airway wall resistance usually above the carina (Fig. 1). Sampling occurs using a rotational movement of the brush rather than a probing movement. Completion of sampling would see the cytology brush being withdrawn from the ETT, the brush tip inserted and detached into cold sterile collection media. The brushing process is repeated with the second cytology

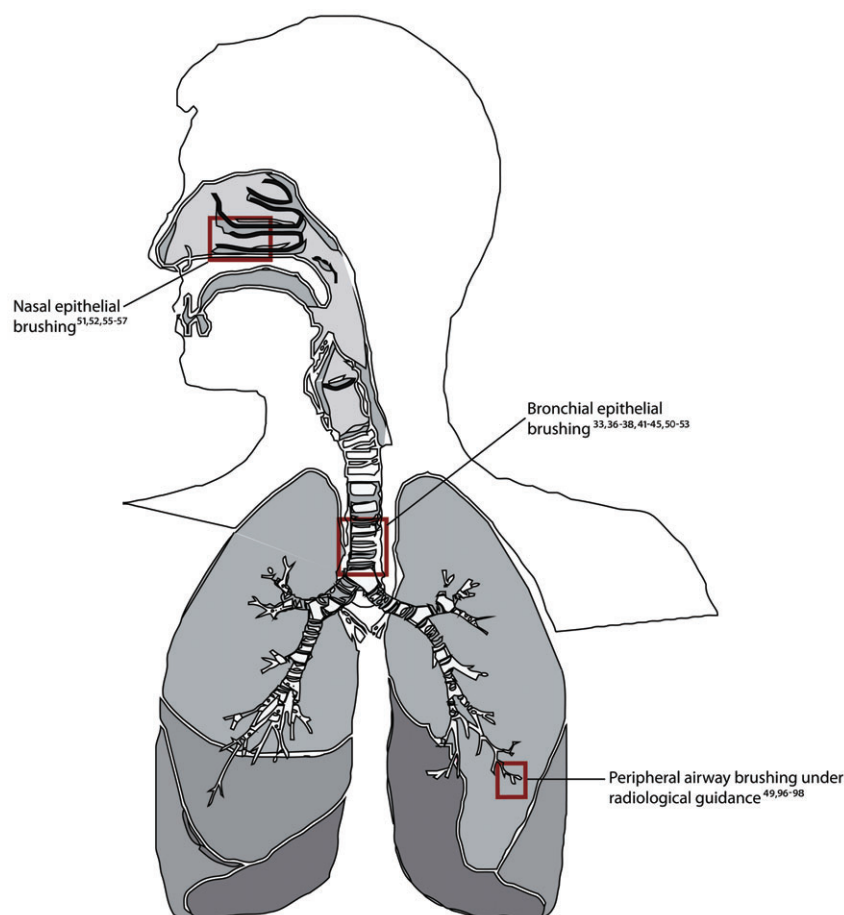


Figure 1 Schematic diagram indicating regions sampled using cytology brushes.

brush tip also being inserted and detached into the same collection tube. The collection tube, together with the brush tips, is then immediately processed by vortexing for a period of time to dislodge the cells off the brush tips as well as breaking up larger cell clumps. The brush tips are then removed into another collection tube and the process repeated. Following that, the two collection media are pooled, centrifuged and resuspended in growth media. The cell suspension is subsequently fractionated for multiple downstream uses, including cytospins for immunohistochemistry, cell culturing, RNA or protein extraction. During our initial assessment of the methodology published by Doherty and colleagues, we found the use of sheathed cytology brushes to greatly diminish the total yield and subsequently the ability to establish epithelial cultures. Further analysis identified this to be due to the loss of cells as the brush bristles directly slide against the sheath lining during its retraction (A. Kicic, unpubl. data, 2004). We compared total yields between sheathed and unsheathed cytology brushes and observed a 2–4 fold increase in total cell number in using unsheathed cytology brushes (A. Kicic, unpubl. data, 2004). As a result of these initial in house studies we have subsequently implemented the use of unsheathed cytology brushes in all our sampling methodologies.

More recently, with the increased frequency of laryngeal mask airways (LMA) being used in paediatric

anaesthesia, we have established, adapted and refined a bronchoscopic cytology brushing technique to allow for the sampling of bronchial epithelial cells.⁴⁸ This technique involves having the unsheathed cytology brush passed down the instrument port of a portable intubation bronchoscope and kept concealed within the bronchoscope tip. The portable bronchoscope is then passed down through the LMA and directed and positioned at a suitable site for brushing beyond the lower edge of the vocal cords. Once positioned below the vocal cords, the cytology brush is then advanced till resistance is encountered typically above the carina region in the same region as the blind brushing technique described above. Rotational movement is performed to sample the cells and subsequently the cytology brush tip is retracted to just beyond the tip of the bronchoscope. Both the bronchoscope and the concealed cytology brush are then withdrawn together. The cytology brush would then be pushed out from the bronchoscope and the brush tip detached into sterile collection media. This procedure is repeated once more with a second brush tip being detached into the same collection tube. The collection tubes containing the brush tips are then similarly processed as mentioned previously.⁴² Results obtained have shown that both the blind, non-bronchoscopic bronchial brushing and portable bronchoscopic brushing techniques are equally well tolerated with only a mild self-limiting cough in

~10–20% of the patients reported as the only reported adverse event after the procedures.^{41,48}

We have also adapted the method of bronchoscopic cytology brushing for use in the sampling of human airway epithelial cells in the small and large airways of lung transplant patients.⁴⁹ Similar to the non-bronchoscopic or the portable bronchoscopic cytology brushing method, the procedure is performed under general anaesthesia, with the cytology brush passed down through the instrument port of the bronchoscope upon successful positioning in a suitable lateral segment of the lower right or left lobe of the lung. The main difference with this methodology is that the cytology brush tip is extended, under radiological guidance, to an area 2–3 cm from the pleural surface for the collection of small airway brushings (Fig. 1). Large airway epithelial cells are obtained from the proximal bronchi by several gentle passages of the cytology brush into each airway. Cells from large and small airways are deposited in separate collection media to prevent cross-contamination and similarly processed as previously mentioned.⁴² Similar to the previously described brushing techniques no distinguishable adverse side-effects have been observed with this methodology.⁴⁹ Despite its advantages, cytology bronchial brushing is limited in that it can only provide cells of epithelial origin. The inability to sample other cell types such as inflammatory and mesenchymal cells, obtained via bronchial biopsies, can then limit the application of this technique to assess airway inflammation and remodelling.

Overall, these methodologies can be confidently used to obtain human airway epithelial cells which can be directly analysed or grown to establish primary cell cultures for more complex experiments.^{38,41,42,48,49} Despite the reported mild, self-limiting cough, the ability to obtain highly viable epithelial cells for culture establishment without overt invasiveness merits its suitable use in both children and adult.

NASAL VERSUS BRONCHIAL BRUSHINGS

Studies using airway epithelial cells obtained either via bronchoscopy, bronchial biopsies, BAL or isolated from non-transplanted lungs have shown the potential for diagnostic or investigative assessments of many airway-related diseases. However, these methodologies are more complex and invasive, often requiring superior surgical competency or infrequently performed due to the low rate of available target organ tissue. The need for a less invasive methodology, which can be routinely performed in either adults or children and potentially yield highly viable epithelial cells, thus brings us to highlight the immense potential of cytology brushings. Concurrently, obtaining and characterizing airway epithelial cells from the nasal cavity via cytology brushings has also been recently explored and in this section, we will compare this technique and resulting cells to those obtained by brushings of the lower airways.

Ideally, human airway epithelial cells provide a model system for the study of the respiratory epithelium in the pathogenesis of many airway diseases and the onset of inflammatory, hyperresponsiveness and remodelling changes. Recent studies using cytological brushings of both the nasal cavity and bronchial airways have allowed investigators to circumvent the major limitation in the past to obtain sufficient cell numbers to allow for large-scale and complex experiments.^{41,42,49–54} The sampling of nasal epithelia involves the use of a cytology brush to gently brush the medial aspect of the inferior turbinate (Fig. 1). The tip of the cytology brush is then detached into a collection tube, which is then agitated to dislodge cells from the brush tip and subsequently processed for further downstream uses including cell culture establishment, epithelial cell lineage verification assessments or immunocytochemistry.

Nasal epithelial cell brushings have been gaining popularity recently due to several merits including: its relatively non-invasive nature, the requirement for trace or no anaesthesia, its simple protocol and short time frame to perform, accessibility of the brush to the sample tissue and potential for repeated sampling of individuals. Previous studies have proven that nasal airway epithelial cells obtained via nasal brushings^{52,55–57} or nasal biopsies^{58,59} showed similar morphology and growth characteristics to bronchial epithelial cells; moreover, they can also be successfully used to establish primary cell cultures. A study by McDougall and co-workers⁵² comparing nasal and bronchial epithelial cells from matched patients suggested that the obtained nasal epithelial cells are suitable surrogates. Their study showed that the inflammatory responses of nasal epithelial cells closely resemble those of bronchial epithelial cells. Furthermore, they showed that the release of inflammatory mediators, including IL-6, RANTES, VEGF, MCP-1, MMP-9 and TIMP-1, and receptor expression (ICAM-1, $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$) in human nasal epithelial cell cultures reflect the bronchial epithelial cell cultures under either resting or cytokine-stimulated conditions.⁵² However, it was also noted by the same authors that the absolute mediator levels produced did in fact differ. Despite this, they conclude that the merit to nasal airway epithelial cell brushings is in its feasibility for use in individuals in an expansive age range ~0.7–80 years.⁵²

Interestingly, another recent study conducted by Lopez-Souza and co-workers⁵¹ has shown different responses between nasal and bronchial derived epithelia. Specifically, they showed that human nasal airway epithelial cells were less susceptible to human rhinovirus (HRV) infection than bronchial epithelial cells. They clearly showed that HRV replicated more easily, had a 20- to 30-fold greater viral load, and infected more cells in bronchial epithelial cells than nasal epithelial cells. In addition, inflammatory markers were similarly greater in bronchial epithelial cells than in nasal epithelial cells.⁵¹ The authors concluded that differentiated human bronchial epithelial cells are more susceptible to HRV infection as compared with similarly differentiated human nasal epithelial cells. Moreover, differentiated human nasal

epithelial cells might also have an increased resistance to HRV infection. Importantly, this study implies possible region-specific responses that may occur by the epithelium and the inability to translate findings between the two due to the variability. Only a few studies including the two highlighted in this review that have attempted to characterize and compare these two epithelial populations. The paucity in this field justifies further investigation to explore in more detail the applicability and purpose of nasal derived epithelial cells in the experimental setting.

Despite the benefits of sampling nasal airway epithelial cells, the use of bronchial brushings still remains a useful and more appropriate technique for morphological and functional studies of these cells in different airway diseases. Bronchial epithelial cells obtained by cytology brushings are considered reflective and representative of the current *in vivo* condition and thus provide valuable insight into the various mechanisms underlying the onset of airway inflammation and remodelling. In asthma research, bronchial brushings are often used in the study of bronchial epithelial cells themselves,^{31,42,60} inflammatory cells such as neutrophils or eosinophils infiltrating the respiratory epithelium^{37,61} or microbiota species such as *Haemophilus*, *Moraxella* or *Neisseria* present at the surface of the bronchial epithelial layer^{62,63} (Table 1).

Although the resulting applications for the cells are extremely varied, the cytology brushing technique should be continually validated in each new subject group sampled. As mentioned previously and in our hands the technique of sampling bronchial airway epithelial cells has been found to be well-tolerated by healthy and mildly asthmatic children^{41,48} and in lung transplant recipients.⁶⁴ Similarly, a study by Romagnoli and co-workers showed bronchial brushings being well-tolerated and a safe method for obtaining bronchial epithelial cells from individuals with severe asthma ranging from mild (Score 1) to severe (Score 4) on the AAS (Aggregate Aggression Score) scores.⁶⁵ While there appears to be an emerging pattern of safety and widespread suitability of this sampling technique, its full applicability in other disease settings still need to be elucidated.

BRONCHIAL BRUSHINGS FOR AIRWAY INFLAMMATION AND REMODELLING ASSESSMENT

This section of the review revisits the use of bronchial brushings of airway epithelial cells for use in establishing cultures in order to investigate the mechanisms underlying airway-related diseases. Among the assessment methods which are presently performed to assess airway inflammation and remodelling, the

Table 1 Collation of experimental analyses utilizing airway epithelial sample obtained by cytology brushing

Brushing technique	Airway epithelial source	Summary of experiments performed on/using source tissues	References
Endotracheal tube	Paediatric	Immunocytochemical staining Measurement of: – Cytokines – Inflammatory mediators DNA microarray Gene expression Gene silencing, promoter activation Protein quantification Total and differential cell counts Epithelial wounding and repair Viral and non-viral stimulation Differentiation into air-liquid interface	38,41,42,44,48,50
Portable bronchoscopic	Paediatric	Immunocytochemical staining Cytokine measurement Flow cytometry	48
	Adult	Immunocytochemical staining Measurement of: – Cytokines – Inflammatory mediators DNA microarray Gene expression Protein quantification Flow cytometry	33,36,37,43,45,51–53
Radiological guided	Paediatric	Not applicable	49,96–98
	Adult	Cytokine measurement Gene expression Therapeutic drug treatment Cellular migration	

technique of bronchial cytology brushing provides an enormous opportunity to perform both diagnostic as well as investigative evaluations of various airway-related diseases. The use of bronchial cytology brushing is not only applicable to adult populations, but is similarly used within the paediatric subset thus providing even greater potential in comprehending disease pathogenesis and mechanisms.

As mentioned earlier, bronchial cytology brushings can be performed differently and with various adaptations to the methodology. Our initial work first showed the large-scale feasibility of using bronchial cytology brushing in children and in particular, successfully obtaining viable cell cultures of epithelial origins.⁴¹ We managed to obtain, on average, $\sim 2.6 \times 10^6$ cells, with 93% of all cultures exhibiting some growth, 80% growing to confluence and 80% surviving a second passage. Furthermore, established cultures were also successfully grown till passage 7. In a separate but parallel investigation utilizing the 'bronchoscope-directed' bronchial brushing technique, we found that this technique consistently yields more cells of epithelial lineage, approximately 5×10^6 cells. All cultures were found to exhibit some growth with 88% of cultures growing to confluence and 100% surviving beyond a third passage. In addition, no gross morphological difference was observed within cultures over successive passage. More recently, we have expanded the potential of this technology by performing cytology brushings of the peripheral airways within the lungs of adult transplant patients under radiological guidance. This technique has yielded reliable numbers of airway epithelial cells, with cell yields of 0.9×10^6 cells on average for small airway epithelial cells and 1.3×10^6 cells for large airway epithelial cells.⁴⁹ Our current cohort now exceeds 600 healthy and asthmatic children as well as children with CF and over 40 adult lung transplant recipients and current yields and culture success rates remain comparable to these initial studies (Table 2). Furthermore, when we have examined the established cultures we find that regardless

of the method used to brush the large airway, the subsequent isolation and purification process yields a primarily pure (>95) epithelial cell culture with similar morphologies (Fig. 2). Collectively, these studies have shown not only the evolution of cytology bronchial brushing in our hands but also the success of the methodology in repeatedly obtaining airway epithelial cells from paediatric and adult-derived healthy and diseased tissues.

TRANSLATIONAL RESEARCH FROM THIS TECHNOLOGY

In light of its high reproducibility and reliability, research investigations are now utilizing this technology in the evaluation and investigation of many airway-related diseases. In this section, we draw attention to the present use of cytology brushings in airway-related diseases, in particular, asthma, CF and lung transplantation but also highlight emerging areas of airway disease research utilizing this technique including COPD and IPF.

Paediatric asthma

We have successfully used the cytology bronchial brushing technique on children with asthma to establish epithelial cell cultures and consequently have been able to perform morphologic, biochemical, molecular and more significantly functional assessments. Our research has determined that cells obtained from children having both asthma and atopy express lower levels of the epithelial lineage specific marker, cytokeratin-19; produce higher levels of anti-inflammatory mediators and epidermal growth factor but significantly less TGF- β 1 and exhibit a faster doubling time than their healthy counterparts. Of significance, these differences were found to be maintained despite repeated passages.⁴²

Table 2 Comparison of yields obtained and success rates for culture establishment on airway epithelial cells derived from the various cytology brushing techniques

Brushing technique	Phenotype	Age range (years)	Yield (mean $\pm$ SE, $\times 10^6$ cells)	Success rate (%)
Endotracheal tube	Healthy	1.7–17.0	2.56 ± 0.14	>85.0 ⁴²
	Healthy Atopic	1.4–14.9	2.61 ± 0.21	
	Atopic Asthmatic	2.9–14.6	4.05 ± 1.08	
	Asthmatic non-atopic	1.8–18.4	2.97 ± 0.29	
Portable bronchoscopic	Healthy	2.6–16.6	3.01 ± 0.33	>88.0 ⁴⁸
	Healthy atopic	4.6–16.9	3.82 ± 0.70	
	Atopic Asthmatic	4.9–15.3	4.42 ± 0.82	
	Asthmatic non-atopic	2.7–11.0	7.88 ± 2.82	
Radiological guided	Cystic fibrosis	0.1–9.4	1.47 ± 0.10	~63.0 (E.N. Sutanto, pers. comm., 2005)
	Adult Lung transplant (small airways)	18.0–64.0	0.96 ± 0.06	
	Adult Lung transplant (large airways)		1.31 ± 0.08	

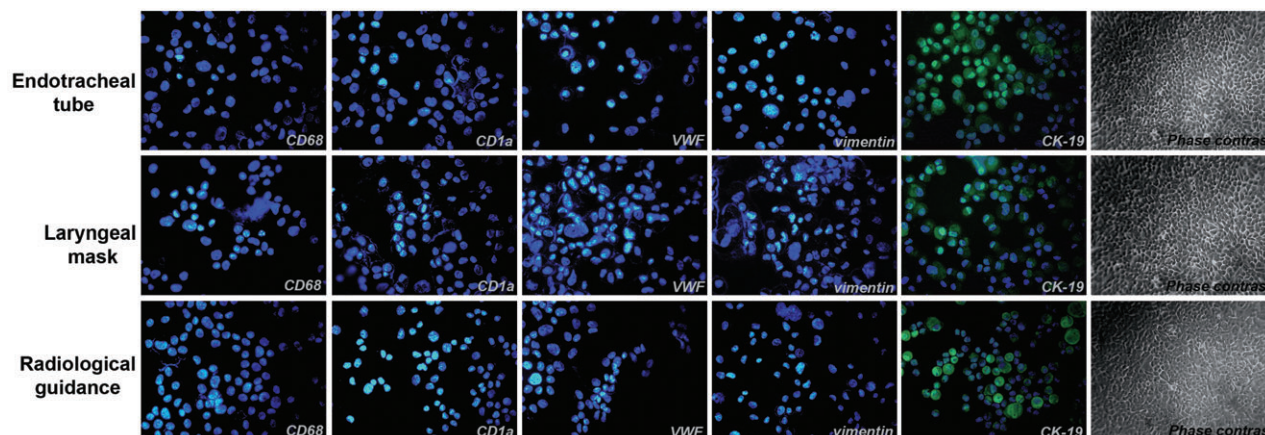


Figure 2 Comparison of airway epithelial brushing techniques. Airway epithelial cells were sampled utilizing various apparatuses and lineage and purity determined and compared. Obtained samples have been shown to be contaminated with other cell types, including macrophages (CD68), dendritic cells (CD1a), endothelial cells (von Willebrand factor; VWF) and mesenchymal cells (vimentin), however stained positively for cytokeratin-19 (CK-19). Resulting cultures all have comparable morphological phenotypes, exhibiting a typical 'cobblestone' appearance.

Collectively, our findings have shown that, even in mild asthma, where overt inflammation is absent, the asthmatic epithelium is intrinsically abnormal. In a separate study, we mechanically induced wounds on established cultures obtained from the brushing technique we have described earlier. We have been able to investigate and assess the repair capacity of asthmatic epithelial cells and also the role plasminogen activator inhibitor-1 (PAI-1) plays in this repair process. Our findings provided the first functional evidence that epithelial cells obtained from children with asthma require a significantly longer time to repair as compared with healthy airway epithelial cells and that this is an intrinsic abnormality to the asthmatic cells. We also showed the pivotal role PAI-1 played in the repair process and the likelihood that it plays a significant role in airway remodelling in asthma.⁵⁰ In a follow-up study using airway epithelial cells obtained from bronchial cytology brushings, we were able to investigate whether a dysregulated production of extracellular matrix contributed to aberrant wound repair in asthma. Here, we found that fibronectin (FN) was necessary in normal wound repair and that asthmatic epithelial cells have reduced FN production which contribute to deficient wound repair *in vitro* in these cells.⁵⁴ In a larger perspective, this sampling technique has been able to marry observational findings such as differential gene expression with functional studies to elucidate their downstream consequences. As such, we have been able to combine other cutting edge technologies as part of this functional context in the form of gene-silencing, promoter activation and epigenetic regulation studies.

Paediatric cystic fibrosis

Despite bronchial cytology brushing being actively utilized as a technique in the evaluation of CF disease in various previous investigations,^{43,44,66–68} no study

until recently⁴⁵ has utilized the establishment of primary epithelial cultures. Instead, the functional properties of CF airway epithelium have been performed using bronchial epithelial cells isolated from lung fragments. These studies have provided critical findings, including but not limited to showing that small percentages of phenotypically normal airway epithelial cells are adequate for the functional correction of CF airway epithelial cells *in vitro*,⁶⁹ identifying the abnormal Cl^- and Na^+ ions transport in CF and its effect on reduction of airway surface liquid (ASL),⁷⁰ and the hyper-inflammatory status of CF airway epithelium.⁷¹ Other studies have also examined the hyper-inflammatory status of CF airway epithelium utilizing lung tissues,^{72–75} animal models,^{76–78} or cell lines such as IB3-1, CFBE41o- and 16HBE14o-.^{79–81} Nasal epithelial cells isolated from nasal polyp explants, nasal brushings or nasal epithelial cell lines JME/CF15 have also been used in similar studies.^{57,82–84} In contrast to the popularity of these alternative sources and despite the advantages of larger study recruitment and repeat sampling that brushings provide, there are few studies that use primary airway epithelial cells from CF patients for biochemical and functional investigations.

A review by Machen⁸⁵ reported that the hyper-inflammatory status of CF airway epithelium is conflicting, likely reflecting the various sources of respiratory epithelial cells being used in different studies. In addition, there are currently limited data available to investigate hyper-inflammatory status in the paediatric setting, upon where dysregulation might be a critical determinant of disease progression into adulthood. Using this cytology brushing technique we have recently reported the innate inflammatory responses of paediatric CF airway epithelial cells to both non-viral stimulation and viral infection.⁴⁵ These findings suggest that paediatric CF airway epithelial cells are not hyper-inflammatory per se and that they have a specific inflammatory response and

greater susceptibility to rhinovirus infection. In a separate study using cells isolated via the same technique, we have also shown low inducible nitric oxide synthase (NOS) expression in children with CF which reflects defective innate immunity.⁸⁶ This *in vitro* model of CF airway epithelial cells can also be used to study the functional correction of CF transmembrane conductance regulator (CFTR) via quantitative fluorescence measurements such as those reported by Verkman and co-workers,^{87,88} to assess gene transfer efficiency of CFTR correction,^{89–91} and to evaluate the efficacy of current treatment^{92,93} and the potential cellular basis for developing new therapeutic targets.

Lung transplantation

Bronchoscopy is a standard procedure, routinely used to assess the health of lung transplant recipients at regular intervals, particularly in the first year post transplant.⁹⁴ Emergency bronchoscopy is often used if a sharp decline in lung function is noted.⁹⁵ Samples such as biopsies, bronchial brushings or washings can be taken during bronchoscopy to assess for rejection, often associated with the presence of a perivascular mononuclear infiltrate and mucosal inflammation; and/or infection, by establishing cultures from the collected bronchial brushings or washings and assessing for bacteria or fungal infection.⁹⁴ Bronchoscopy, thus, provides an excellent opportunity for the collection of bronchial and bronchiolar brushings for research with minimum risk or discomfort to the patient.⁶⁵

Similar techniques have been used by a number of studies to obtain bronchial brushings from lung transplant recipients.^{49,96–98} This involves extending a cytology brush down the channel of the bronchoscope and brushing an area of the segmental bronchi under direct visual guidance and is carried out under general⁴⁹ or localized anaesthesia.⁹⁶ A number of passes are made with the unsheathed brush, to ensure optimal collection of cells. To further improve cell yield, the same area is sometimes brushed multiple times, by removing and collecting cells of the brush between each brushing.

An emerging brushing technique of the airways in lung transplant patients incorporates the use of radiological guidance with current sampling methodology to collect cells from the bronchiolar region of the lung for the study of airway remodelling in peripheral airways. In brief, the small airways are sampled by extending the cytology brush down the bronchoscope under radiological guidance, to ensure that airway 2–3 cm from the pleural surface is sampled.⁴⁹ The small airways have been shown to be the source of developing obliterative bronchiolitis (OB), which is a fibroproliferative disease causing over 80% of post-transplant deaths after the first year.⁹⁹ Bronchial or bronchiolar epithelial cells sampled from the small airways can then be used in different types of research including cytokine production measurement in response to immunosuppression agents such as cyclosporine, tacrolimus and mycophenolate mofetil;¹⁰⁰ measurement of mRNA

levels of different cytokines including IL-8, IL-10, TNF- α , TGF- β 1 in patients with bronchiolitis obliterans syndrome (BOS)¹⁰¹ and evaluation of rejection markers such as B7-1 and B7-2.¹⁰² Furthermore, the ability to sample epithelial cells opens up the possibility of studying the controversial role of epithelial-mesenchymal transition (EMT) in BOS. Studies have utilized epithelial brushings from BOS patients co-expressing mesenchymal markers,⁹⁸ or with increased matrix metalloproteinase (MMP) activity and cell migration capacity.⁹⁷ The small airway brushing is therefore the ideal tissue to study OB pathogenesis.^{49,98} Recently, studies have also used this technique in establishing primary cell cultures from bronchial⁹⁶ and bronchiolar⁴⁹ brushings for assessing EMT changes when stimulated, such as decrease in epithelial markers, increase in mesenchymal markers, increase in MMP-2 and 9 activity and increased cell migration.^{64,97} In addition, primary epithelial cell cultures have also been utilized to study the *in vitro* effects of therapeutic drugs such as azithromycin¹⁰³ and simvastatin¹⁰⁴ on epithelial cells, including production of cytokines and changes in mRNA.

Smoking and chronic obstructive pulmonary disorder (COPD)

Tobacco use or smoking is often associated with a higher incidence of chronic obstructive pulmonary disorder. Although we see a recent increase in the use of non-invasive sampling techniques such as induced sputum and exhaled breath condensate^{105,106} in the identification of airway obstruction and inflammation markers, the steadfast use of the 'gold standard' bronchial cytology brushing method to sample the respiratory airways remains routinely performed in many diagnostic and research studies involving airway inflammation.^{107,108} Inhaled cigarette smoke has the capacity of causing extensive injury to various orders of the respiratory airway,^{109–114} hence, the method of bronchial cytology brushing has enabled us to enhance our understanding and also elucidate the various effects inhaled cigarette smoke has on the bronchial epithelium, such as the promotion and perpetuation of inflammatory responses in the airways.¹¹⁵ An adapted method of obtaining peripheral airway cells utilizing an ultrathin bronchofiberscope, together with a modified cytology brush inserted through the biopsy port of a regular bronchoscope and under fluorographic guidance, has allowed for the sampling of epithelial cells within the small airways. Results from this study showed a possible link between tobacco smoke and the expression of inflammatory mediators such as interleukin-8 (IL-8) and intracellular adhesion molecule (ICAM-1) in the sequestration of neutrophils to the smaller airways of tobacco smokers. This study also showed the capability of bronchial cytology brushing of small airways by recovering $\sim 2 \times 10^6$ cells, which were highly viable and of epithelial origins.¹¹⁶ Furthermore, Harvey and co-workers¹¹⁷ have used fibre-optic bronchoscopy and brushing for the comparison of gene expression and morphology of small and large airway epithelium

obtained from smokers and non-smokers. Determination of genetic profiles within the epithelium of smokers using microarray has shown the upregulation and downregulation of relevant genes associated with COPD.^{117–119} These smoking-modulated genes could be used as a potential candidate in developing new therapeutic treatment for COPD.

IPF

Cytology specimens obtained from bronchoscopy brushing has also been commonly used in evaluating suspected lung carcinoma as well as cancer diagnosis. A study using Affymetrix HG-U133A microarrays has successfully identified 80-gene biomarkers from epithelial cells collected via bronchial cytology brushing which differentiated smokers and non-smokers with and without lung cancer.¹²⁰ In combination with fluorescence *in situ* hybridization, the sensitivity of routine bronchial cytology brushing in the detection of lung cancer has significantly improved.¹²¹ Furthermore, other cellular components such as alveolar macrophages or airway epithelial cells obtained from patients with other lung diseases such as IPF could also be utilized in array-based assessments to elucidate the pathobiology of IPF.¹²² As a result, the strategy of using cytology brushing samples in both clinical research and diagnosis can prove to be effective in the development of new therapeutic paradigms to treat various lung diseases.

In this section of the review we have highlighted only a few research fields, which are currently utilizing the bronchial cytology brushing methodology, and highlighted the fact that the subsequent derived epithelial samples are more than sufficient in number, viability and purity to enable downstream translational research. Despite the emergence of this technique in obtaining bronchial epithelial cells, few studies have progressed beyond the cellular characterization or diagnostic phase and established epithelial cell cultures. It is only when we advance into culture establishment that we then fully harness the potential of bronchial cytology brushings through the ability to perform functional evaluation.

CONCLUDING COMMENTS

Multiple assessment methodologies exist for the elucidation of airway inflammation and remodelling of various airway-related diseases. Although it is clear that in the routine clinical diagnosis of airway inflammation and remodelling, invasive methods are seldom utilized, for research purposes, methods such as fibre-optic bronchoscopy can be safely performed. Each assessment method has its strengths as well as its limitations and the reader must bear in mind that there is no optimal method and the decision to employ one sampling technique depends entirely upon the aims of the investigation. In this review, we have provided an overview into the various methodologies currently in use and have also discussed in detail, the merits of bronchial cytology brushings and

the immense potential this recent sampling technique has in providing the means to answer the many gaps in our knowledge relating to airway inflammation and remodelling within the respiratory epithelium.

REFERENCES

- 1 Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA); 2010. [Accessed 9 Jun 2011.] Available from URL: <http://www.ginasthma.org/>
- 2 Braman SS. The global burden of asthma. *Chest* 2006; **130**: 4–12S.
- 3 Australian Centre for Asthma Monitoring 2008. Asthma in Australia 2008. AIHW Asthma series no. 3. Cat. no. ACM 14. Canberra: AIHW.
- 4 Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 2008; **372**: 1107–19.
- 5 Erjefält JS, Erjefält I, Sundler F *et al.* *In vivo* restitution of airway epithelium. *Cell Tissue Res.* 1995; **281**: 305–16.
- 6 White SR, Tse R, Marroquin BA. Stress-activated protein kinases mediate cell migration in human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 2005; **32**: 301–10.
- 7 Vignola AM, Mirabella F, Costanzo G *et al.* Airway remodeling in asthma. *Chest* 2003; **123**: 417–22S.
- 8 Kraft M, Martin Richard J, Wilson S *et al.* Lymphocyte and eosinophil influx into alveolar tissue in nocturnal asthma. *Am. J. Respir. Crit. Care Med.* 1999; **159**: 228–34.
- 9 Hackett T-L, Warner SM, Stefanowicz D *et al.* Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor- β 1. *Am. J. Respir. Crit. Care Med.* 2009; **180**: 122–33.
- 10 Hackett TL. Characterization of side population cells from human airway epithelium. *Stem Cells* 2008; **26**: 2576–85.
- 11 Prakash U, Mehta A. Newer and miscellaneous applications of bronchoscopy. In: Prakash U (ed.) *Bronchoscopy*. Raven Press Ltd, New York, 1994; 433–41.
- 12 Prakash UB. Bronchoscopy in North America: the ACCP survey. *Chest* 1991; **100**: 1668–75.
- 13 Burns DM, Shure D, Francoz R *et al.* The physiologic consequences of saline lobar lavage in healthy human adults. *Am. Rev. Respir. Dis.* 1983; **127**: 695–701.
- 14 Ettensohn DB. Bronchoalveolar lavage in the normal volunteer subject. 2. Safety and results of repeated BAL and use in the assessment of intra-subject variability. *Chest* 1988; **94**: 281–5.
- 15 Jarjour NN, Calhoun WJ. Bronchoalveolar lavage in stable asthmatics does not cause pulmonary inflammation. *Am. Rev. Respir. Dis.* 1990; **142**: 100–3.
- 16 Krug N. Safety aspects of local endobronchial allergen challenge in asthmatic patients. *Am. J. Respir. Crit. Care Med.* 1996; **153**: 1391–7.
- 17 Nussbaum E. Pediatric fiberoptic bronchoscopy: clinical experience with 2,836 bronchoscopies. *Pediatr. Crit. Care Med.* 2002; **3**: 171–6.
- 18 Pue CA. Complications of fiberoptic bronchoscopy at a university hospital. *Chest* 1995; **107**: 430–2.
- 19 Wood R, Daines C. Bronchoscopy and bronchoalveolar lavage in pediatric patients. In: Chernick V, Boat T, Wilmott R *et al.* (eds) *Kendig's Disorders of the Respiratory Tract in Children*, 7th edn. Elsevier, Philadelphia, PA, 2006; 94–109.
- 20 Veeraghavan S, Latsi PI, Wells AU *et al.* BAL findings in idiopathic nonspecific interstitial pneumonia and usual interstitial pneumonia. *Eur. Respir. J.* 2003; **22**: 239–44.
- 21 Casale TB. Direct evidence of a role for mast cells in the pathogenesis of antigen-induced bronchoconstriction. *J. Clin. Invest.* 1987; **80**: 1507–11.
- 22 Callister ME. Extracellular thioredoxin levels are increased in patients with acute lung injury. *Thorax* 2006; **61**: 521–7.

- 23 Crohns M, Saarelainen S, Laine S *et al*. Cytokines in bronchoalveolar lavage fluid and serum of lung cancer patients during radiotherapy—association of interleukin-8 and VEGF with survival. *Cytokine* 2009; **50**: 30–6.
- 24 Striz I, Wang YM, Svarcova I *et al*. The phenotype of alveolar macrophages and its correlation with immune cells in bronchoalveolar lavage. *Eur. Respir. J.* 1993; **6**: 1287–94.
- 25 Ishii H. High serum concentrations of surfactant protein A in usual interstitial pneumonia compared with non-specific interstitial pneumonia. *Thorax* 2003; **58**: 52–7.
- 26 Ward C. Evaluation of albumin as a reference marker of dilution in bronchoalveolar lavage fluid from asthmatic and control subjects. *Thorax* 1993; **48**: 518–22.
- 27 De Jong PM, Van Sterkenburg MAJA, Kempenaar JA *et al*. Serial culturing of human bronchial epithelial cells derived from biopsies. *In Vitro Cell Dev. Biol. Anim.* 1993; **29A**: 379–87.
- 28 Ackerman V. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* 1994; **105**: 687–96.
- 29 Laitinen LA, Laitinen A, Altraja A *et al*. Bronchial biopsy findings in intermittent or “Early” asthma. *J. Allergy Clin. Immunol.* 1996; **98**: S3–6.
- 30 Bradley BL, Azzawi M, Jacobson M *et al*. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* 1991; **88**: 661–74.
- 31 Bayram H. Comparison of ciliary activity and inflammatory mediator release from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients and the effect of diesel exhaust particles *in vitro*. *J. Allergy Clin. Immunol.* 1998; **102**: 771–82.
- 32 Devalia JL, Bayram H, Abdelaziz MM *et al*. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int. Arch. Allergy Immunol.* 1999; **118**: 437–9.
- 33 Campbell AM, Chanez P, Vignola AM *et al*. Functional characteristics of bronchial epithelium obtained by brushing from asthmatic and normal subjects. *Am. Rev. Respir. Dis.* 1993; **147**: 529–34.
- 34 Vignola AM, Chanez P, Campbell AM *et al*. Quantification and localization of HLA-DR and intercellular adhesion molecule-1 (ICAM-1) molecules on bronchial epithelial cells of asthmatics using confocal microscopy. *Clin. Exp. Immunol.* 1994; **96**: 104–9.
- 35 Ketterer MR. Infection of primary human bronchial epithelial cells by *Haemophilus influenzae*: macropinocytosis as a mechanism of airway epithelial cell entry. *Infect. Immun.* 1999; **67**: 4161–70.
- 36 Lordan JL, Bucchieri F, Richter A *et al*. Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. *J. Immunol. Methods* 2002; **169**: 407–14.
- 37 Bucchieri F. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am. J. Respir. Cell Mol. Biol.* 2002; **27**: 179–85.
- 38 Doherty GM, Christie SN, Skibinski G *et al*. Non-bronchoscopic sampling and culture of bronchial epithelial cells in children. *Clin. Exp. Allergy* 2003; **33**: 1221–25.
- 39 Wallace JM, Deutsch AL, Harrell JH *et al*. Bronchoscopy and transbronchial biopsy in evaluation of patients with suspected active tuberculosis. *Am. J. Med.* 1981; **70**: 1189–94.
- 40 Sarkar SK. Fiberoptic bronchoscopy in the diagnosis of pulmonary tuberculosis. *Tubercle* 1980; **61**: 97–9.
- 41 Lane C. The use of non-bronchoscopic brushings to study the paediatric airway. *Resp. Res.* 2005; **6**: 53–63.
- 42 Kicic A, Sutanto EN, Stevens PT *et al*. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am. J. Respir. Crit. Care Med.* 2006; **174**: 1110–18.
- 43 Trapnell BC, Chu CS, Paakko PK *et al*. Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc. Natl Acad. Sci. USA* 1991; **88**: 6565–69.
- 44 Hamosh A. Severe deficiency of cystic fibrosis transmembrane conductance regulator messenger RNA carrying nonsense mutations R553X and W1316X in respiratory epithelial cells of patients with cystic fibrosis. *J. Clin. Invest.* 1991; **88**: 1880–5.
- 45 Sutanto EN, Kicic A, Foo CJ *et al*. Innate inflammatory responses of pediatric cystic fibrosis airway epithelial cells: effects of non-viral and viral stimulation. *Am. J. Respir. Cell Mol. Biol.* 2011; **44**: 761–7.
- 46 Campbell AM. Low-affinity receptor for IgE on human bronchial epithelial cells in asthma. *Immunology* 1994; **82**: 506–8.
- 47 Guo FH. Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. *J. Immunol.* 2000; **164**: 5970–80.
- 48 McNamara PS, Kicic A, Sutanto EN *et al*. Comparison of techniques for obtaining lower airway epithelial cells from children. *Eur. Respir. J.* 2008; **32**: 763–8.
- 49 Banerjee B, Kicic A, Musk M *et al*. Successful establishment of primary small airway cell cultures in human lung transplantation. *Respir. Res.* 2009; **10**: 99–108.
- 50 Stevens PT, Kicic A, Sutanto EN *et al*. Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1. *Clin. Exp. Allergy* 2008; **38**: 1901–10.
- 51 Lopez-Souza N, Favoreto S, Wong H *et al*. *In vitro* susceptibility to rhinovirus infection is greater for bronchial than for nasal airway epithelial cells in human subjects. *J. Allergy Clin. Immunol.* 2009; **123**: 1384–90.
- 52 McDougall CM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *Am. J. Respir. Cell Mol. Biol.* 2008; **39**: 560–8.
- 53 Lopez-Souza N, Dolganov G, Dubin R *et al*. Resistance of differentiated human airway epithelium to infection by rhinovirus. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2004; **286**: L373–81.
- 54 Kicic A. Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium. *Am. J. Respir. Crit. Care Med.* 2010; **181**: 889–98.
- 55 Devalia JL. Culture and comparison of human bronchial and nasal epithelial cells *in vitro*. *Respir. Med.* 1990; **84**: 303–12.
- 56 Devalia JL. Human nasal and bronchial epithelial cells in culture: an overview of their characteristics and function. *Allergy Asthma Proc.* 1991; **12**: 71–9.
- 57 Mosler K, Coraux C, Fragaki K *et al*. Feasibility of nasal epithelial brushing for the study of airway epithelial functions in CF infants. *J. Cyst. Fibros.* 2008; **7**: 44–53.
- 58 Calderón MA, Devalia JL, Prior A *et al*. A comparison of cytokine release from epithelial cells cultured from nasal biopsy specimens of atopic patients with and without rhinitis and non-atopic subjects without rhinitis. *J. Allergy Clin. Immunol.* 1997; **99**: 65–76.
- 59 Chanez P, Vignola Antonio M, Vic P *et al*. Comparison between nasal and bronchial inflammation in asthmatic and control subjects. *Am. J. Respir. Crit. Care Med.* 1999; **159**: 588–95.
- 60 Vignola AM, Campbell AM, Chanez P *et al*. HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. *Am. Rev. Respir. Dis.* 1993; **148**: 689–94.
- 61 Gibson PG. Chronic cough resembles asthma with IL-5 and granulocyte-macrophage colony-stimulating factor gene expression in bronchoalveolar cells. *J. Allergy Clin. Immunol.* 1998; **101**: 320–6.
- 62 Hilty M, Burke C, Pedro H *et al*. Disordered microbial communities in asthmatic airways. *PLoS One* 2010; **5**: e8578–86.
- 63 Huang YJ, Nelson CE, Brodie EL *et al*. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J. Allergy Clin. Immunol.* 2011; **127**: 372–81.

- 64 Borthwick LA, Parker SM, Brougham KA *et al.* Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation. *Thorax* 2009; **64**: 770–7.
- 65 Romagnoli M, Vachier I, Vignola AM *et al.* Safety and cellular assessment of bronchial brushing in airway diseases. *Respir. Med.* 1999; **93**: 461–6.
- 66 Vega-Carrascal I, Reeves EP, Niki T *et al.* Dysregulation of TIM-3 Galectin-9 pathway in the cystic fibrosis airways. *J. Immunol.* 2011; **186**: 2897–09.
- 67 Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J. Allergy Clin. Immunol.* 1999; **104**: 72–8.
- 68 Carson JL, Collier AM, Gambling TM *et al.* Ultrastructure of airway epithelial cell membranes among patients with cystic fibrosis. *Hum. Pathol.* 1990; **21**: 640–7.
- 69 Dannhoffer L, Blouquit-Laye S, Regnier A *et al.* Functional properties of mixed cystic fibrosis and normal bronchial epithelial cell cultures. *Am. J. Respir. Cell Mol. Biol.* 2009; **40**: 717–23.
- 70 Blouquit S, Regnier A, Dannhoffer L *et al.* Ion and fluid transport properties of small airways in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 2006; **174**: 299–5.
- 71 Ribeiro CM, Paradiso AM, Schwab U *et al.* Chronic airway infection/inflammation induces a Ca²⁺ i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia. *J. Biol. Chem.* 2005; **280**: 17798–806.
- 72 Terajima M, Yamaya M, Sekizawa K *et al.* Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1 β . *Am. J. Physiol.* 1997; **273**: L749–59.
- 73 Wojnarowski C, Frischer T, Hofbauer E *et al.* Cytokine expression in bronchial biopsies of cystic fibrosis patients with and without acute exacerbation. *Eur. Respir. J.* 1999; **14**: 1136–44.
- 74 Perez A, Issler AC, Cotton CU *et al.* CFTR inhibition mimics the cystic fibrosis inflammatory profile. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2007; **292**: L383–95.
- 75 Hybiske K, Fu Z, Schwarzer C *et al.* Effects of cystic fibrosis transmembrane conductance regulator and Δ F508CFTR on inflammatory response, ER stress, and Ca²⁺ of airway epithelia. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2007; **293**: L1250–60.
- 76 Heeckeren A, Walenga R, Konstan MW *et al.* Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with *Pseudomonas aeruginosa*. *J. Clin. Invest.* 1997; **100**: 2810–15.
- 77 Gosselin D. Impaired ability of CFTR knockout mice to control lung infection with *Pseudomonas aeruginosa*. *Am. J. Respir. Crit. Care Med.* 1998; **157**: 1253–62.
- 78 Gavilanes X, Huaux F, Meyer M *et al.* Azithromycin fails to reduce increased expression of neutrophil-related cytokines in primary-cultured epithelial cells from cystic fibrosis mice. *J. Cyst. Fibros.* 2009; **8**: 203–10.
- 79 Kube D, Sontich U, Fletcher D *et al.* Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2001; **280**: L493–502.
- 80 Stecenko AA, King G, Torii K *et al.* Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation* 2001; **25**: 145–55.
- 81 Aldallal N, McNaughton EE, Manzel LJ *et al.* Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 2002; **166**: 1248–56.
- 82 Verbeek E. Chloride transport in cultured nasal epithelium of cystic fibrosis patients. *Pflügers Archiv* 1990; **415**: 540.
- 83 Carrabino S, Carpani D, Livraghi A *et al.* Dysregulated interleukin-8 secretion and NF- κ B activity in human cystic fibrosis nasal epithelial cells. *J. Cyst. Fibros.* 2006; **5**: 113–19.
- 84 Norez C, Antigny F, Noel S *et al.* A cystic fibrosis respiratory epithelial cell chronically treated by miglustat acquires a non-cystic fibrosis-like phenotype. *Am. J. Respir. Cell Mol. Biol.* 2009; **41**: 217–25.
- 85 Machen TE. Innate immune response in CF airway epithelia: hyperinflammatory? *Am. J. Physiol. Cell Physiol.* 2006; **291**: C218–30.
- 86 Moeller A, Horak F Jr, Lane C *et al.* Inducible NO synthase expression is low in airway epithelium from young children with cystic fibrosis. *Thorax* 2006; **61**: 514–20.
- 87 Verkman AS. Development and biological applications of chloride-sensitive fluorescent indicators. *Am. J. Physiol.* 1990; **259**: C375–88.
- 88 Galletta LV, Jayaraman S, Verkman AS. Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists. *Am. J. Physiol. Cell Physiol.* 2001; **281**: C1734–42.
- 89 Li W, Zhang L, Johnson JS *et al.* Generation of novel AAV variants by directed evolution for improved CFTR delivery to human ciliated airway epithelium. *Mol. Ther.* 2009; **17**: 2067–77.
- 90 Granio O. Improved adenovirus type 5 vector-mediated transduction of resistant cells by piggybacking on coxsackie B-adenovirus receptor-pseudotyped baculovirus. *J. Virol.* 2009; **83**: 6048–66.
- 91 Kwilas AR, Yednak MA, Zhang L *et al.* Respiratory syncytial virus engineered to express the cystic fibrosis transmembrane conductance regulator corrects the bioelectric phenotype of human cystic fibrosis airway epithelium *in vitro*. *J. Virol.* 2010; **84**: 7770–81.
- 92 Cigana C, Assael BM, Melotti P. Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. *Antimicrob. Agents Chemother.* 2007; **51**: 975–81.
- 93 Ribeiro CM, Hurd H, Wu Y *et al.* Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia. *PLoS One* 2009; **4**: e5806–21.
- 94 Kukafka DS, O'Brien GM, Furukawa S *et al.* Surveillance bronchoscopy in lung transplant recipients. *Chest* 1997; **111**: 377–81.
- 95 Matsuo T, Colt HG. Evidence against routine scheduling of surveillance bronchoscopy after stent insertion. *Chest* 2000; **118**: 1455–9.
- 96 Forrest IA, Murphy DM, Ward C *et al.* Primary airway epithelial cell culture from lung transplant recipients. *Eur. Respir. J.* 2005; **26**: 1080–85.
- 97 Ward C, Forrest IA, Murphy DM *et al.* Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients. *Thorax* 2005; **60**: 865–71.
- 98 Hodge S, Holmes M, Banerjee B *et al.* Posttransplant bronchiolitis obliterans syndrome is associated with bronchial epithelial to mesenchymal transition. *Am. J. Transplant.* 2009; **9**: 727–33.
- 99 Christie JD, Edwards LB, Kucheryavaya AY *et al.* The registry of the international society for heart and lung transplantation: twenty-seventh official adult lung and heart-lung transplant report—2010. *J. Heart Lung Transplant.* 2010; **29**: 1104–18.
- 100 Borger P, Kauffman HF, Timmerman JAB *et al.* Cyclosporine, FK506, mycophenolate mofetil and prednisolone differentially modulate cytokine gene expression in human airway-derived epithelial cells. *Transplantation* 2000; **69**: 1408–13.
- 101 Elssner A, Jaumann F, Dobmann S *et al.* Elevated levels of interleukin-8 and transforming growth factor- β in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. *Transplantation* 2000; **70**: 362–7.
- 102 Elssner A, Jaumann F, Wolf WP *et al.* Bronchial epithelial cell B7-1 and B7-2 mRNA expression after lung transplantation: a role in allograft rejection? *Eur. Respir. J.* 2002; **20**: 165–9.
- 103 Murphy DM. Effect of azithromycin on primary bronchial epithelial cells derived from stable lung allografts. *Thorax* 2007; **62**: 834–5.
- 104 Murphy DM, Forrest IA, Corris PA *et al.* Simvastatin attenuates release of neutrophilic and remodelling factors from primary

- bronchial epithelial cells derived from stable lung transplant recipients. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2008; **294**: L592–9.
- 105 Biernacki WA, Kharitonov SA, Barnes PJ. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax* 2003; **58**: 294–8.
- 106 Kharitonov SA, Barnes PJ. Exhaled biomarkers. *Chest* 2006; **130**: 1541–6.
- 107 Fabbri LM, Romagnoli M, Corbetta L *et al.* Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 2003; **167**: 418–24.
- 108 Rutgers SR, Postma DS, ten Hacken NHT *et al.* Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000; **55**: 12–18.
- 109 Powell CA. Loss of heterozygosity in epithelial cells obtained by bronchial brushing: clinical utility in lung cancer. *Clin. Cancer Res.* 1999; **5**: 2025–34.
- 110 Wistuba II. Molecular damage in the bronchial epithelium of current and former smokers. *J. Natl Cancer Inst.* 1997; **89**: 1366–73.
- 111 Franklin WA. Widely dispersed p53 mutation in respiratory epithelium. A novel mechanism for field carcinogenesis. *J. Clin. Invest.* 1997; **100**: 2133–7.
- 112 Guo M. Promoter hypermethylation of resected bronchial margins. *Clin. Cancer Res.* 2004; **10**: 5131–6.
- 113 Miyazu YM. Telomerase expression in noncancerous bronchial epithelia is a possible marker of early development of lung cancer. *Cancer Res.* 2005; **65**: 9623–7.
- 114 Spira A. Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc. Natl Acad. Sci. USA* 2004; **101**: 10143–8.
- 115 Schulz C, Kratzel K, Wolf K *et al.* Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD. *Chest* 2004; **125**: 1706–13.
- 116 Takizawa H, Tanaka M, Takami K *et al.* Increased expression of inflammatory mediators in small-airway epithelium from tobacco smokers. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2000; **278**: L906–13.
- 117 Harvey BG, Heguy A, Leopold PL *et al.* Modification of gene expression of the small airway epithelium in response to cigarette smoking. *J. Mol. Med.* 2007; **85**: 39–53.
- 118 Hackett NR, Heguy A, Harvey BG *et al.* Variability of antioxidant-related gene expression in the airway epithelium of cigarette smokers. *Am. J. Respir. Cell Mol. Biol.* 2003; **29**: 331–43.
- 119 Zhang X, Sebastiani P, Liu G *et al.* Similarities and differences between smoking-related gene expression in nasal and bronchial epithelium. *Physiol. Genomics* 2010; **41**: 1–8.
- 120 Spira A, Beane JE, Shah V *et al.* Airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer. *Nat. Med.* 2007; **13**: 361–6.
- 121 Voss JS, Kipp BR, Halling KC *et al.* Fluorescence in situ hybridization testing algorithm improves lung cancer detection in bronchial brushing specimens. *Am. J. Respir. Crit. Care Med.* 2010; **181**: 478–85.
- 122 Crystal RG. Future research directions in idiopathic pulmonary fibrosis: summary of a national heart, lung, and blood institute working group. *Am. J. Respir. Crit. Care Med.* 2002; **166**: 236–46.