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Establishing a wound healing model in well differentiated cultures of primary human nasal epithelial cells — Source link

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Published on: 13 Sep 2019

Topics: Wound healing, Respiratory infection and Respiratory epithelium

THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Establishing a wound healing model in well differentiated cultures of primary human nasal epithelial cells

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Bachelor of Science (Honours)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2018

School of Medicine

Abstract

The nasal epithelium is the initial contact between the external environment and the respiratory tract. Its response to noxious stimuli and how it repairs after damage is important in many respiratory diseases, including Asthma. Growing airway epithelial cells in culture at the air-liquid interface allows for a physiologically relevant model of the human upper airways. The aim of this thesis was to characterize human primary nasal epithelial cells grown at the air-liquid interface and establish a model for use in wound healing assays.

This study determined the time required for full differentiation of nasal epithelial cells in an air-liquid interface culture to be at least 7 weeks using the standardised ALI differentiation techniques in two media types B-ALI and PC-ALI. Using this model, nasal epithelial cultures from healthy, atopic, non-atopic asthmatic and atopic asthmatic subjects were differentiated at air-liquid interface and manually wounded. Wounds were monitored over time until complete closure using a time lapse imaging microscope and with cultures identified to have a rate of wound healing (%/hour) independent of initial wound size. No significant difference in the wound healing rate in cells differentiated in B-ALI (3.7 ± 1.8 %/hour) and PC-ALI (5.3 ± 1.9 %/hour) when compared. Testing robustness of the model by EGFR inhibition caused the rate of wound healing to drop a significant 3.6%/hour with there being no closure of the wound after 48 hours. There was significant difference in the rate of repair for atopic subjects (2.9 ± 1.8 %/hour) when compared to healthy controls (4.3 ± 1.9 %/hour), and for non-atopic asthmatic (4.1 ± 1.1 %/hour) and with a significant difference in barrier polarization between non-atopic asthmatic and atopic asthmatic subject cultures.

Restoration of the faulty repair mechanisms were monitored by the addition of EGF to the ALI cultures over time of wound healing. EGF was added 7 days prior to wounding and caused no change in the rate of wound closure. Remodelling of the epithelium was reduced by the addition of IL-13 but inhibition of the cytokine had no effect on wound repair. Lastly, respiratory infection that occurs at the airway epithelium was tested by infecting the ALI cultures with green fluorescent-tagged RSV-A2 at MOI 1 and 0.1 and monitored for the effect on wound healing. A significant decrease in rate of wound healing was seen for all phenotypes at a MOI 1 after a 6 day infection with no significant difference in viral titre in subject groups over the infection period, leading to further analysis into a steroid responsive, Th-2 driven defect in wound healing.

The robust wound healing model established in this study will be essential for studying factors influencing wound healing, including host disease status and environmental exposures in the future.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications included in this thesis

Peer-reviewed Articles

Schagen J, Sly PD, Fantino E., Characterizing well-differentiated culture of primary human nasal epithelial cells for use in wound healing assays, Lab Invest. 2018 Nov;98(11):1478-1486. doi: 10.1038/s41374-018-0100-1. Epub 2018 Aug 8.

Conference Presentations

1. 15-minute oral presentation at the Epithelial Cell Workshop, Newcastle, Australia, 2015.
2. Poster presentation at the Lorne Infection and Immunity conference, Lorne, Australia, 2017.
3. 3-minute oral presentation at the Lorne Infection and Immunity conference, Lorne, Australia, 2017.
4. 3-minute oral presentation at the 3MT competition University of Queensland, Brisbane, 2017.
5. 10-minute oral presentation at the Children's Health Research Centre High Degree Research Student Symposium, Brisbane, Australia, 2018.

Submitted manuscripts included in this thesis

I have chosen to incorporate one peer-reviewed publication into my thesis as per UQ policy (PPL 4.60.07 Alternative Thesis Format Options).

Schagen J, Sly PD, Fantino E., Characterizing well-differentiated culture of primary human nasal epithelial cells for use in wound healing assays, Laboratory Investigation, (Not yet printed).

Contributor	Statement of contribution
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Author: Fantino E	Conceptualized the study (40%) Data analysis (5%) Edited the manuscript (30%)

Other publications during candidature

No other publications.

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This study and thesis received scientific wisdom, guidance and thoughtful feedback from two supervisors, Dr. Emmanuelle Fantino (UQ) and Professor Peter Sly (UQ).

Recruitment and nasal epithelial cell collection from adults and children were carried out by Sally Galbraith, Carmen Jones and Anna Henningham at the Children Health Queensland (Royal Children Hospital/Lady Cilento Children's Hospital), Brisbane Australia.

Voluntary participation of all donors was greatly appreciated.

The studies reported in this thesis were supported by grants from the National Health and Medical Research Council, Australia, and Asthma Foundation Queensland Charles Mitchell PhD Scholarship, The Asthma Foundation, Australia.

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

The project was approved by the human research ethics committees of Children's Health Queensland (2011000058) and of The University of Queensland (2017000520).

Acknowledgements

Firstly, I would like to thank my supervisor's Dr Emmanuelle Fantino and Professor Peter Sly, for giving me the opportunity to conduct my PhD under their guidance and as part of the CHEP team. Your never ending support, knowledge and kindness towards my PhD helped guide me in the right direction throughout my project.

I would also like to thank all the members in the CHEP team for helping me complete experiments, provide laughs and most importantly feed my cells.

From this experience I have made two very great friends and I would like to thank both Claire and Karin for always being a fit outlet to keep any unwanted stress at bay.

Lastly I would like to thank my family for just being them and always helping me see the bigger picture. And Gen, you bore the brunt of all the stress, late nights and missed events. Thanks for keeping me fed and happy.

Financial support

This research was supported by an Australian Government Research Training Program Scholarship, and the Asthma Foundation Australia.

Keywords

Epithelial cells, Wound healing, Repair, Air-liquid Interface, Respiratory Syncytial Virus, Epidermal Growth Factor.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110203, Respiratory Diseases. 50%

ANZSRC code: 110707, Innate Immunity, 25%

ANZSRC code: 060502, Infectious Agents, 25%

Fields of Research (FoR) Classification

FoR code: 1107, Immunology, 70%

FoR code: 0601, Biochemistry and Cell Biology, 15%

FoR code: 0605, Microbiology, 15%

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List of Abbreviations

3D	Three-dimensional
°C	Degrees Celsius
µl	Micro liter
ALI	Air-liquid interface
B-ALI	Bronchial Air Liquid Interface Media
BEGM	Bronchial epithelial growth medium
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
CHEP	Children's Health and Environment Program
COAST	The childhood origins of asthma
COPD	Chronic Obstructive Pulmonary Disease
CT	Cycle threshold
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FOXA2	Forkhead box protein A2
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO	Growth-regulated oncogene
GWAS	Genome-wide association studies
HBE	Human bronchial epithelial
HCl	Hydrochloride
hEGF	Human epidermal growth factor
HMGB1	High mobility group box 1
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
iLCs	Innate lymphoid cells
IP-10	Interferon gamma-induced protein 10
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MCP-3	Monocyte-chemotactic protein 3
MDC	Macrophage-derived chemokine
MIP	Macrophage Inflammatory Proteins
mL	Millilitre
MOI	Multiplicity of infection
MUC5B	Mucin 5B
MyD88	Myeloid differentiation primary response 88
NTHi	Haemophilus influenza
ORMDL3	Orosomucoid like 3

PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid–Schiff stain
PBS	Phosphate buffered saline
PC-ALI	PneumaCult™-ALI Medium
PC-ExPlus	PneumaCult™-Ex Plus Medium
PDGF	Platelet-derived growth factor
pfu	Plaque forming units
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT	Real time
RV	Rhinovirus
SP	Streptococcus pneumonia
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor
STAT6	Signal transducer and activator of transcription 6
TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor beta
Th2	T helper 2
TLRs	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TSLR	Thymic stromal lymphopoietin
VAMPS	Vesicle-associated membrane protein

Chapter 1 Literature Review

1.1 Thesis Outline

This thesis is presented in three stages with the first being model development where a reliable and repeatable model for wound healing was established. After this, the model was tested using cells from healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects, with the final stage testing the model against RSV infection. Each stage of the thesis is outlined in each chapter with the first being a comprehensive review of the literature and the last an informed discussion on the data developed in this thesis.

1.2 Background

Asthma is a chronic inflammatory disease of the airways that effects people of all ages with symptoms of shortness of breath and wheezing. This is caused by obstruction due to hyper reactivity, inflammation and remodelling, causing a narrowing of the airway (Figure 1-1).

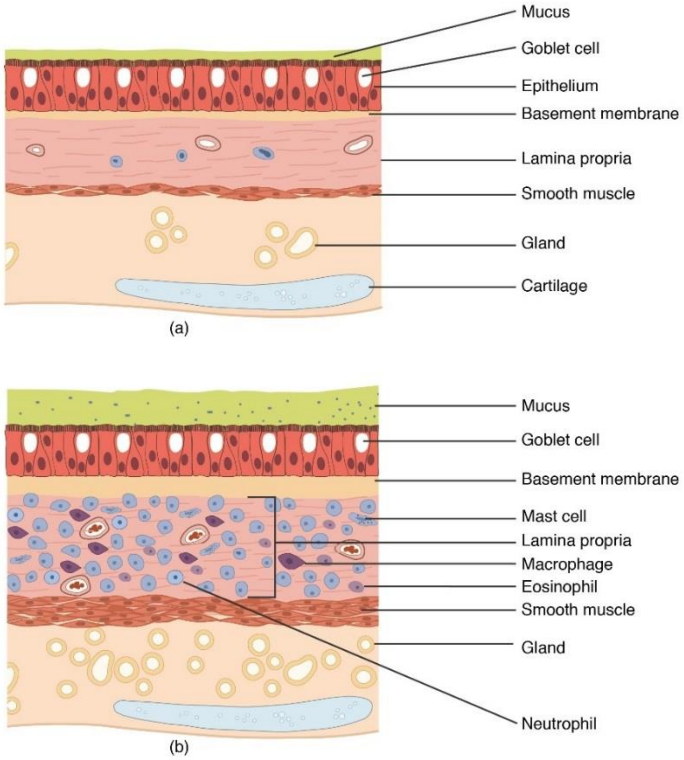


Figure 1-1 Airway tissue. a) Normal airway tissue and b) airway tissue during an asthma attack, with a thickened mucosa, increased mucus-producing goblet cells, and eosinophil infiltrates(1).

Asthma most commonly develops in early childhood, and more than three-quarters of children develop asthma symptoms before age 7. However, asthma can develop at any stage in life, including adulthood and often causes a reduced quality of life(2). Diagnosis of asthma in children 5 years and younger is difficult because even those without asthma can present with episodic respiratory symptoms(3, 4).

Asthma occurrence in Australia is at epidemic proportions with 10.2% of the population diagnosed with asthma in 2011-2012, and 9% in children alone(5), causing an economic burden on the Australian community especially as its negative impact on productivity is as large as its direct costs.

The initiation of asthma is through the exposure to pathogens that cause respiratory tract infection and involve a number of factors including single-to-complex combination of environmental, immunological and host genetic factors. In epidemiological studies a key correlation has been detected between asthma and infection by common respiratory viruses or bacteria(6, 7). This infection attacks the airway epithelium triggering a rapid antiviral response against these pathogens.

The airway epithelium lining the respiratory tract has many important and complex functions that include; the production of cytokines and mediators that recruit and activate inflammatory cells(8, 9), the removal of environmental pollutants (10), and secretion of factors regulating fibroblasts and smooth muscle function(11). Recent technical developments into airway epithelial cell culture has provided a new, more physiological model that will provide further advances. Epithelial cells grown at air-liquid interface (ALI) have replaced submerged cell culture as the experimental standard for asthma and respiratory research as a more physiological relevant model. ALI culture conditions are modified to enable cellular polarization and causes a powerful phenotypic conversion that more closely mirrors the normal *in vivo* morphology of the airways.

In this project, I propose to establish and characterize a wound healing model of airway epithelial cells and how viral exposure can alter this response, especially in asthmatics.

1.3 Asthma

1.3.1 Asthma Prevalence

The estimated number of Australians affected by asthma is set to rise to 3 million sufferers by 2030(12) and in higher income countries, like Australia and the United States of America, the prevalence is higher (>10%) compared to developing and lower income countries(13). Males between the ages of 5 to 9, have the highest prevalence rate of reported asthma and currently in Australia 9.9% of the population suffer, with 54% female and 46% male(12).

On a more global scale 14% of the world's children experience asthma symptoms, with asthma being the 14th most important disorder in the world in terms of extent and duration of disability as reported by the Global Asthma Network(2). Mortality rates are comparatively low relative to other chronic conditions with asthma contributing to less than 1% of all deaths in most countries worldwide(12). This low number of asthma related deaths is because many are preventable, however asthma is an ongoing and recurrent disease that can cause long term disability and reduced quality of life

1.3.2 What we don't know about asthma

Sly et al, 2010 has identified two important scientific gaps in the knowledge of asthma development. These include whether viral infections in early life cause asthma through the damaging of respiratory and immune system development, or if the infections just identify susceptibility for asthma in genetically predisposed infants. Also, of interest is the involvement of viruses and if the associations simply reflect the epidemiology of respiratory viral infections in early life(14).

Studies have been conducted that answer similar questions such as The Childhood Origins of Asthma (COAST) study that looked at the contribution and interactions and patterns of cytokine secretion and virus infection to understand the development of asthma. A cohort of 287 children were recruited that had an increased risk of developing asthma and found that lower respiratory tract infection with rhinovirus had an association with asthma in children at 6 years of age(15, 16). The Childhood Asthma Study also looked at a similar cohort of children in the first year of life finding rhinovirus and respiratory syncytial virus associated wheezing a significant risk factor for asthma(17).

1.3.3 Causes/Risk factors for asthma

Asthma can be the result of a wide variety of factors either biological or environmental. The underlying cause of asthma is still not identified and can be the result from complex interactions of different genetic, environmental and lifestyle factors(18). Asthma risk factors include genetic predisposition, environmental exposures, family history, cytokines in uterine environment, environmental protection against Th2-mediated sensitization, infection and allergen interaction(19, 20). Environmental and genetic factors interact in a complex manner to alter disease susceptibility and expression and will be explored in more depth in the following sections.

1.3.4 Genetics

Many genetic predispositions for phenotypic features of asthma include low lung function, increased susceptibility to lower respiratory infections in early life, delayed immune maturation, allergic sensitization, and airway modelling with decline in lung function(20). A common genetic predisposition for asthma is linked with variations in the genes that encode Toll-like receptors, CD14, and genes expressed in the epithelium and innate immune pathways(14). Many studies have been aimed at the discovery of genes that identify individuals with the predisposition of asthma hoping to lead to improvements in diagnosis, prevention and treatment(21). Previously the focus has been on genes associated with atopy and serum IgE, such as HLA-DQ locus, FCER1A, STAT6 and IL13(22, 23). Identifying these genetic risk factors will be useful in identifying subtypes of asthma and determining the phenotypes of the disease.

1.3.5 Allergy and environmental factors

The risk of developing chronic asthma is increased through early life exposures to environmental factors. This disturbs the normal lung growth and delays the maturation of the immune system, causing higher susceptibility to wheeze and increased frequency and severity of viral infection(16, 24). The disruption of tight junctions and the activation of protease-activated receptors has been shown to be linked to components of proteolytic allergens such as, house dust mite, cockroach, animal and fungal allergens(25-28). Other environmental stimuli that impair barrier function include respiratory viruses and air pollutants such as ozone, particulates and tobacco smoke(29, 30). Sensitization to common aeroallergen in children is likely to cause ongoing wheezy symptoms and lower lung function at school age.

Along with the association between allergy and asthma, atopic children are frequently seen to have atopic dermatitis preceding asthma. This suggests that there could an epicutaneous allergen transfer that contributes to the development of asthma(31) and also be an indication of a causal link. Research is now being focused on how these strategies will help identify and treat children at high risk of asthma.

Atopy occurs when the immune system is dysregulated causing allergic inflammation. This dysregulation is caused by genetic and environmental factors which lead to the development of an atopic disease. Atopic asthma is the most common form of asthma in children and is characterized by eosinophilic airway inflammation associated with specific immunoglobulin E (IgE) antibodies sensitization to various allergens(32). There are problems with defining atopy as it is defined as either sensitized or non-sensitized, with no range of the severity disease being measured. This is difficult when in combination with asthma because recent data indicate that atopy may also encompass distinct endotypes characterized by different patterns of association with asthma(33). This makes it hard to define the relationship between asthma and atopy and this had been discussed in the review article by Comberiati, looking at the burden and heterogeneity of both diseases(34). Having an understanding of each disease individually and in combination might better define the cause of atopy in relation to asthma.

1.3.6 The hygiene hypothesis

Asthma in young adults and children is often associated with allergies (atopic asthma), with this type of asthma seen most commonly in the westernized and urbanized societies. The true cause of this is not known but many theories have been established to explain this phenomenon. One of these is the "Hygiene Hypothesis" in which data from a publication by Strachan suggests that infant exposure to an environment rich in microbial organisms helps to establish resistance to asthma(35). Factors such as living with pets, several brothers and sisters, and living on a farm have been shown to be protective against asthma and atopy(36-40). However, development of asthma appears to be much more complex with the hygiene hypothesis unlikely to be the sole explanation. Exposure and interaction to pathogens, microbial compounds and genetics have roles in the innate and adaptive immune responses and the development of asthma.

1.3.7 Innate immunity

The defensive response against pathogens and microorganisms, such as virus, bacteria and fungi, and epithelial injury, is initiated by the innate immune system through phagocytosis and secretion of inflammatory and antimicrobial mediators. Epithelial cells of the airways react in parallel with inflammatory and chemotactic mediators to produce and secrete antimicrobial peptides and surface proteins (Figure 1-2)(41, 42).

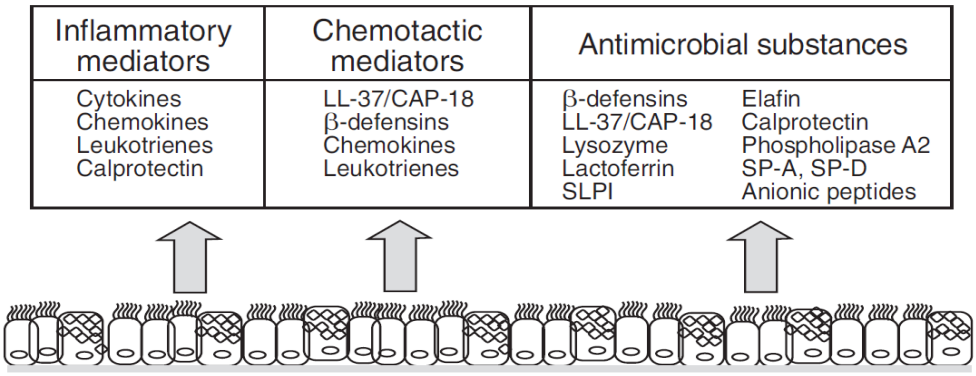


Figure 1-2 Molecules secreted by the airway epithelium in defence against infection. Some of the molecules are secreted primary to the basolateral side (chemokines) or apically secreted (antimicrobial peptides)(10).

Several innate immunological signalling pathways, cytokines and chemokines augment the airway adaptive immunity and inflammation(10), such as toll-like receptors (TLRs). These are pattern-recognition receptors (PRRs) that mediate recognition and response to pathogen-associated molecular patterns (PAMPs) through microbial, fungal and viral products and their ligands(43, 44). Toll-like receptors are an important family of proteins involved in the recognition of microorganisms with 11 TLRs identified in humans and all being expressed in the epithelium. Some of these TLRs are used by the airways to sense and initiate innate and adaptive immunity response to pathogens. TLRs are either located on the cell surface (TLR1, TLR2, TLR4-6) or in the cytoplasm (TLR3, TLR7-9)(45) and are activated after interaction with pathogens causing the production of thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, with TSLP preferentially driving Th2 inflammation linking it to the severity of allergic disease.(46). TLR3 and TLR7 is key in the detection of viruses and its transcription is induced when there is an infection(47-49), with TLR2(50) shown to regulate mucin gene expression through pro-inflammatory signalling, activating MAPK(51) and EGFR(52, 53).

Studies by Wark and Contoli also show that when viral load is high and during virus induced asthma exacerbation, type I (IFN-β) and III interferon (IFN-λ) production are impaired in epithelial cells and macrophages(54, 55). Cytokines and chemokines are also important in the development and progression of inflammation, mucus production and airway remodelling, with IL-4, IL-5, IL-13 and TNF-α being the focus in asthma pathogenesis(44). This is seen by IL-4 regulating Th2 cell survival, and with IL-13 regulating B cell isotype by switching to IgE synthesis(56). These studies into the innate immunity of asthma demonstrate the complexity of pathways regulating inflammation, immunity and wound repair.

On a cellular level, airway dendritic cells are trigged by the release of TSLP and IL-13 from epithelial cells. This, with IL-25 and IL33, act on mast cells, basophils and innate type 2 lymphocytes to cause mucous metaplasia and airway smooth muscle contraction, causing asthma(57). Numerous other cell processes and interactions occur during an asthma exacerbation, such as airway smooth muscle contraction, but for this thesis the airway epithelium will be the focus.

1.4 The Airway Epithelium

The airway epithelium acts as a physical barrier protecting the host from inhaled external environment contaminants from chemical and particulate pollutants to pathogens. It coordinates the physical trapping and removal of these harmful substances by secretion of mucus, antimicrobial factors, antioxidants, and protease inhibitors, with recruitment of nonspecific inflammatory cells like neutrophils and monocytes to act as the airway innate host defence(58). The airway epithelium also plays important roles in the modulating of inflammation and adaptive immunity through dendritic cell function and specific T and B cells. Studies from Nussbaum et al, Locksley, Bando et al, Scanlon and McKenzie, and Barlow et al report that the secretion of cytokines TSLP, IL-25, and IL-33 only occur when the airway epithelial cells are activated by inhaled stimuli such as proteases, inhaled allergens, respiratory viruses and air pollutants(59-63). These cytokines recruit both innate and adaptive hematopoietic cells through mast, dendritic and innate lymphoid cells (iLCs), and initiate the release of T helper 2 (Th2) cytokines, mainly IL-5 and IL-13(57).

Two important barrier and defence functions are essential to the airway epithelium, the formation of tight junctions and cell polarisation. As reported by Matsui, tight junctions regulate the permeability

barrier by limiting the passive flow of molecules between the apical and basolateral compartments. This is coupled with cell polarisation to allow directional transport of molecules through the epithelial layer(64). The epithelium is divided into different classes based on cell morphology, with the upper airway being focused on in this thesis (Figure 1-3) because it is the first point of contact for inhaled external contaminants. In the upper airways the trachea and bronchi are composed of pseudostratified columnar epithelium(65), consisting of three cell types; ciliated cells, basal cells, and goblet cells that produce mucin. The pseudostratified layer is complex with all cells having contact with the basement membrane but with only the ciliated and goblet reaching the epithelium surface.

Research is now finding that the epithelial defences are critical in containing infection and triggering an acquired immune response, with epithelial disruption believed to contribute in the pathogenesis of several lung diseases including asthma as Jakiela et al found in their study of rhinovirus (RV) and its effect on bronchial epithelial cells(66). Toll-like receptors (TLRs) and intracellular viral sensors recognise a variety of pathogen-associated molecular patterns (PAMPs) by cells of the innate immune system. As mentioned above, TLRs are widely being studied with many papers being published showing how RSV interacts with TLR4 and signals an immune response through the MyD88 pathway(67-71). This has sparked others to research the role of RSV interaction with TLR2 and TLR6(71). This is however only just scraping the surface of the complex nature of the airway epithelium function. Interesting areas of research in the epithelium include;

- signalling pathways that induce cytokines and chemokines where Ip et al has looked at the production of IL-31(72),
- inflammatory response to pathogens as seen by Aldallal et al in which cells from patients with cystic fibrosis were exposed to bacteria(73),
- similarities and differences between upper and lower airways, highlighted in many studies but with a comprehensive comparison written in the book chapter by Ball et al(74),
- loss of epithelial integrity, where Man et al reports on the manipulation of E-cadherin function and how it can alter epithelial integrity(75)
- and rate of epithelial repair, as studied by Perotin et al that reported abnormal bronchial epithelial wound closure process in severe COPD(76).

Finding out how all of these aspects of the airway epithelium relate to the innate immunity when influenced by viral exposure will extend the knowledge and understanding of asthma initiation in early life.

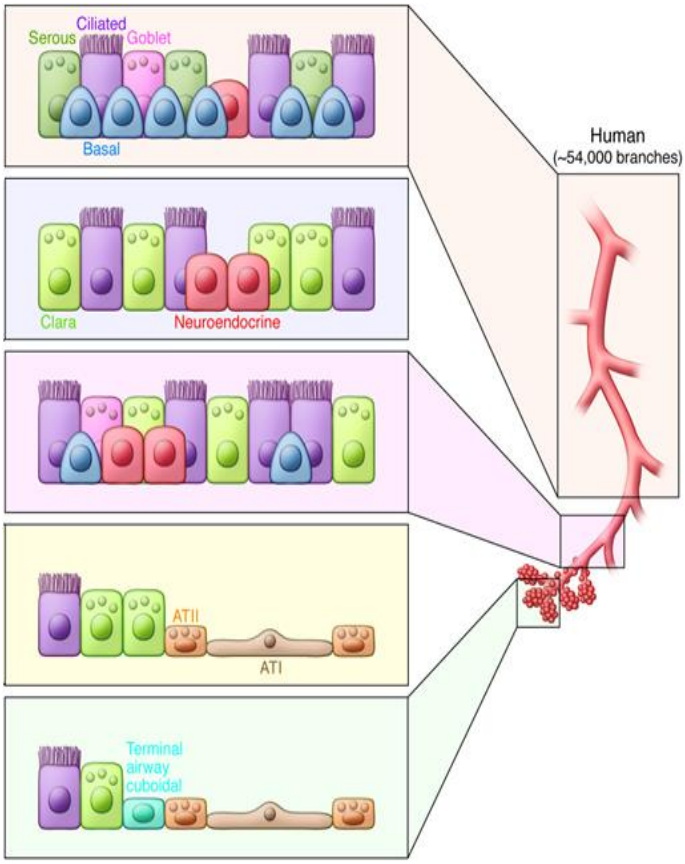


Figure 1-3 Lung epithelium. Regional differences in the cellular composition of the lung epithelium (Adapted from Rackley and Strip(65))

1.4.1 Components and function

Three main cell types have been identified in the pseudostratified columnar epithelium of the upper airways. Ciliated, goblet and basal cells and the role of tight junctions are highlighted in the next section. Club cells (previously Clara cells) are nonciliated, nonmucous secretory cells and are also sometimes present in the upper airways. However, they are predominantly in the terminal and respiratory bronchioles of humans and will be delved into much detail in this thesis.

1.4.1.1 Basal Cells

Basal cells are attached to the basal membrane and are highly abundant in the upper airway(77). They play key roles in the airway and contribute to disease susceptibility, initiation and progression. There is substantial evidence proving that basal cells are pluripotent stem cells that organise regeneration of the epithelium after injury by driving homeostasis of the normal epithelium(77-82).

1.4.1.2 Goblet Cells

Goblet cells are mucus secreting cells providing protection of the mucosa by creating a semipermeable barrier that enables the exchange of nutrients, water, and gases while still being impermeable to most pathogens(83). The production of mucin is induced by many bacteria, soluble factors and respiratory viruses, including RSV, with enhanced mucus production leading to trapping and clearance of viruses, as papers from Tamura and Zhu show(53, 84). Mucus production is a delicate balance with more goblet cells present in the airways of subjects with asthma and overproduction of mucin leading to obstruction of the airways and causing exacerbation(85). This has been known for many years with a paper published back in 1984 by Lumsden et al that reports on mucus cell hyperplasia and metaplasia occurring in asthmatic patients that leads to excessive sputum production(86).

1.4.1.3 Ciliated cells

Ciliated cells are critical for propelling the mucus produced by the goblet cells up the airway for removal by rhythmic waving and beating motions. The cilia are coated with membrane spanning mucins to separate it from the mucus by the formation of a periciliary space, both allowing for the beating of the cilia and the movement of the mucus away from the lungs (87). Ciliated cells play an important role in how the epithelial layer is infected with recent studies focusing on the particular susceptibility of ciliated cell infection by pathogens, with Zhang et al already proving this the case for RSV(88). Future research is now needed to determine the role these cells play in virus infections and how it effects epithelial integrity, with papers already reporting the relevance ciliated cells play in infection, but with more research needed to follow up these studies(88-90).

1.4.1.4 Tight Junctions

In the epithelium there are many types of junctions connecting the cells. These include Desmosomes and gap junctions the form patch-like lateral connections between the cells, and hemidesmosomes that anchor the cells to the basal lamina. Belt like tight junctions and adherens junctions encircle the apex of the cell, with tight junctions being the focused on in this thesis. Tight junctions are the most apically located of the intercellular junctional complexes. They inhibit solute and water flow through the paracellular space and separate the apical from the basolateral cell surface domains. These function as a barrier and allow for cell polarity(91). Tight junctions provide strong adhesion between cells and form adhesive bonds to give mechanical strength to the pseudostratified epithelial layer,

through interacting proteins and receptors. This ensures impermeability of the barrier and also enable communication between adjacent cells and regulate intercellular transport(92). The barrier formed by tight junctions hinder virus access to receptors on the epithelial basolateral membrane which is an important entry site for respiratory viruses as shown by two papers published by Bergelson(93, 94). Studies using *in vitro* models have identified that cytokines such as IL-13 and TNF-α can cause disruption of epithelial tight junctions(95, 96) with environmental factors like respiratory syncytial virus also having an detrimental effect(97).

1.4.2 Damage to the epithelium

Damage to the epithelium occurs frequently and recovery of an intact epithelium is critical for restoration of lung homeostasis. The airway epithelium is in direct contact with environmental factors frequently causing damage and forcing repair, with persistent injury contributing to the pathology of diseases such as asthma. In comparison to other cell types, little is known regarding the mechanisms of airway epithelial cell migration. Injury to the epithelium can be caused by bacterial or viral infection, inflammation, allergic reactions, environmental particulate matter and many others, all which has been detailed in a review by Crosby and Waters(98). Research on the enhancement of epithelial repair and regeneration may lead to new therapeutic strategies allowing for the reconstitution of well-differentiated and functional airway epithelium. This thesis will focus on the effect of viral infection and the inhibition on wound healing.

1.4.2.1 Viral Infection

Viral infections occur in the airway and infects the bronchial airway epithelial cells with these infections, specifically rhinovirus and respiratory syncytial virus(99) responsible for most asthma exacerbations in adults and children. Many studies and papers have been published detailing the important role respiratory virus's play in asthma. One key discovery comes from Lambert and Minor detailing the link between viral infection causing asthma exacerbations(99, 100), with these studies showing that infections can trigger an antiviral response aimed at containing and eliminating the infection and are more severe and long lasting in asthmatics(101). Asthma patients that are also atopic are more susceptible to respiratory infection, especially rhinovirus, with studies showing them having deficient production of IFN-β(54) and IFN-λ(55). However, a paper by Spann debates this and reports that it might be virus specific with no intrinsic defect in the production of IFN-β or –λ in children with wheeze(102). This needs to be proven further in infants and young children, with children younger than 3 years accounting for the most hospital admissions for bronchiolitis from

rhinovirus and RSV infection(14). Two of the most important viruses in relation to asthma are human rhinovirus and human respiratory syncytial virus.

1.4.2.2 Respiratory syncytial virus

Human respiratory syncytial virus (RSV) is a member of the *Paramyxoviridae* family of lipid membrane encapsulated, single-strand, negative sense RNA viruses(103). RSV is an important human respiratory viral pathogen of the lower respiratory tract and has been described as the single most important virus causing acute respiratory tract infections in children and a common cause of bronchiolitis in infants(104-107). There is currently no licensed vaccine available for RSV, even though nearly all children are infected by RSV at least once by the age of two(108), with 0.5% of these infants requiring hospitalization from airway dysfunction resulting in bronchiolitis(109). The link between severe RSV infection and an increased likelihood of developing recurrent wheezing or asthma has been confirmed by numerous studies(17, 110-116). One study by Henderson et al indicates that in the first year of life, children admitted to hospital for RSV-induced bronchiolitis were at an increased risk of recurrent wheeze up to 7 years of age. Even with all these studies using various models there remains a scientific gap of the mechanisms resulting in disease in humans.

RSV is composed of two subgroups, A and B, which often cocirculate in annual epidemics (117) and are distinguishable by antigenic and genetic characteristics. The variation between the two is within the G glycoprotein, a surface-expressed glycoprotein putatively associated with attachment of the virus(118). Studies have looked at the differences between the two subgroups revealing that while some show no significant clinical differences between the two, there is some indication that infection from subgroup A isolates are associated with a more severe illness(119). RSV infection can cause destruction of the airway epithelium as reported by Wright et al. and Zhange et al. (88, 120) with infection mostly targeted to the ciliated cells(88, 121-123) and not goblet or basal cells. Mirroring studies have looked at the secretion of cytokines and chemokines released in nasal aspirates, bronchoalveolar lavages, and blood in infants *in vivo*(124, 125) comparing them to secretions in apical and basolateral compartments of RSV infected ALI epithelial cells *in vitro*, with CXCL8, CXCL10, CCL5, and IL-6 present in both models(122, 126-128). When infection occurs it leads to loss of ciliated cells, excess mucus production, and blocking of airways by dead epithelial cells, fibrin, mucus, and inflammatory cells(129).

The upper airways are important in RSV infection as Hall et al shows, with the infection then descending to the lungs(117). Knowing this, nasal epithelium is the initial contact between the epithelium barrier and virus, with RSV infection responsible for a large range of diseases including rhinorrhoea, bronchiolitis, and pneumonia and for this thesis the relationship between RSV and asthma was examined. This link has been widely reported with studies from Stein and Sigurs outlining that RSV influences the mechanisms involved in the development of asthma and allergy in children(116, 130). RSV also appears in non-contiguous or small clumps of infected cells that have been evident in the large airway and along with loss of ciliated cells and excess mucus production, it causes occlusion of some airways by sloughed epithelial cells, fibrin, mucus and inflammatory cells, such as macrophages and neutrophils, along with apoptosis and occasional syncytia(131). RSV does induce an immune response from neutrophils, macrophages, T cells and to a lesser extent eosinophils. These cells can all cause widespread destruction to the airways and act as a secondary effect of RSV infection(124). These unique features of RSV infection have made it hard to study viral interaction and its role on the wounded epithelium *in vivo*. A study from as early as 1957, from Chanock, have looked at the RSV infection mechanisms but there is still a lot that remains unknown(132).

1.4.2.3 Bacteria

Associations have been reported between bacterial infection and the initiating events of early asthma. Clinical studies have detected the etiologic agents in children infected with both *Streptococcus pneumonia* (SP) and *Haemophilus influenza* (NTHi), which are the most prevalent bacterial pathogens(133, 134). Evidence reported by Korppi found that bacterial infection was found in 21% of 188 wheezing children younger than 6 years of age(135), with supporting data from Bisgaard et al reporting 21% of infants colonized with NTHi or SP, individually or in combination, had significant association with wheeze(136). Teo et al also reports early asymptomatic colonization with SP increasing the risk of asthma in early life(137). A detailed look at the viral and bacterial co-detection during paediatric respiratory infections and the effect of disease severity has been outlined in the paper by Brealey(138). They outlined that co-detection of virus and bacteria can increase the likelihood of asthma exacerbation in children, with a lack of data present in children with wheeze. Further understanding into how early colonization with bacteria in early life increases the risk of asthma is still needed.

1.4.3 Repair of the epithelium

It is believed that epithelium repair after injury is highly regulated and involves a series of temporally regulated steps; spreading, migration, proliferation and differentiation. Healing is initiated by cells next to the wound edge that dedifferentiate and migrate to cover the denuded wound area to form a confluent layer of undifferentiated cells. The wound area is covered and the cells start to proliferate and differentiated back form a pseudostratified cell layer (Figure 1-1) with this process widely reported in numerous reviews(98, 139-141).

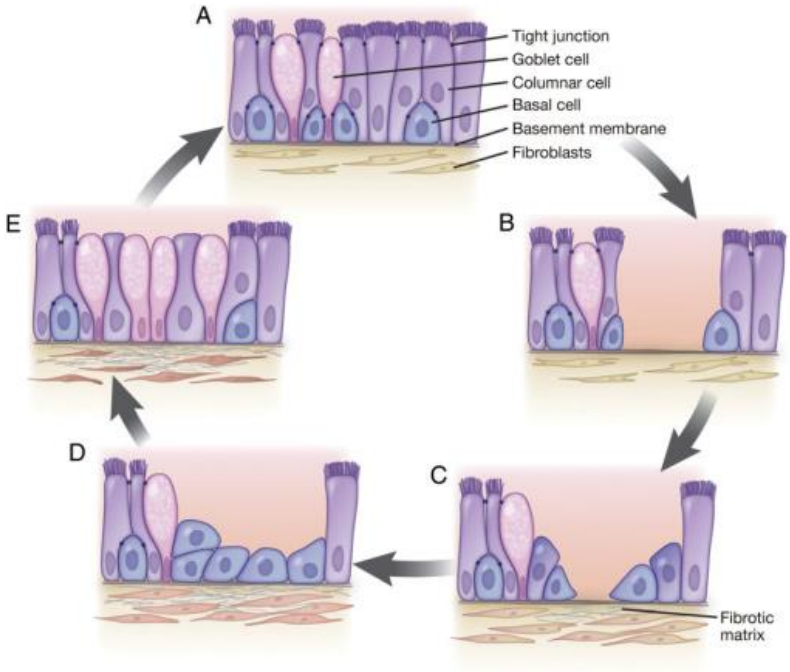


Figure 1-4 Normal wound healing of the epithelium after injury(141).

The mechanisms of epithelial repair are complex depending on how it was damaged, with exposure to tobacco smoke, allergens, airborne particulates, infectious agents and noxious gases all inducing injuries and requiring a regulated repair mechanism to restore its functionality(142). Like a switch, the process of cell migration can be turned on and off by regulatory changes of molecular components, including soluble factors (growth factors, cytokines, etc.), adhesion molecules, and extracellular matrix (ECM) molecules. Cytokines and growth factors coordinate the effects of positive and negative cellular responses to inflammation that are both reversible (synthesis/secretion of specific proteins) and irreversible (cell division or apoptosis). These include proinflammatory cytokines IL-1β, TNF-α, and growth factors, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)(143). Failure in any of these pathways can lead to dysregulated repair.

Not only does repair after wounding occur in the epithelial cells, there are messages between fibroblasts, smooth muscle and nerve cells to generate signals, both secreted and intrinsic, to initiate repair. Holgate has reported an epithelial-mesenchymal communication between the damaged and stressed epithelium, and underlying fibroblasts(11). After wounding by an external trigger, growth factors and cytokines, like TGF- β, activate mesenchymal cell proliferation that can lead to remodelling of the epithelium. Multiple pathways are likely to be involved in abnormal wound repair and with TGF-β being implicated in this process. Hackett has shown that asthmatic epithelial cells undergo more induction of TGF-β causing a slower repair even though they have an increase of repair markers(144). In asthmatics, abnormal healing and remodelling to the epithelium is due to impairment or upregulation of the epidermal growth factor receptor (EGFR)(145). *In vitro* studies suggested that barrier properties of the asthmatic epithelium can be improved by supplemental epidermal growth factor (EGF) applied apically (mimicking inhaled drug administration) as EGF plays a role in the development of tight junctions(23).

1.5 Airway epithelial injury and repair

1.5.1 Epithelial Cell Cultures

Culturing of human airway epithelial cells has increased understanding mechanisms underlying normal function and disease pathology of the airway epithelium. Epithelial cells are isolated by several different methods including explant cultures, enzymatic dissociation, bronchial brushings and nasal scrapings. Advancements in primary epithelial cell cultures has led to the development of transformed airway epithelial cell lines and establishment of conditions that enhance the proliferative capacity of airway epithelial cells, increasing understanding of the pathology associated with asthma.

Studying the epithelium has been restricted by the limited access to the lungs without invasive procedures. Culturing of samples of the epithelium allows for a less invasive way to examine the epithelium at a cellular level *in vitro*. Cells are collected from the epithelium, dissociated and seeded onto a plastic substrate. They are then maintained with specific media to enhance growth and cell division. This has historically been the method used to amplify epithelial cells *in vitro*, with many significant studies using submerged epithelial cells to report significant findings(146, 147). These included studies by Lechner et al, who developed the media formulations prolonged cell survival in culture, and Kelsen et al that pioneered the technique of collecting epithelial cells by bronchial brushings(148-152). Of most relevance in asthma research was the study by Campbell et al that looked at the response to inflammation and damage which was found to be abnormal in asthmatics(153).

The many positives of growing epithelial cells this way was less time in culture before experimentally ready (< 3 weeks), cheaper costs of flasks and media, and culture robustness. However, as many studies are now reporting both independently(154) and studies backed by the media manufactures (155), submerged culturing of epithelial cells is not a true representation of the *in vivo* lung epithelium. As shown in a review by Stewart et al there is only on cell type present, the culture is not polarised and does not produce mucus(156). Also, there is no presence of tight junctions even when cultures are at 100% confluence. These factors highlight the flaws of submerged cultures and although this method has been used successfully in the past, was no considered in this thesis. To

continue the development of culture conditions to allow for cell differentiation, the use of air-liquid interface has been a significant advance(157).

1.5.2 Air-Liquid Interface

Human airway epithelial cell culture has taken a massive leap forward with the replication of the *in vivo* biology by the method of growing epithelial cells at air-liquid interface (ALI). For the past 30 years epithelial cell culture has been based around growing cells in a submerged monolayer with poor differentiation, but early experiments from Whitsitt et al showed that epithelial cells growing on a porous membrane at an air-liquid interface would undergo mucociliary differentiation(158). This study explains that when the culture conditions are modified it enables cellular polarization and causes a powerful phenotypic conversion that more closely mirrors the normal *in vivo* morphology (Figure 1-5). The basis for experimental protocols of ALI originate from Lachner and Laveck, with strong influence from Gray et al.(154). Cells grown at ALI differentiate in approximately 21 days in culture and form a pseudostratified epithelium with cilia projecting from the apical surface, with goblet cells producing mucus, and basal cells on the basolateral surface(159, 160). Differentiation of nasal epithelial cells at ALI takes at least 21 days after "lift" as recommended by the media manufacturer(161) but much conjecture about this differentiation period exists in the literature. Studies have used ALI cultures from as early as 14 days for human rhinovirus infections(162) with cells harvested from the trachea and nose, to as late as 42 days to see mucus production(162) and cilia beat frequency from nasal cells(163). These studies harvested bronchial epithelial cells from the trachea whereas this thesis proposes to use epithelial cells harvested from the nasal cavity. Ong et al has already reported that nasal epithelial cells take longer to differentiate (seven weeks) than bronchial epithelial cells (three weeks) and explains the wide differences seen in time required to form a pseudostratified epithelial culture at ALI(164). Some studies also compare the epithelial cells harvested from the nose to those lower down the airways such as bronchial epithelial cells(165). McDougall shows that there was little difference seen between epithelial cells harvested from the nasal cavity to those from the trachea with them both having identical epithelial morphology, uniform expression of cytokeratin 19, along with many other surface receptors(166).

ALI is now used for a broad range of epithelial research, including a study by Parker comparing the growth of paediatric bronchial epithelial cells at ALI from both asthmatics and non-asthmatics(167), where they demonstrate the intrinsic differences between the two groups. Also, Zhang et al has revealed cell-type specific infection by viruses with RSV targeting ciliated cells that

are more susceptible to infection(88). ALI epithelial cell cultures are vital in the understanding of virus infection with most studies in the past using animal models, continuous cell lines or submerged cultures, which are not reflective of the range of pathologies described in viral epithelial infection(168). Monolayer cell cultures of epithelial cells are considerably less susceptible to infection with many receptors and cell types not present when cells are not differentiated(131). Therefore, using ALI is a physiologically relevant platform to study the fundamental mechanisms associated with disease states like asthma and represents an excellent and more realistic model for airway epithelial function experiments with many studies now using this as the gold standard.

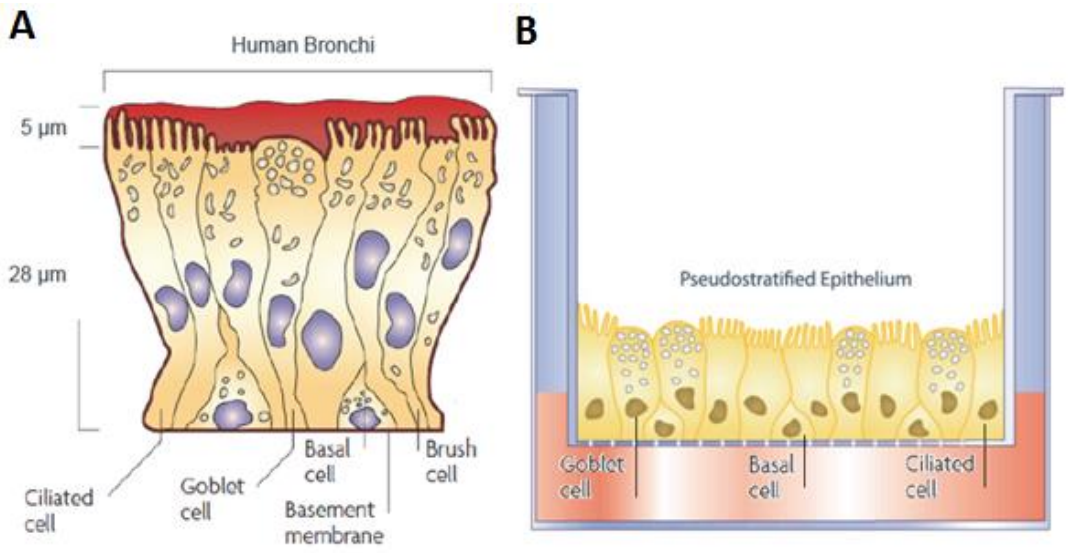


Figure 1-5 Epithelium *in vivo* and *in vitro*.Cells in the upper airways *in vivo* (A) compared to epithelial cells grown at ALI (B)(169).

1.5.3 Epithelial wound healing model

Maintaining the epithelial barrier is vital for maintaining the effective airway function, to prevent initial or progressive infection, and to also prevent further damage. *In vitro* modelling of wound healing is a vital next step in discovering the mechanisms of wound healing. The complexity of cell-to-cell interactions, along with pathways involved, makes a do-all model for wound healing impossible. For each aspect there needs to be considerable thought as to what needs to be tested and what outcomes could be generated. In the past wound healing of the airway epithelium has been modelled in by *in vitro* systems using cell lines or primary cells, submerged culture, air-liquid interface culture, explanted tissues and 3D culture systems(139). There has also been *ex vivo* models looking at pulmonary (animal) or non-pulmonary (skin) wounding, human observation and explant lungs. Lastly, there have been *in silico* models of genotyping and organ modelling that allow for

analysis or modelling of organ systems. Air-liquid interface cultures stand out as a favourable wound healing model as it allows for polarity in culture and better modelling of epithelial conditions in the lung using primary epithelial cells.

1.5.4 Treatments for healing

In the asthmatic airway, repeated epithelial damage and repair occur and there is no current therapy that directly targets this process. There have been studies that have used 16HBE and primary cells for expression but none using the ALI method of growing cells. The nasal epithelial cells are an effective starting point as there is potential for immediate translation to treatments. To add to what is mentioned above, the epithelium has a large surface area in the nasal cavity (50-200cm^2, about four times that of the trachea)(170), avoids the first pass metabolism, has a relatively high blood flow which promotes rapid absorption(171) and most importantly provides a non-invasive delivery for both local and systemic treatment.

Currently treatments include inhaled corticosteroids which have been shown to prevent but not repair epithelial damage in asthmatics(172, 173)and resolution of airway hyper reactivity showing restoration of the epithelium(174, 175) and many of the nasal delivery drugs for rhinitis and allergies (corticosteroids, decongestants and antihistamines). New nasal and inhaled treatments could be formulated for specific respiratory diseases and tested for effectiveness on nasal epithelial cells replicating the lung physiology(164).

Studies have already started to look at direct treatments for epithelial repair with Michael et al showing that *Saccharomyces cerevisiae* mediates a direct healing effect on human bronchial epithelial cell wounds. This is done through mannose receptors and the increase of transcription factors important in epithelial cell repair(176). The main benefit of using nasal epithelial cells in this model is that they directly represent the native phenotype with simultaneous studies from a single experiment, even if they fail to provide a high throughput capacity the same as cell lines. It also better represents the initial interaction between environmental exposures and the airway epithelium.

1.6 Hypothesis and specific aims

1.6.1 Hypothesis

Airway epithelium repair will be delayed after virus infection, especially in asthmatics.

1.6.2 Specific aims

1. To develop a well differentiated air-liquid interface model from primary nasal epithelial cells.
2. To establish a wound healing assay for differentiated airway epithelial cells from healthy, non-atopic asthmatic, atopic asthmatic and atopic subjects.
3. To determine the effect virus infection has on wound healing in airway epithelial cells

1.7 Summary and Implications

Asthma is a complex disease of the airways that effects people of all ages. Inflammation of the airways is a symptom that can be the results of exposure to environmental stimuli with RSV the cause of respiratory tract infections. This thesis will aim to develop a model for wound healing in the airway epithelium and see the interactions and inhibition virus infection has on the epithelium, especially in asthmatics. With this model, future studies into drug production and disease treatment can be fastracked, along with the model being adapted into other respiratory diseases, such as COPD.

1.8 Thesis Outline

This thesis was divided into three main parts; method development and evaluation, wound healing with disease comparison and pathogen exposure. The method development and evaluation were conducted at the beginning of the project and spanned over at large part of the complete project. All methods and wound healing experiments were evaluated before progression onto disease models and pathogen exposure. The following section describes the materials and methods used during the project.

Chapter 2 Establishing a model

2.1 Introduction

The airway epithelium is the first line defence of the airways and is exposed to inhaled external contaminants. The pseudostratified columnar epithelium of the upper airways can effectively be modelled using epithelial cells grown at ALI. Many studies have used this method to look at gene markers, cytokine secretion, and comparing disease types like asthma or COPD. All of these studies show different culturing techniques from harvest site (nasal or bronchial), media used in differentiation, and the time spend in culture, to name just a few. Not only are there these differences, once the ALI culture is established and fully differentiated there are still differences in experimental procedures from measuring the gene expression, cytokine release to wound healing. For that reason, this thesis had to establish an effective ALI model optimizing the conditions and test the capabilities of the ALI model. This included growing nasal epithelial cells at ALI and testing it against known outcomes, like; staining the culture for basal, goblet, ciliated cells and tight junctions; gene expression for known markers; and cytokine secretion. Establishment of an ALI model took many planned experiments to target all aspects effectively. This method development has been detailed in this chapter.

2.2 Methodology and Research Design

2.2.1 General methods

2.2.1.1 Participants

Adults and children were recruited through the Royal Children's Hospital (Brisbane, Queensland), The Lady Cilento Hospital (Brisbane, Queensland), Queensland Children's Medical Research Institute (Brisbane, Queensland) and Centre for Children's Health Research (Brisbane, Queensland). All donors were asked to complete a questionnaire to identify asthmatics and allergy disease history (Appendix 6.1.2) and gave written consent for the study. The project was approved by the human research ethics committees of Children's Health Queensland (2011000058) and of The University of Queensland (2017000520). Primary human nasal epithelial cells were collected from 74 subjects, 71 from adults aged 18-60 years, and 3 from children aged 4-15 years.

2.2.1.2 Nasal scraping

Primary normal human nasal epithelial cells were isolated from the epithelial cell layer lining the nasal cavity. This was done by scraping the inferior surface of the anterior turbinate using a purpose designed curette (ASI Rhino-Pro, Arlington Scientific, USA) and transported in 2ml of RPMI (Roswell Park Memorial Institute) media.

2.2.1.3 Cell culture

Nasal epithelial cells removed from curette and pelleted by centrifugation. Cells were then seeded in 24-well plates in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza, Cat# CC-3171) or Pneumacult-Ex-Plus™ Media (PC-ExPlus)(Cat# 05040). Cells were expanded until 70% confluence, split with TrypLE Express Enzyme (1X) (Cat# 12604013), centrifuged at 2000 rpm for 5 minutes at 23^{o}C, and seeded in a T25 flask. Cells were grown in submerged culture for 3 weeks until they reached passage 2 and cryopreserved.

2.2.1.4 Cryopreservation

When cells reached 70% confluence at passage 2 they were lifted again with TrypLE Express Enzyme (1X) and centrifuged at 2000 rpm for 5 minutes at 23^{o}C to pellet cells. Pellets were resuspended in media and counted using a haemocytometer with trypan blue (1:1). Cells were then spun again to

pellet cells. Lastly they were resuspended in 10% DMSO in FBS at an appropriate cell concentration (average 100,000 cells/100µL).

2.2.1.5 Air-Liquid interface

When required, cells were thawed and seeded (25,000 cells/insert) on collagen coated Transwell permeable polyester membrane inserts with a 0.4um pore size in 24 well plates (Corning Costar, Cambridge, MA, USA) and grown in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza) or Pneumacult™-ExPlus (PC-ExPlus). After approximately three days of cell division the cells are "lifted" with media removed from the apical chamber and media in the basal chamber replaced with B-ALI™ Bronchial Air Liquid Interface BulletKit™ (B-ALI, Lonza, Cat# 00193514)(155) or Pneumacult™-ALI Medium (PC-ALI)(Cat# 05001). All media was prepared following the manufacturer's instructions. Cells were maintained for at least 3 weeks until a pseudostratified epithelium with a high transepithelial electrical resistance (TEER) was established. A successful ALI culture was determined to contain basal cells, tight junctions, goblet cells and ciliated epithelial cells in which the cilia are seen to be beating. Cilia were observed under light microscope and detected by their movement.

2.2.1.6 Trans-Epithelial Electrical Resistance Measurements

The health of the epithelial cell cultures was validated using the non-invasive technique of measuring Trans Epithelial Electrical Resistance (TEER). This technique measures the resistance to an electric current travelling across the cell culture. TEER was monitored with the EVOM2 Epithelial Voltohmmeter (World Precision Instruments Inc., Sarasota, FL, USA) connected to STX2 Chopstick Electrode (World Precision Instruments Inc.). PBS (200µl) is added to the apical chamber and TEER is measured be placing the chopstick probe into the culture with one in the apical chamber and the other in the basolateral chamber. The resistance in OHMS is then recorded once the value is stable. A blank well with no cells is also recorded as the control well. The measurements were conducted at specific time point in line with media changes or relevant time points. Prior to measurements, the device was cleaned with ethanol and PBS, and PBS (200µL) was added to the apical surface of the cells for a ~5 minute incubation. TEER was calculated by subtracting the resistance of a blank insert then using the formula as follows to correct for the surface area of the insert:

Equation 1: $\Omega.cm^2 = \Omega$ (OHMS) x Transwell area insert ($0.33cm^2$)

2.2.1.7 Data handling and storage

All volunteer information collected at the time of the enrolment was entered into a database in a non-identifiable way and stored on a secure system at The University of Queensland. All data from laboratory analyses was stored in the same location.

2.2.1.8 Statistics

Data were analysed with GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA, USA) using appropriate statistical test. Non-parametric test included Kruskal-Wallis and Mann-Whitney test, and correlation was used with a $p < 0.05$ taken to be statistically significant.

2.2.2 Results

2.2.2.1 Participants

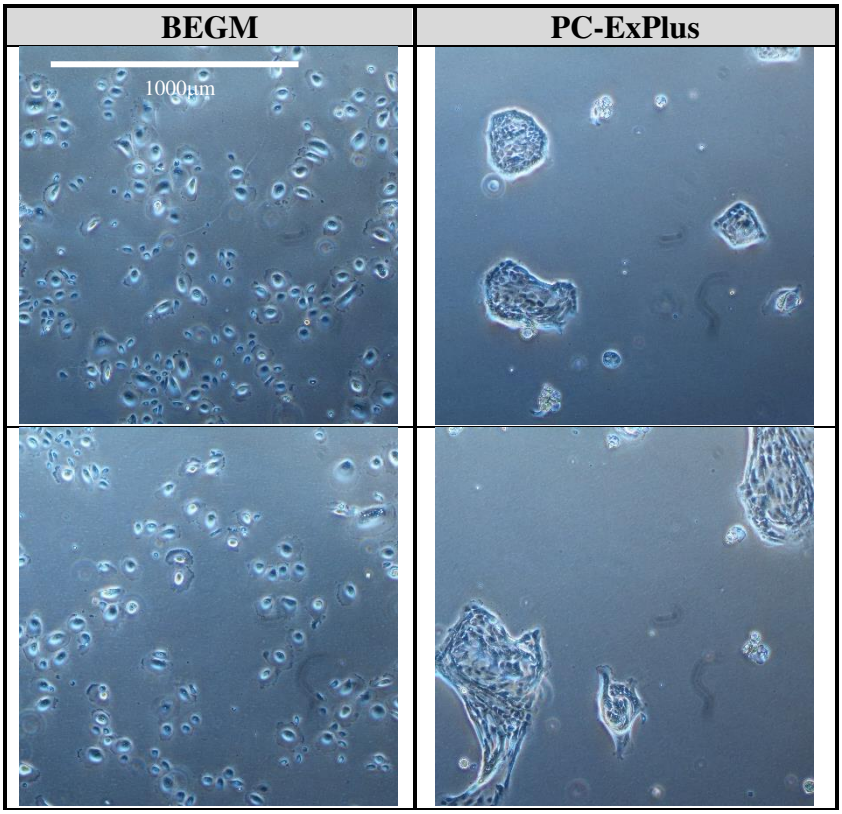
Table 2-1 Successful nasal epithelial cell subjects

Subject	Disease Status	Sex		Media
1	Healthy	Female	Adult	BALI
2	Healthy	Male	Adult	BALI
3	Healthy	Male	Adult	BALI
4	Healthy	Female	Adult	BALI
5	Healthy	Male	Adult	BALI
5	Healthy	Male	Adult	PC
6	Healthy	Female	Adult	BALI
6	Healthy	Female	Adult	PC altered
7	Healthy	Male	Adult	BALI
8	Healthy	Female	Adult	BALI
9	Healthy	Female	Adult	BALI
10	Healthy	Female	Adult	BALI
11	Healthy	Male	Adult	BALI
12	Healthy	Female	Adult	BALI
13	Healthy	Female	Adult	PC
14	Healthy	Male	Adult	PC
15	Healthy	Female	Adult	PC
16	Healthy	Female	Adult	PC
17	Healthy	-	Adult	PC
18	Healthy	Female	Adult	PC
19	Healthy	Male	Adult	BALI
20	Healthy	Female	Adult	BALI
21	Healthy	Female	Adult	BALI
22	Healthy	Male	Child	BALI
23	Healthy	Female	Child	BALI
24	Healthy	Male	Child	BALI
25	Healthy	Female	Adult	PC altered
26	Healthy	Male	Adult	PC altered
27	Healthy	Female	Adult	PC altered
28	Non-atopic asthmatic	Female	Adult	BALI
29	Non-atopic asthmatic	Female	Adult	BALI
30	Non-atopic asthmatic	-	Adult	BALI
31	Non-atopic asthmatic	Female	Adult	BALI
32	Non-atopic asthmatic	Male	Adult	BALI
33	Non-atopic asthmatic	Male	Adult	BALI
33	Non-atopic asthmatic	Male	Adult	PC

Subject	Disease Status	Sex		Media
34	Non-atopic asthmatic	Female	Adult	BALI
35	Non-atopic asthmatic	Male	Adult	PC
36	Non-atopic asthmatic	Male	Adult	PC
37	Non-atopic asthmatic	Male	Adult	PC
38	Non-atopic asthmatic	Male	Adult	PC
39	Non-atopic asthmatic	-	Adult	PC
40	Non-atopic asthmatic	Male	Adult	PC
41	Atopic	Female	Adult	BALI
41	Atopic	Female	Adult	PC
42	Atopic	Female	Adult	BALI
43	Atopic	Female	Adult	BALI
43	Atopic	Female	Adult	PC
44	Atopic	Male	Adult	BALI
45	Atopic	Female	Adult	BALI
46	Atopic	Male	Adult	BALI
47	Atopic	Female	Adult	BALI
47	Atopic	Female	Adult	PC
48	Atopic	Female	Adult	BALI
48	Atopic	Female	Adult	PC
49	Atopic	Female	Adult	BALI
49	Atopic	Female	Adult	PC
50	Atopic	Female	Adult	BALI
50	Atopic	Female	Adult	PC
51	Atopic	Female	Adult	PC
51	Atopic	Female	Adult	PC altered
52	Atopic	Female	Adult	PC
53	Atopic	Female	Adult	PC
53	Atopic	Female	Adult	PC altered
54	Atopic	-	Adult	PC altered
55	Atopic asthmatic	Female	Adult	BALI
56	Atopic asthmatic	Female	Adult	BALI
56	Atopic asthmatic	Female	Adult	PC
57	Atopic asthmatic	-	Adult	PC
58	Atopic asthmatic	Female	Adult	PC
59	Atopic asthmatic	Male	Adult	PC
60	Atopic asthmatic	Male	Adult	PC
61	Atopic asthmatic	-	Adult	PC
62	Atopic asthmatic	Female	Adult	PC
63	Atopic asthmatic	Female	Adult	PC
64	Atopic asthmatic	Female	Adult	PC

2.2.2.2 *Differentiation Media*

Figure 2-1 Cell morphology of nasal epithelial cellsgrown in Lonza (BEGM) and Stemcell (PC-ExPlus) expansion media in submerged monolayer cultures, at passage 2.



2.2.3 Discussion

2.2.3.1 Participants

Nasal epithelial cells were collected from 74 subjects with 71 adults and 3 children. Ideally for this study child nasal epithelial cells would have been used; however, sampling and recruitment was limited by the availability of children for sampling. The first step of developing an ALI model for wound healing needed to first be established using adult samples before using precious and limited children samples. Although some experiments (Cytokine secretion after TLR agonist exposure) have used nasal epithelial cells from children we don't propose it has had a great effect on the results and end conclusion(144). The main experiments and model development have been done in nasal epithelial cells harvested from adults.

The present study used epithelial cells sampled from the nasal cavity of healthy adults. The nasal epithelium is the "site of first contact" for pathogens and environmental stimuli. Nasal airway epithelial cells are easily accessible and resemble the upper airway epithelium morphology with similar gene expression and mucus production(177). We are deliberately studying the response of the nasal epithelium in its own right and not as a substitute for lower airway epithelium. As such, the results of the present study may not be applicable in the lower airways. However, as the structure and cell types are similar to those in the larger conducting airways, similar results are likely for cells obtained from those airways(166).

Adult subjects completed a simple questionnaire to determine their asthmatic and atopic status. The questions did not involve questions about other medical histories not related to respiratory diseases. This was important to not limit the willingness to donate from potential volunteers.

2.2.3.2 Differentiation media

Two types of differentiation media was used in this study as a result of B-ALI media becoming unavailable for purchase from Lonza. Contamination at the manufacturing stage in one of the media additives saw the production of B-ALI stop. This forced the change of differentiation media to PC-ALI from StemCell. Although both media claim to achieve the same outcome, a pseudostratified epithelium layer at the air-liquid interface, there were some observed differences. The change in

differentiation media did cause some changes in the cell growth and morphology as seen in Figure 2-1 from cells grown in a monolayer submerged culture in the two different expansion medias, BEGM from Lonza and PC-ExPlus from Stemcell. We can see from Figure 2-1 that even just the appearance was different between the two media with PC-ExPlus resulting in higher cell counts at each passage and faster cell growth. This would be beneficial in the culturing of delicate nasal epithelial cells in culture by providing higher cells counts over a shorter period of time.

The change in epithelial media caused the crossover of media between expansion and differentiation for some subjects. The large bank of cryopreserved nasal epithelial cells collected over many years in the CHEP lab saw numerous cell cultures expanded using BEGM and cryopreserved at passage 2. Once these cells were needed for this thesis they were thawed and seeded in transwell inserts in PC-ALI. This is a mix of Lonza media for expansion and Stemcell media for differentiation. We saw no obvious difference between the growth of these cells compared to straight Stemcell cells when seeded at the same density, although statistic and direct comparisons were not performed. One parameter of developing a successful ALI culture that did need to be strictly followed was if cells were seeded below the 25,000 cells/well there was a much higher chance that the culture could be unsuccessful, as seen from experience. Defining the seeding densities allows for direct comparison between cultures and can define the growth of individual cultures. One aspect not fully compared between media in this thesis was looking at the time to differentiation in PC-ALI. Experience dealing with epithelial cells at the ALI lead us to determine a fully differentiated pseudostratified epithelium by eye, identifying cilia and mucus cells, using a light microscope. Under 4x, 10x and even 20x when needed, cilia can be seen beating and moving the mucus around the culture. TEER values were also generated to measure the polarization of the cultures. In a more comprehensive study comparing the two media types, a time course experiment with supporting immunofluorescence staining would be done. Time restraints were a factor and these data could not be generated for this thesis.

Lastly, the media formula between the two ALI medias could not directly be compared as the Lonza B-ALI media was clearly defined by its additives (BPE, insulin, hydrocortisone, gentamicin, retinoic acid, transferrin, triiodothyronine, epinephrine and hEGF) on a DMEM base media, with only its concentration of these additives proprietary. Pneumacult media, however, does not disclose in as much detail the additives or concentrations used in its formula with the media only defined as basal medium, 10X base supplement and 100X maintenance supplement. This made a direct comparisons and conversions over to the available PC-ALI media much more difficult.

2.3 Time to full differentiation

2.3.1 Methods

2.3.1.1 Immunofluorescence Staining and Confocal Imaging

ALI cultures were maintained using B-ALI media, with hydrocortisone, for a period of 7 weeks with TEER values recorded at 3, 5, and 7 weeks. Wells were harvested at each time point for immunofluorescence staining and confocal imaging.

Epithelial ALI cell cultures were washed with PBS three times and fixed in paraformaldehyde (3%) for 10 minutes at room temperature and rinsed in PBS. Cell membranes were permeabilized with Triton X100 (0.5% in PBS) for 10 minutes at room temperature and blocked with BSA (2%) in TritonX100 (0.2%) for 1 hour at room temperature. Cells were incubated with primary antibodies at 4ºC overnight and washed 3 times with Triton X100 (0.5% in PBS). Secondary antibodies were added and incubated for 1 hour at 37ºC. After two washes with Triton X100 (0.5% in PBS) and PBS each, cells were stained with DAPI (Hoechst) for 10 mins before mounting with Prolong Gold Antifade reagent (Life Technologies). Anti-ZO-1/TJP1 (40-2200) antibody and Acetyl-alpha-tubulin (32-2700) antibody were purchased from Thermo Scientific. Anti-MUC5B (HPA008246) was purchased from Sigma and anti-cytokeratin 14 (ab192694) purchased from Abcam. Alexa fluor 594 goat anti-mouse, alexa fluor 488 chicken anti-rabbit, alexa fluor 594 goat anti-rabbit and alexa fluor 488 goat anti-guinea pig obtained from Life Technology (Carlsbad, CA). Images were captured from two fields per well at 20× magnification with a Nikon inverted fluorescent microscope (Model HB-10101AF), filters suitable for DAPI (blue), 488 (green) and 594 (red) fluorescence were used with a Spot CCD camera (Model 1.1.0).

2.3.2 Results

Epithelial cells were grown at ALI for a maximum of 7 weeks and stained for the presence of tight junctions with basal, goblet and ciliated cells. Five ALI cultures were stained at 3, 5, and 7 post air-lift. Basal cells and goblet cells were present at 3 weeks in all cultures with tight junctions present in three cultures at 3 weeks. The remaining two that did not have tight junctions by week 3 died before week 7. Ciliated cells appeared by week 7 in all three remaining subjects with representative data are shown in **Error! Reference source not found.**. The TEER value for all three subjects shows an i ncrease from week 3 to week 7 with a peak at week 5 (Figure 2-3).

Figure 2-2 Cells grown at ALI in B-ALI media for 7 weeks. Cultures subjected to immunofluorescence labelling as in Methods. Cultures were labelled with basal (cytokeratin 14), goblet (MUC5B) and ciliated (Acetyl-alpha-tubulin) cells and tight junctions (ZO-1/TJP1) with DAPI staining. Representative images form Subject 10 are shown. Scale bar represents 100µm

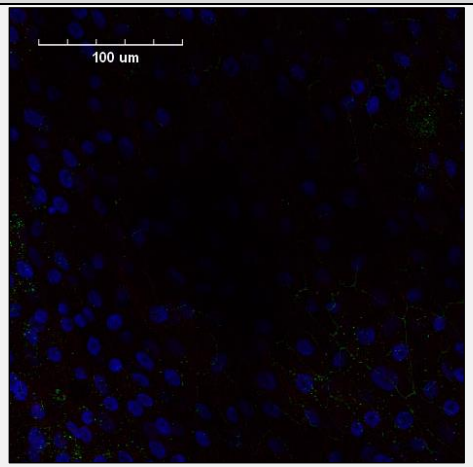
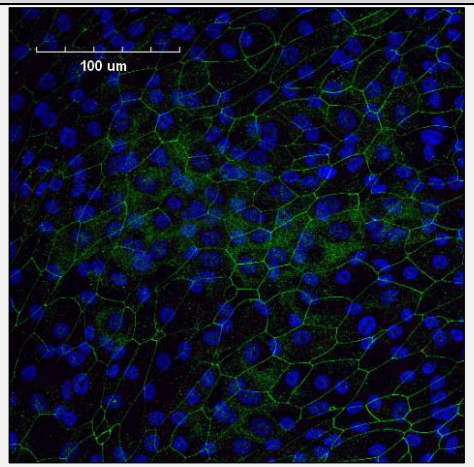
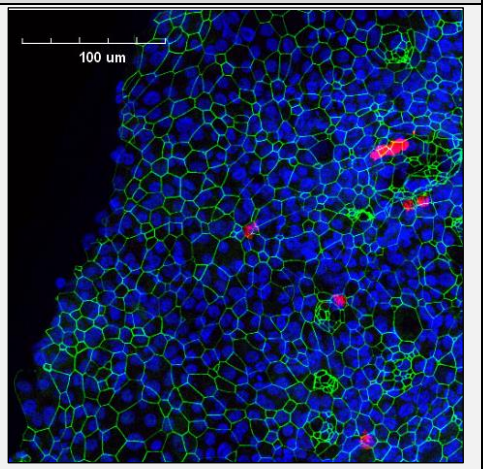
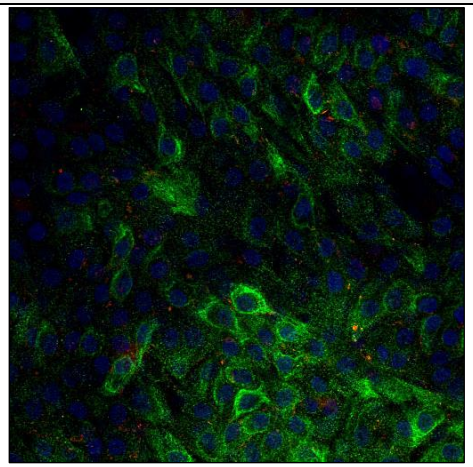
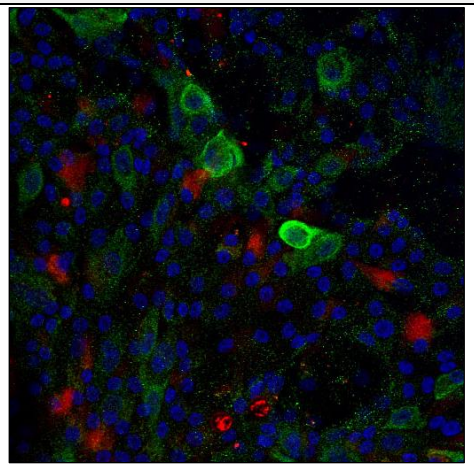
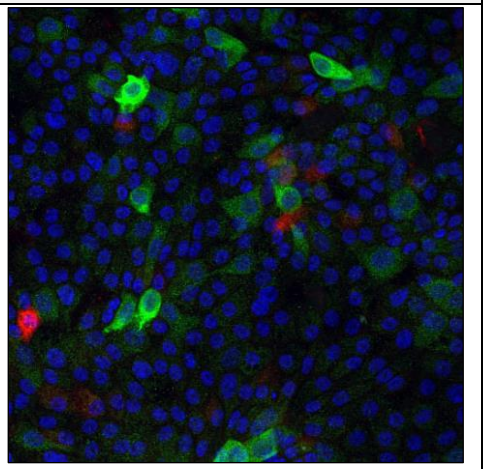
SUBJECT 10	**WEEK 3**	**WEEK 5**	**WEEK 7**
CILIA - RED (ACETYL-ALPHA-TUBULIN) TIGHT JUNCTIONS – GREEN (ZO-1/TJP1)			
GOBLET – RED (MUC5B) BASAL – GREEN (CYTOKERATIN-14)			

Table 2-2 Summary of the observed fluorescence staining of the epithelial cells at ALI over a 7 week differentiating period. Presence or absence of immunofluorescence stain for three subjects. Ciliated cells were only seen for 3 subjects at week 7.

	Subject	Week 3	Week 5	Week 7
Basal	55	Yes	Yes	No
	56	Yes	No	Dead
	22	Yes	Yes	Yes
	10	Yes	Yes	Yes
	57	Yes	Yes	Yes
Goblet	55	Yes	Yes	Yes
	56	Yes	No	Dead
	22	Yes	Yes	Yes
	10	Yes	Yes	Yes
	57	Yes	Yes	Yes
Cilia	55	No	No	No
	56	No	No	Dead
	22	No	No	Yes
	10	No	No	Yes
	57	No	No	Yes
Tight Junctions	55	Few	Yes	Yes
	56	Few	No	Dead
	22	Yes	Yes	Yes
	10	Yes	Yes	Yes
	57	Yes	Yes	Yes

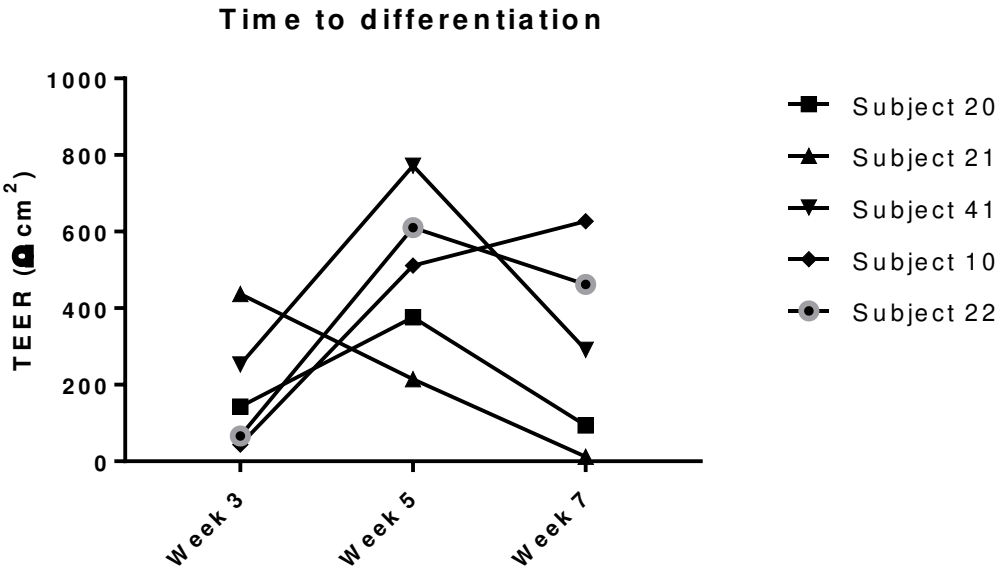


Figure 2-3 Transepithelial electrical resistance (TEER) of ALI cultures. TEER measurements were taken over the 7-week differentiation period at 3, 5 and 7 weeks after lift (n=5).

2.3.3 Discussion

Epithelial cells were grown at ALI for 7 weeks and stained for the presence of tight junctions with basal, goblet and ciliated cells at 3, 5 and 7 weeks post air-lift. Basal cells and goblet cells were present at 3 weeks in all cultures (Table 2-2), with tight junctions in three cultures at 3 weeks. The remaining two cultures that failed to develop tight junctions died before week 7 as indicated by the low TEER value. These two cultures also failed to develop cilia and were deemed to be failed ALI cultures. These were excluded from further analysis. For the surviving three cultures, ciliated cells appeared by week 7 in three subjects (Table 2-2). Representative data are shown in **Error! Reference source not found.** from subject 10. The TEER value for all three subjects shows an increase from week 3 to week 7 with a peak at week 5 (Figure 2-3).

The lineage of each cell type and structure of the epithelium developed at differing rates over the 7 weeks. Basal cells develop early with studies identifying them as the progenitor cells of the epithelium(178). They were seen from the earliest time point of 3 weeks post lift with studies confirming that the monolayer of submerged cells at seeding stage of ALI are basal cells(179). Tight junctions were also present from week 3 but with development increasing by week five as confirmed by the high TEER value for all subjects (Figure 2-3). Goblet cells were identifiable at three weeks post lift but became much more evident after five and seven weeks (**Error! Reference source not found.**). Mechanisms involved in the differentiation of goblet cells are more complex and include the activation of the EGFR(180) and transcription factors, FOXA2 and SPDEF(181), resulting a longer differentiation period. The last cell type to differentiate in the ALI culture were ciliated cells. For three subjects the cilia was evident at seven weeks post lift (**Error! Reference source not found.**). In the airway epithelium, ciliated cells are essential for the transport of mucus and removal of external pathogens(182). Waiting seven weeks for cilia cells to develop in the ALI model is essential because of their importance in maintaining the upper airway homeostasis.

A pseudostratified columnar airway epithelium can be established in an ALI culture, however, for adult nasal primary cells this must be monitored on a subject-to-subject basis with a minimum 7 weeks required for full differentiation under B-ALI conditions. This period allows for the development of basal, goblet and ciliated cells with a stable TEER value. Studies using ALI should be encouraged to report the state of the cultures used.

2.4 Wound healing

2.4.1 Methods

Fully differentiated ALI cultures were starved of hydrocortisone in basolateral media for three days prior to wounding. Mechanical injury was performed by scraping a P10 sterile pipette tip across the cell layer creating a wound with a diameter ranging from 600µm to 1500µm After wounding, the apical surface of the culture was washed with 200µL PBS to remove cellular debris. The leading edge of the wound was tracked with the aid of time-lapse microscopy (CytoSmart, Lonza, and Eclipse Ti, Nikon) with images taken every 60 minutes at 4x magnification and image analysis software used to track the wound area over time. Image editing software (Adobe Photoshop) was used to remove the wound area and convert image files to .TIFF. Wound area was calculated each hour using ImageJ by converting pixels to area, where 1 pixel = 2µm^2 (Cytosmart) and 1 pixel = 0.6µm^2 (Eclipse Ti). The initial wound area was expressed as 100% to remove variability from wounds of different size. Wounds were considered to be closed when the calculated area fell below 3%, the effective limit of detection of the image processing. Wound closure was calculated as follows:

Equation 2: Wound closure (%) =100 - ((Area/Initial Area)*100)

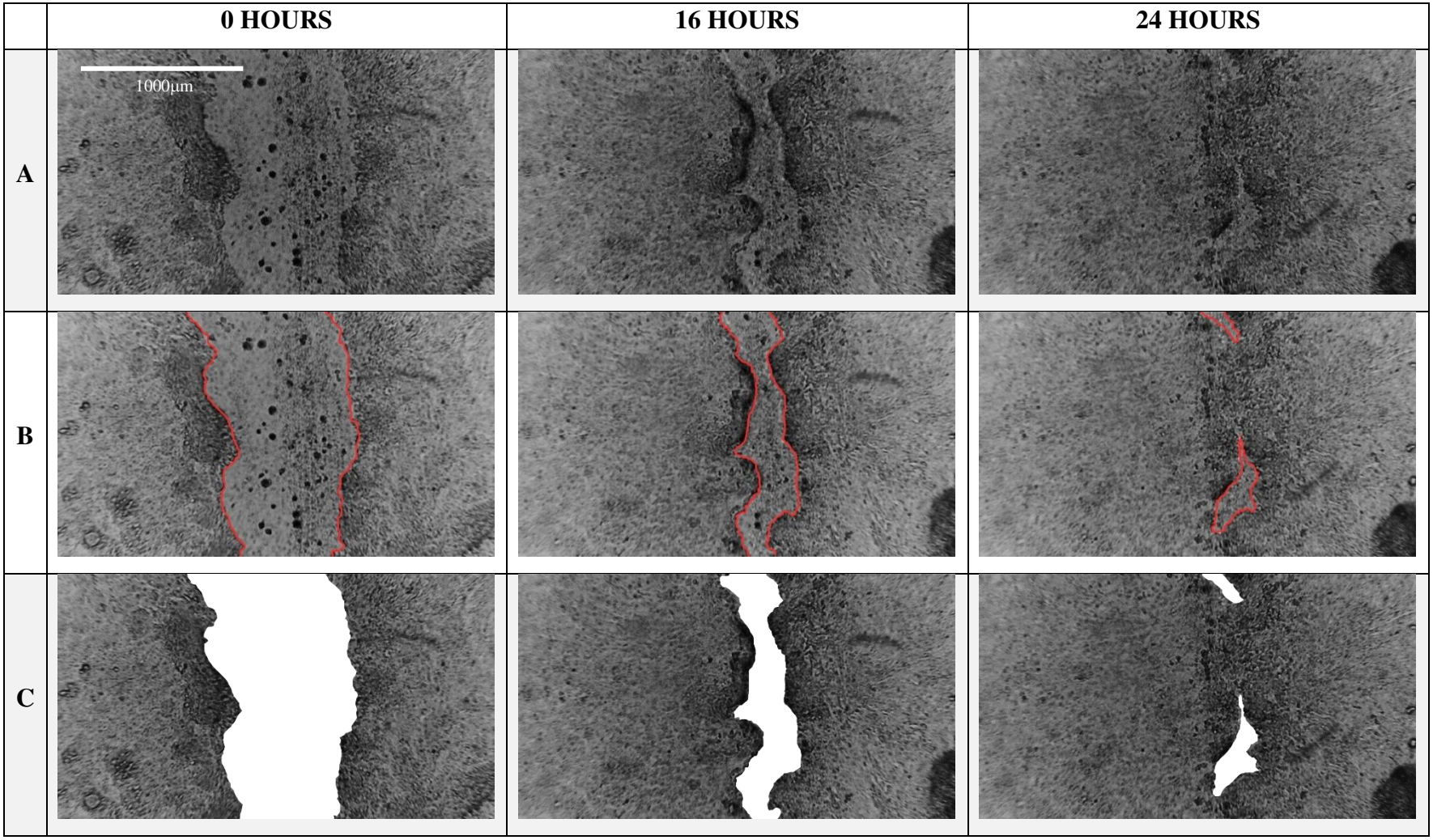
Wound closure (%), plotted as a function of time (hour), was used to calculate the rate of wound closure (%/hour)

2.4.2 Results

2.4.2.1 Rate of wound healing

Wound closure was complete within 35 hours for 16/18 (89%) of cultures (Figure 2-5, Table 2-3). The group mean rate of healing for those subjects was 28.85 ± 9.8%/hour, with all healed subjects achieving a rate above 2%/hour (Table 2-3). Two non-healed subjects had a rate below 2%/hour and eight medium healers had a rate between 2 and 4%/hour. The remaining nine were identified as fast healers with a rate above 4%/hour (Table 2-3). There was no correlation between the rate of epithelial rate of wound closure in healthy and initial wound size (µm^2) with r^2=0.06 (Figure 2-6).

Figure 2-4 Wound closure assay in epithelial cells at ALI. Representative photographs at 0 hours, 16 hours and 24 hours after wounding in Subject 8 (A) with wound area identified (B) and removed (C) for ImageJ analysis.



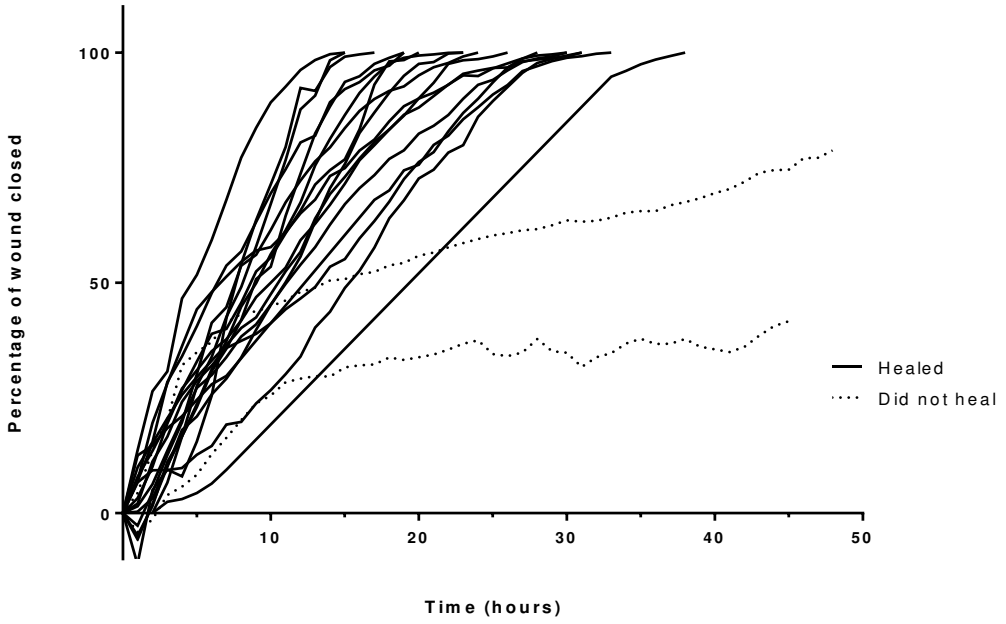


Figure 2-5 Rate of wound closure. Leading edge of the wound was tracked, and the area of the wound determined every 60 minutes until closure of wound. Wound areas were graphed for each participant as a percentage of wound closure (wound closed = 100%) over time (n=19).

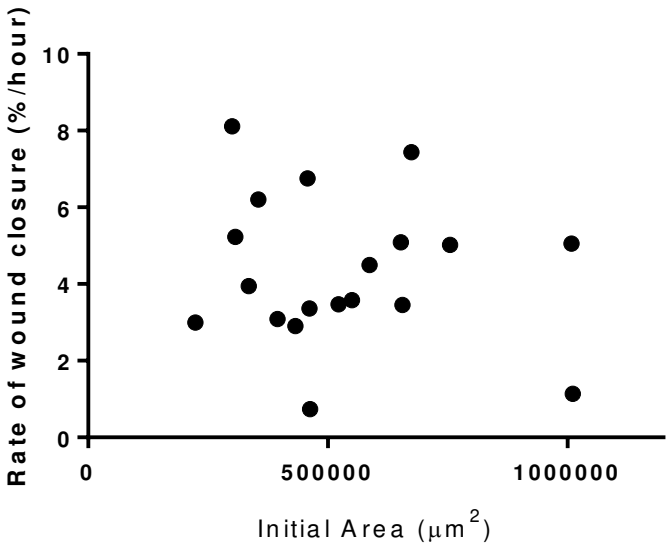


Figure 2-6 Rate of healing (%/hour) compared against the initial area (μm^2) of each wound. At time 0, the area of the initial wound was calculated as initial area (μm^2). There was no correlation seen between initial area (μm^2) and rate of healing (%/hour) for all donors at the 95% confidence interval (n=19).

Table 2-3 Rate of wound closure in ALI of healthy subjects.

Subject	Disease Status	Sex	Days to lift	Days from lift	TEER (Ω.cm^2)		Hours	Wound width (um^2), %[1]			Rate of wound closure (%)	R squared	Media
					Control	Wounded		Initial	Final				
1	Healthy	Female	4	45	1369	1072	25	586748	4820	0.8%	4.49	0.93	BALI
2	Healthy	Male	6	51	247	280	31	461552	1790	0.4%	3.36	0.95	BALI
3	Healthy	Male	9	35	445	445	16	457662	1626	0.4%	6.75	0.94	BALI
4	Healthy	Female	3	58	940	1270	46*	462954	270058	58.3%	0.73	0.67	BALI
5	Healthy	Male	4	47	1765	1699	27	335258	2804	0.8%	3.95	0.98	BALI
5	Healthy	Male	3	72	0	0	29	655548	8504	1.3%	3.45	0.99	PC
6	Healthy	Female	2	37	280	280	32	550584	3532	0.6%	3.58	0.96	BALI
7	Healthy	Male	2	44	280	280	21	307284	8772	2.9%	5.23	0.99	BALI
8	Healthy	Female	2	49	544	544	31	522444	4378	0.8%	3.47	0.97	BALI
9	Healthy	Female	3	56	1699	1699	32	432494	3562	0.8%	2.91	0.90	BALI
10	Healthy	Female	1	59	940	181	33	395260	874	0.2%	3.09	0.98	BALI
11	Healthy	Male	5	42	16	16	20	354688	3992	1.1%	6.20	0.98	BALI
12	Healthy	Female	4	42	198	148	50*	1010808	213974	21.2%	1.14	0.83	BALI
13	Healthy	Female	2	27	82	36	21	652452	10466	1.6%	5.09	0.99	PC
14	Healthy	Male	2	33	478	478	18	674236	2592	0.4%	7.43	0.95	PC
15	Healthy	Female	2	33	141	445	24	1008040	470	0.0%	5.06	0.96	PC
16	Healthy	Female	2	40	141	125	24	754790	1642	0.2%	5.02	0.99	PC
17	Healthy	-	4	31	477	331	15	300616	1399	0.5%	8.11	0.99	PC
18	Healthy	Female	4	27	544	125	39	223369	2292	1.0%	2.99	1.00	PC

[1]final wound area expressed as a percent with the initial wound area normalized to 100%

*Experiment discontinued at time point. Wound failed to close.

2.4.2.2 *Repeatability*

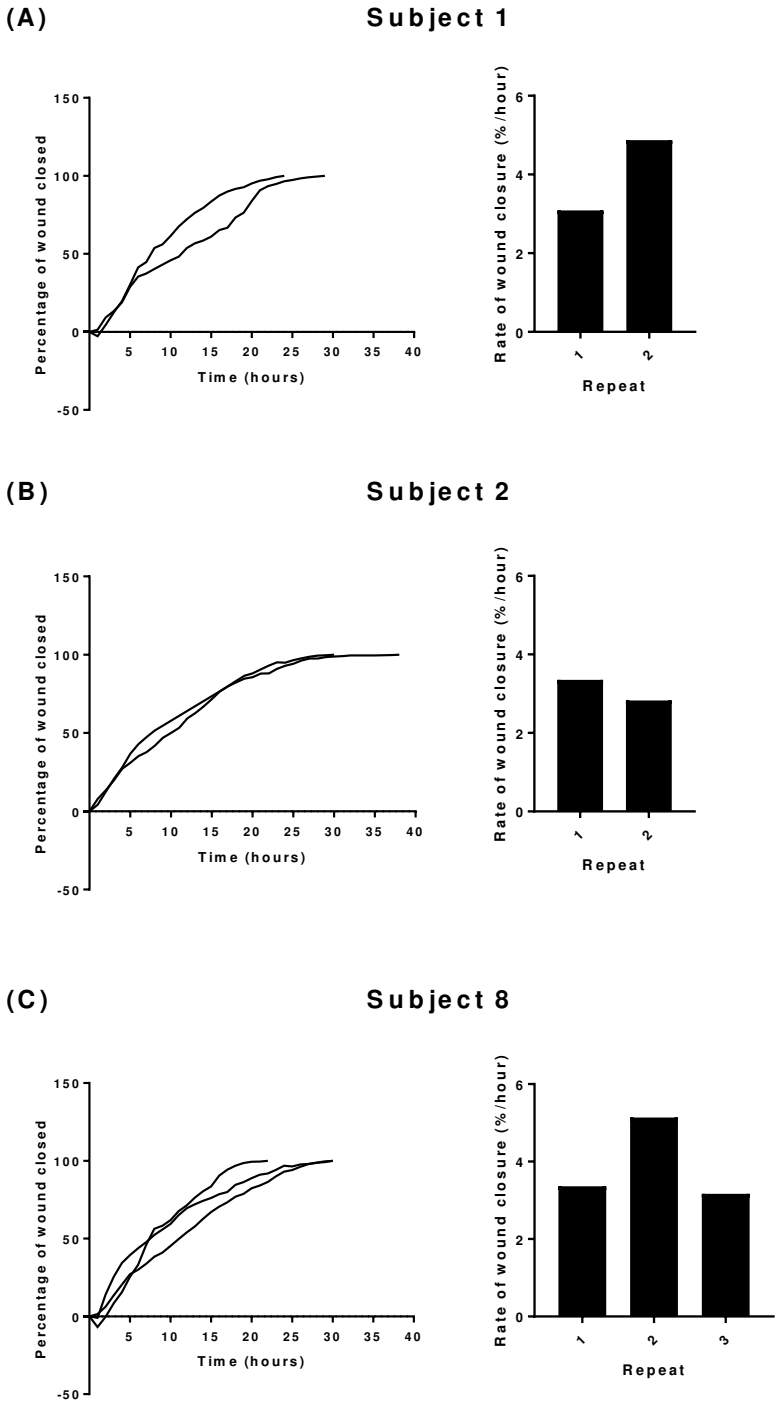


Figure 2-7 Rate of wound closure repeats from the same subject. No significant difference was seen between repeats. The average rate of wound closure for 3 subjects showed 4.34% ± 0.75/hour for Subject 1 and 3.09% ±0.37/hour for Subject 2 both after 1 repeat, and 3.89% ± 1.08/hour for Subject 8 after 2 repeats.

2.4.2.3 *Age of culture*

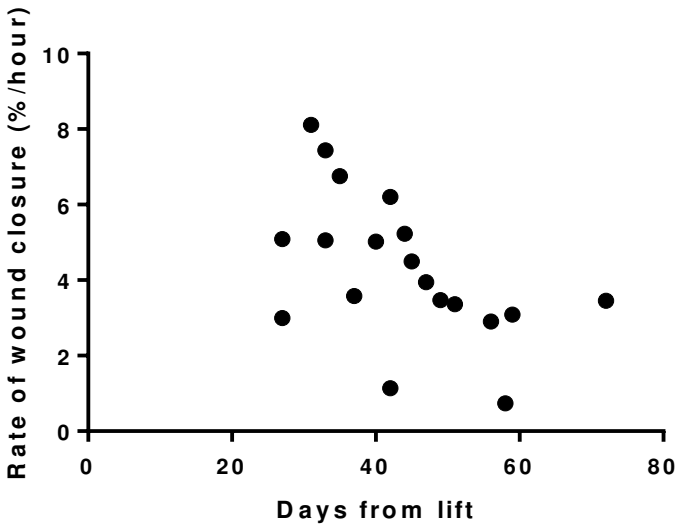


Figure 2-8 The number of days after lift compared to the rate of wound closure (%/hour). There was significant correlation (p-value= 0.02) between age of culture and rate of wound closure where older cultures had lower rates of wound closure (%/hour)(n=19).

2.4.2.4 *Media comparison*

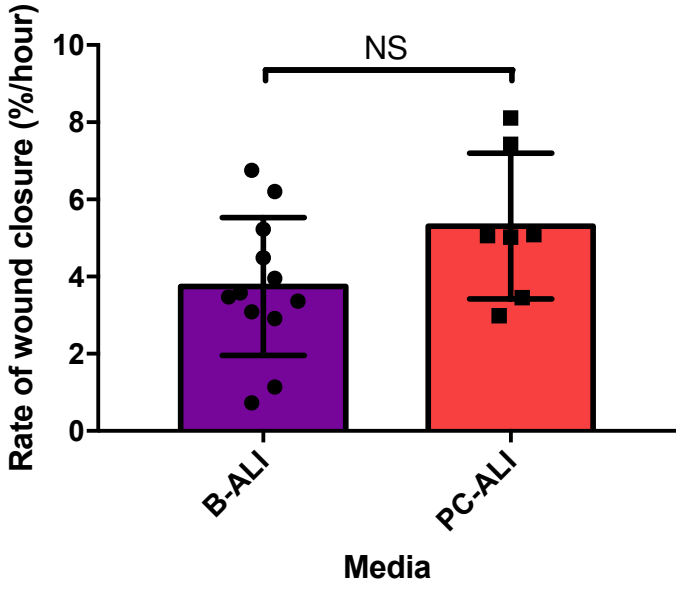


Figure 2-9 Comparison of the rate of wound closure between B-ALI (n=12) and PC-ALI (n=7) media. No significant difference seen (p-value = 0.2) in a Mann Whitney test.

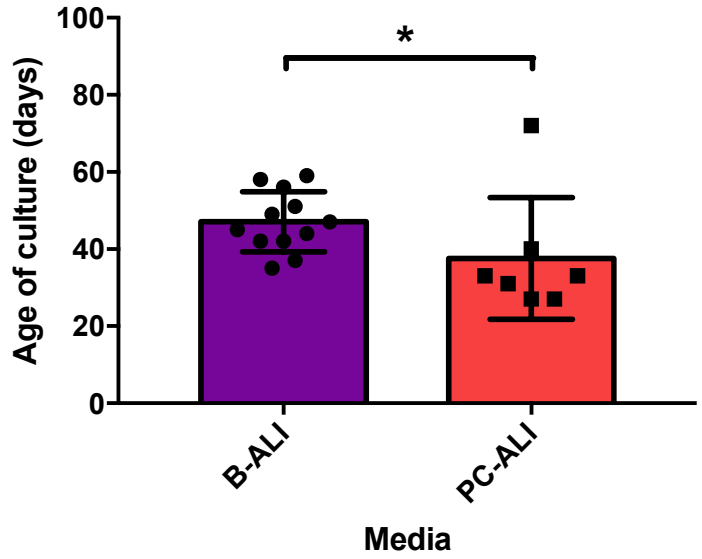


Figure 2-10 Comparison of the age of ALI cell cultures between B-ALI (47.08 ± 7.8, n=12) and PC-ALI (37.6 ± 15.8, n=7) media. Slight significant difference was seen with PC-ALI cultures establishing a pseudostratified epithelium sooner in a Mann Whitney test (p-value = 0.02)

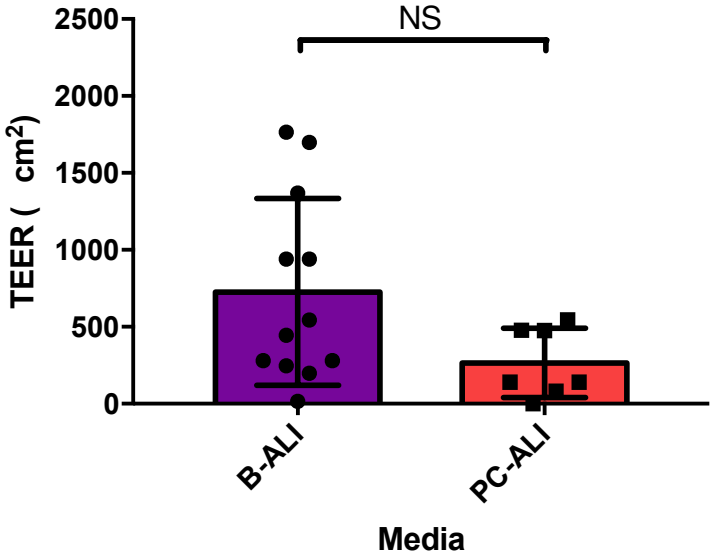


Figure 2-11 Comparison of the TEER values of ALI cell cultures between B-ALI (727 ± 607, n=12) and PC-ALI (266 ± 225, n=7) media. No significant difference was (p-value = 0.09) in a Mann Whitney test, but some ALI cultures had a higher TEER values in B-ALI media.

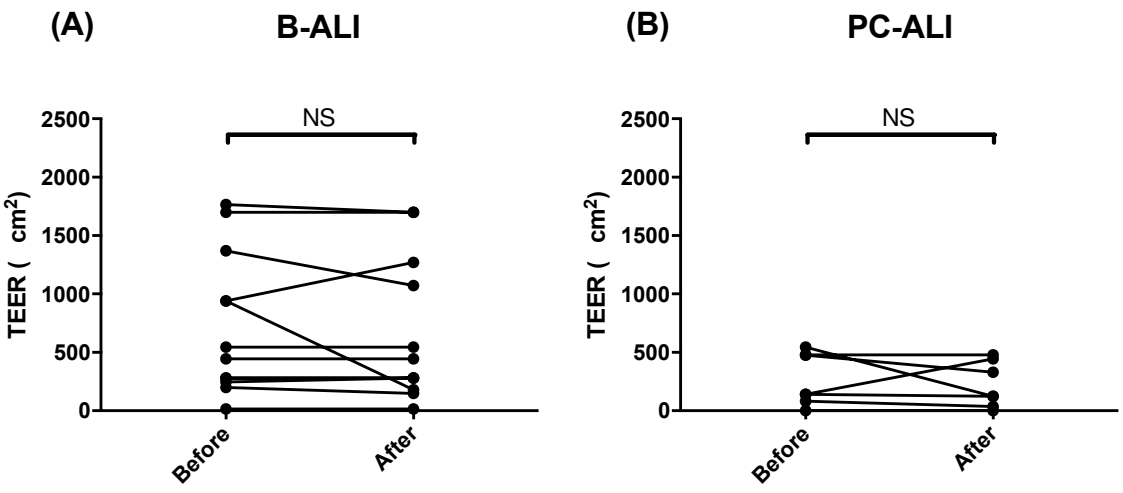


Figure 2-12 Comparison of the TEER values before and after wounding of ALI cell cultures from B-ALI (A) (n=12) and PC-ALI (B) (n=7) media. No significant difference was seen for all ALI cell cultures from B-ALI before (727 ± 607) and after (660 ± 611) in a paired Wilcoxon test (p-value = 0.44). No significant difference was also seen for all ALI cell cultures from PC-ALI before (266 ± 225) and after (220 ± 196) in a paired Wilcoxon test (p-value = 0.44).

2.4.3 Discussion

2.4.3.1 Wound healing model

Previous reports on wound healing have used cells cultured in monolayer, submerged cultures with only basal cells present(76, 183). This thesis used the method of scratch assays similar to these previous studies but adapted it for ALI culture. Live imaging is a non-invasive method that was used to track the cellular migration with the cell culture. Using the CytoSmart and Eclipse Ti microscopes, the cell culture could be monitored by the system while in the ideal incubator conditions. The 3D nature of the ALI system creates obstacles in the wounding, imaging and even sampling of the cultures. Common software programs previously used for analysis of monolayer scratch assays are not able to identify the wound edge (TScratch and Robust Quantitative Scratch Assay) in ALI cultures. The present study used simple programs such as Adobe Photoshop and ImageJ that allow identification and calculation of the wound area (Figure 2-4). The initial wound size did not correlate with the rate of wound closure (Figure 2-6) showing no effect on the initial size of the wound to the overall time it took to close the wound. This allowed normalization of the initial wound to 100% with healing plotted as a percentage of the initial wound and the rate of wound closure taken from the slope (Figure 2-5). Only two subjects failed to close the wound and had a rate of wound healing less than 2%/hour. From the remaining subjects that did close, we report a rate of wound healing of above 2%/hour for all except one subject (Table 2-3). Subject 11 had a rate of closure below 2%/hour and was slow to heal at 43 hours. Thus, we suggest 2%/hour as a baseline rate of wound healing for healthy subjects to be used in future experiments investigating factors that disturb normal wound healing in the airway epithelium and to identify any slow/non healing cultures. The robustness of this wound healing assay was measured by repeated wound healing of the same subject on different wells and on different days, for three subjects. Each subject showed a minimal assay-to-assay variation (<28%) (Figure 2-7). All remained above the recommended 2%/hour allowing for confidence in the wound healing assay and ALI model.

Epithelial wound healing is thought to occur by four processes: dedifferentiation, migration, proliferation and redifferentiation(98, 139-141). The wound healing assay shows that ALI cultures of primary nasal epithelial cells from healthy subjects heal within 36 hours (Table 2-3). However, this period may only represent the one stage of wound repair, migration. Proliferation and redifferentiation are likely to occur once the wound is visually closed but may be inferred from the TEER at 48 hours post wounding using this model (Table 2-3) with dedifferentiation unable to be measured with such fast healing. The use of fluorescence staining and cytokine release could confirm

this, however because of the limited cell numbers collected was not possible for these ALI cultures. For 13 subjects, TEER returned to around the pre-wounding value by 48 hours, which is likely to indicate that the epithelium has polarized with tight junction present and indicate complete repair of the wound. Three subjects with low TEER values at the pre wounding also reported the same low TEER values at 48 hours when wound was closed. The TEER value was taken at these time points to minimize the environmental changes to the cell culture and to not disturbed the wound healing process and is an effective measurement of a fully differentiated ALI culture

2.4.3.2 Age of culture

There was a significant correlation seen between the age of the cultures and the rate of wound closure, with the older cultures having a reduced rate of wound closure. These cultures took longer for these cultures to develop a pseudostratified epithelium with basal, goblet and ciliated cells all present and as a consequence also had reduce wound healing capabilities.

2.5 Cytokine detection in ALI cultures

2.5.1 Methods

2.5.1.1 Luminex screening

Apical wash was collected from nasal epithelial cells grown at ALI for 21 days in B-ALI, from 3 healthy subjects. The apical surface was washed with 100ul PBS and analysed for cytokine secretion using the Luminex Assay (Millipore) against a panel of 9 cytokines.

Table 2-4 Cytokines in Luminex screening assay

Cytokine		**Assay**	**Lower detection limit (pg/ml)**	**Catalogue number**
EGF	Epidermal Growth Factor	Luminex	2.8	HCYTMAG60K
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Luminex	7.5	HCYTMAG60K
GRO	CXCL1	Luminex	9.9	HCYTMAG60K
MCP-3	CCL7, Monocyte-chemotactic protein 3	Luminex	3.8	HCYTMAG60K
MDC	CCL22, Macrophage-derived chemokine	Luminex	3.6	HCYTMAG60K
IL-13	Interleukin 13	Luminex	1.3	HCYTMAG60K
IP-10	CXCL10, Interferon gamma-induced protein 10	Luminex	8.6	HCYTMAG60K
MIP-1α	Macrophage inflammatory protein 1α	Luminex	2.9	HCYTMAG60K
MIP-1β	Macrophage inflammatory protein 1β	Luminex	3	HCYTMAG60K

2.5.1.2 AlphaLISA

Supernatants of epithelial cells from 3 subjects was collected by washing the apical surface of the ALI culture with PBS and the basal media at 0, 2, 24, 48, 72 hours and 6 days. Washes and media were tested for cytokine detection using AlphaLISA kit (Perkin Elmer) following manufacturer's recommendations. This same procedure was done for ALI cultures exposed to TLR agonists.

Table 2-5 Cytokines in AlphaLISA assay

Cytokine		**Assay**	**Detection Limit (pg/ml)**	**Catalogue number**
IFN-β	Interferon beta	AlphaLISA	9.6 – 100,000	AL265
IL-8	Interleukin 8	AlphaLISA	1.1 – 30,000	AL224
IP-10	CXCL10, Interferon gamma-induced protein 10	AlphaLISA	3.3 – 300,000	AL326C
TNF-α	Tumour necrosis factor alpha	AlphaLISA	2.2 – 30,000	AL208

2.5.2 Results

2.5.2.1 Luminex Screening

Table 2-6 Luminex screening against 9 cytokines from apical wash (n=3).

		EGF (pg/ml)	**GM-CSF (pg/ml)**	**GRO (pg/ml)**	**MCP-3 (pg/ml)**	**MDC (pg/ml)**	**IL-13 (pg/ml)**	**IP-10 (pg/ml)**	**MIP-1α (pg/ml)**	**MIP-1β (pg/ml)**
Healthy	Minimum	1.149	8.981	23.52	3.634	2.249	2.002	3.904	-	-
	Maximum	20.39	152.8	29065	16.19	254.4	2.63	110.8	-	-
	Mean	11.23	37.34	6005	9.912	59.3	2.316	48.44	-	-
	Std. Deviation	7.175	47.96	9602	8.878	109.4	0.4438	49.04	-	-

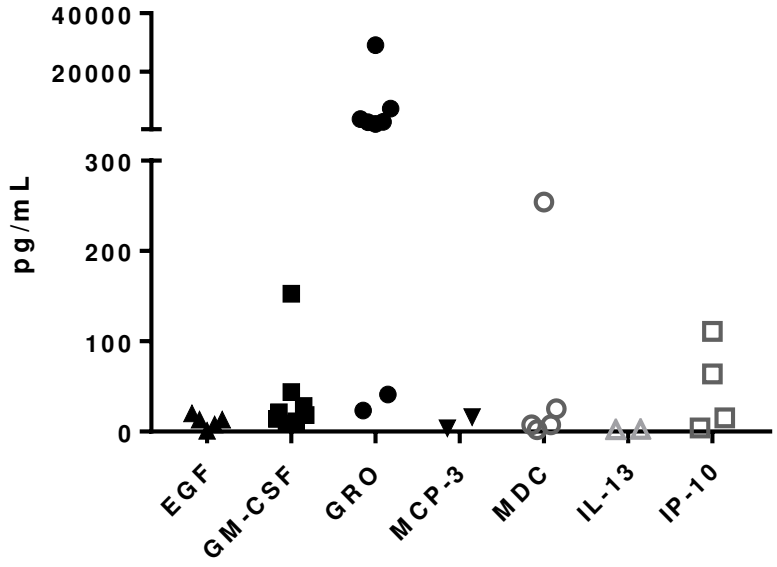


Figure 2-13 Luminex screening. From 9 cytokines the rage of detection for each cytokine in the apical wash was: (A) EGF 1-20 pg/ml, GM-CSF 8-150 pg/ml, MDC 2-255 pg/ml, IP-10 4-110 pg/ml and with (B) GRO having extremely high levels of detection from 23-30,000 pg/ml. Two cytokines have low levels detected; (A) MCP-3 3-16 pg/ml and IL-13 ~2pg/ml. MIP-1α and MIP-1β were not detected in the apical wash.

2.5.2.2 *Cytokine secretion over time*

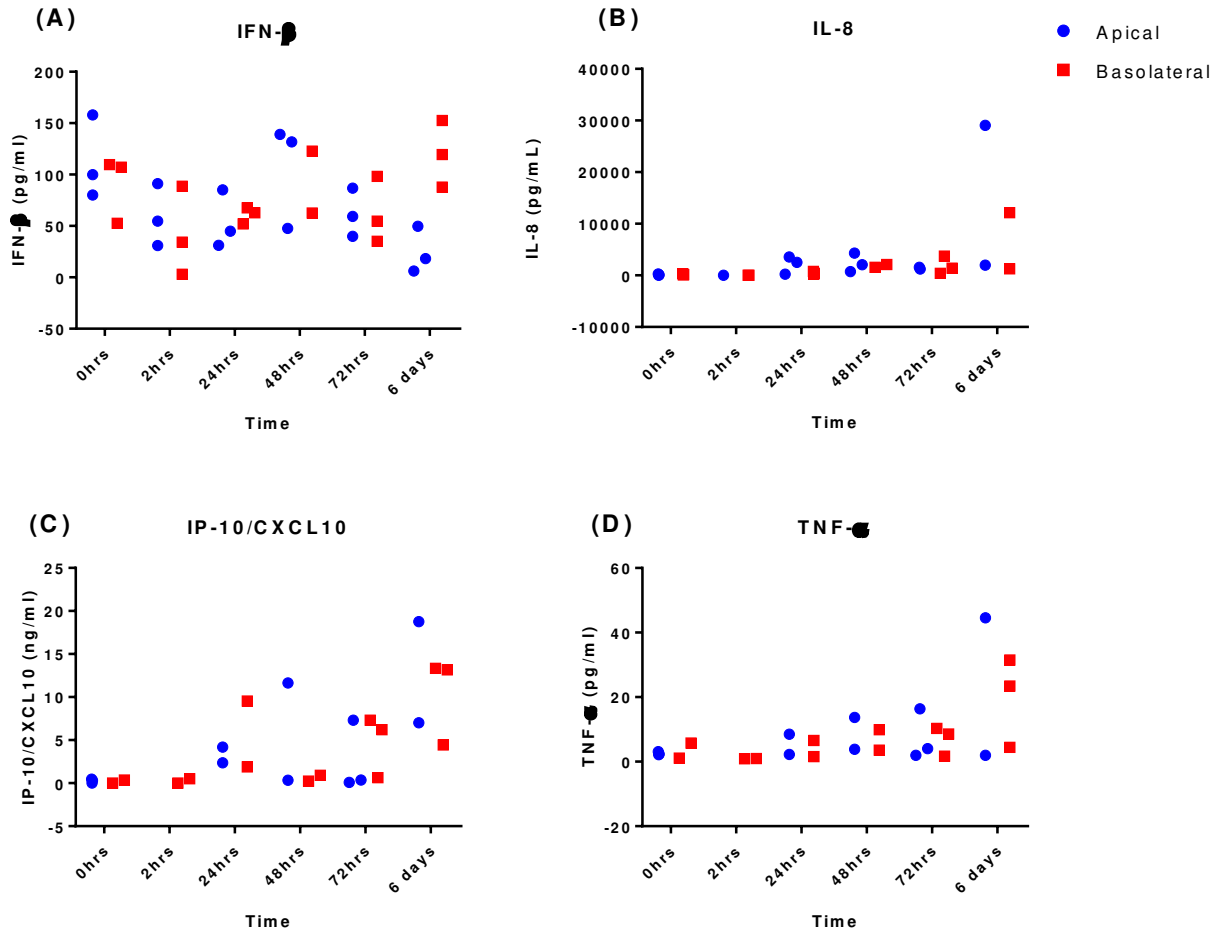


Figure 2-14 Cytokine release from the apical wash of ALI cultures detected by AlphaLISA assay. Wash and media collected over a 6 day time period for 3 subjects.

Apical wash and basolateral media of ALI cultures was collected at 0, 2, 24, 48 and 72 hours, with a final sample taken at 6 days (n=3). (A) There was no significant difference seen between the apical and basolateral release of IFN-β over the sampling period of 6 days, however there was detectable levels of IFN-β in the both the washes. (B) No significant difference of IL-8 was seen between apical and basolateral release for 72 hours, however there was an increase seen at 6 days, with high levels of IL-8 detected in both washes. (C) For IP-10 there was no significant difference was seen between apical and basolateral washes. There was a slight increase of IP-10 detected over the 6 day time period but this was not significant. (D) Lastly, for TNF-α there was no significant difference between apical and basolateral washes for 72 hours. Levels of TNF-α was increasing at 6 days for apical and basolateral.

2.5.3 Discussion

Cytokine levels are a reliable and useful measurement of inflammation and innate immunity (184-186). However, most previous reports used submerged monolayer cultures, where sampling of the culture is easier and less disruptive to the epithelial cells. The situation appears more complex with ALI cultures. In the present study we initially measured the cytokine levels on the apical surface for the Luminex screening, as this is the most applicable site. The Luminex assay was only used as a screening tool and we were able to detect all selected cytokines in the apical wash samples at varying concentrations, except for MIP-1α and MIP-1β. Knowing that specific cytokines could be detected from the ALI cultures was essential before moving forward and using homogenous proximity assays, like the AlphaLISA assay.

From the Luminex screen we had very high levels of GRO/CXCL1 across the healthy ALI cultures with extremely high levels from healthy subject 8. There was very little or no detection of MIP-1α, MIP-1β and IL-13 from most of the cultures. This was surprizing for IL-13 because cells from allergic individuals with asthma should show elevated expression of IL-13(187, 188), with Allhaverdian et al showing that IL-13 can be detected in differentiated ALI cultures(189). The levels of IL-13 will be of more relevance when compared to epithelial cells from subjects with respiratory diseases like asthma. Other cytokines in the screen had high levels of detection which was expected because all were selected for their relation to inflammatory or immune response. As stated above, high release of GRO was seen, with Cao et al reporting release of GRO from submerged bronchial epithelial cells at around 600pg/ml(190), EGF is shown to accelerate wound closure in airway epithelial cells(191), and GM-CSF reported to increase eosinophil survival(186). Although not showing quantitative and comparable results for the cytokines that were detected in the Luminex screening, we were able to identify a small number of cytokines that should not be continued on for future experiments. These included MIP-1α, MIP-1β and MCP-3 because of the small levels of detection.

From this Luminex screening we can conclude that cytokines can be measured from the apical wash, but a more sensitive and selective assay should be used. Samples were taken from ALI cultures from 3 healthy children subjects, from the apical wash and basolateral media. There was no difference seen between the apical or basolateral release of IFN-β, IL-8, IP-10 and TNF-α, but we did see

detection of all four cytokines. This allowed us to pursue this method further to see what effect TLR agonists would have on the ALI culture and how it would affect cytokine release.

2.6 Toll-like receptor agonists on ALI cultures

2.6.1 Methods

Cells collected from 3 healthy children donors and grown at ALI for a minimum of 21 days in B-ALI media. The cultures were exposed to TLR agonists for 2 hours at concentrations in Table 2-7, in 200µL PBS. After incubation, the apical wash and the basolateral media were collected and basolateral replaced with fresh media. Samples were collected at 0, 2, 24, 48, and 72 hours, with a final sample taken at six days post exposure. TLR agonists were purchased from InvivoGen(192) with concentrations listed in Table 2-7.

Table 2-7 Concentrations of TLR agonists

TLR	Agonist	Concentration
2	Pam3CSK; Synthetic triacylated lipoprotein	1µg/ml
3	High-molecular weight poly (I.C)	10µg/ml
4	LPS; Lipopolysaccharide from *E.coli* 0111:B4	10µg/ml
7	CL075; Thiazoloquinolone derivative	5µg/ml

2.6.2 Results

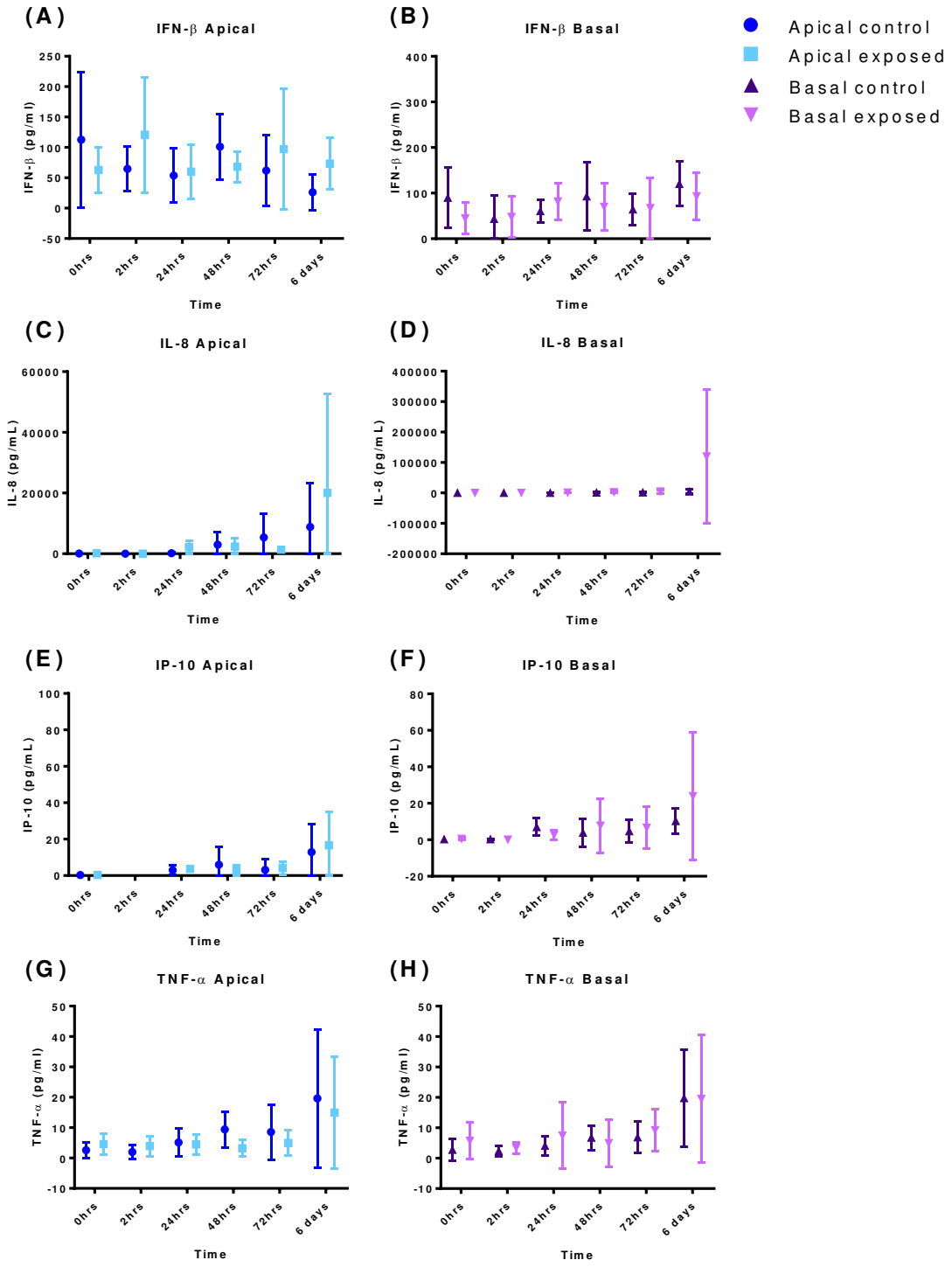


Figure 2-15 TLR 2 agonist exposure. Wash and media was collected over a 6 day period and analysed for cytokine release (n=3). (A, C and D) For three of the cytokines tested after exposure to TLR2 agonist there was no difference seen compared to the control. (B) IL-8 did show a slight increase for the basolateral exposed but was not significant.

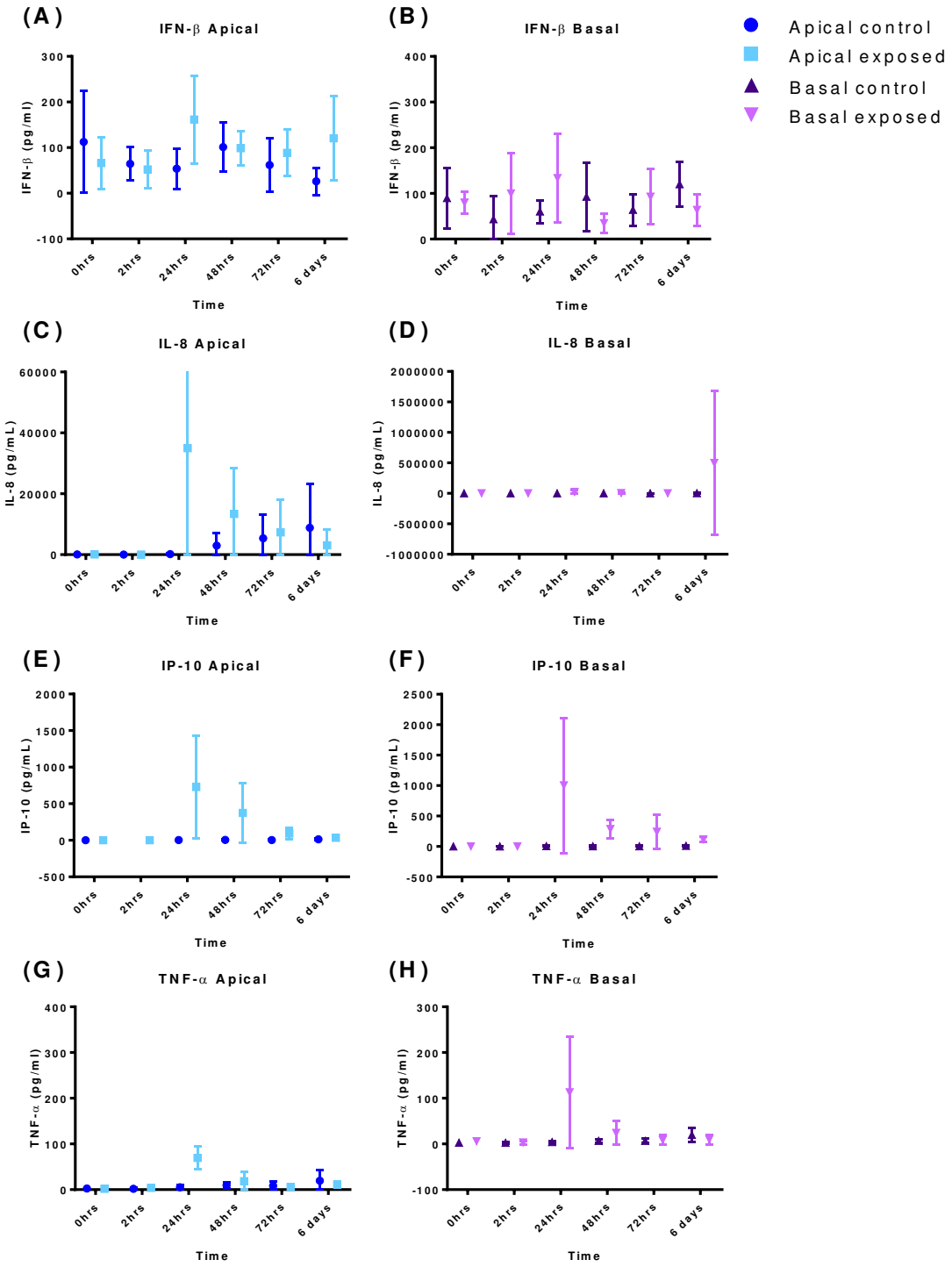


Figure 2-16 TLR 3 agonist exposure. Wash and media was collected over a 6 day period and analysed for cytokine release (n=3). (A) When exposed to the TLR3 agonist there was no difference in the IFN-β expression compared to the control over time. (B) There was a slight increase of IL-8 in the basolateral exposed at 6 days. (C and D) Both IP-10 and TNF-α had an increase at 24hrs for apical and basolateral exposed, although not significant.

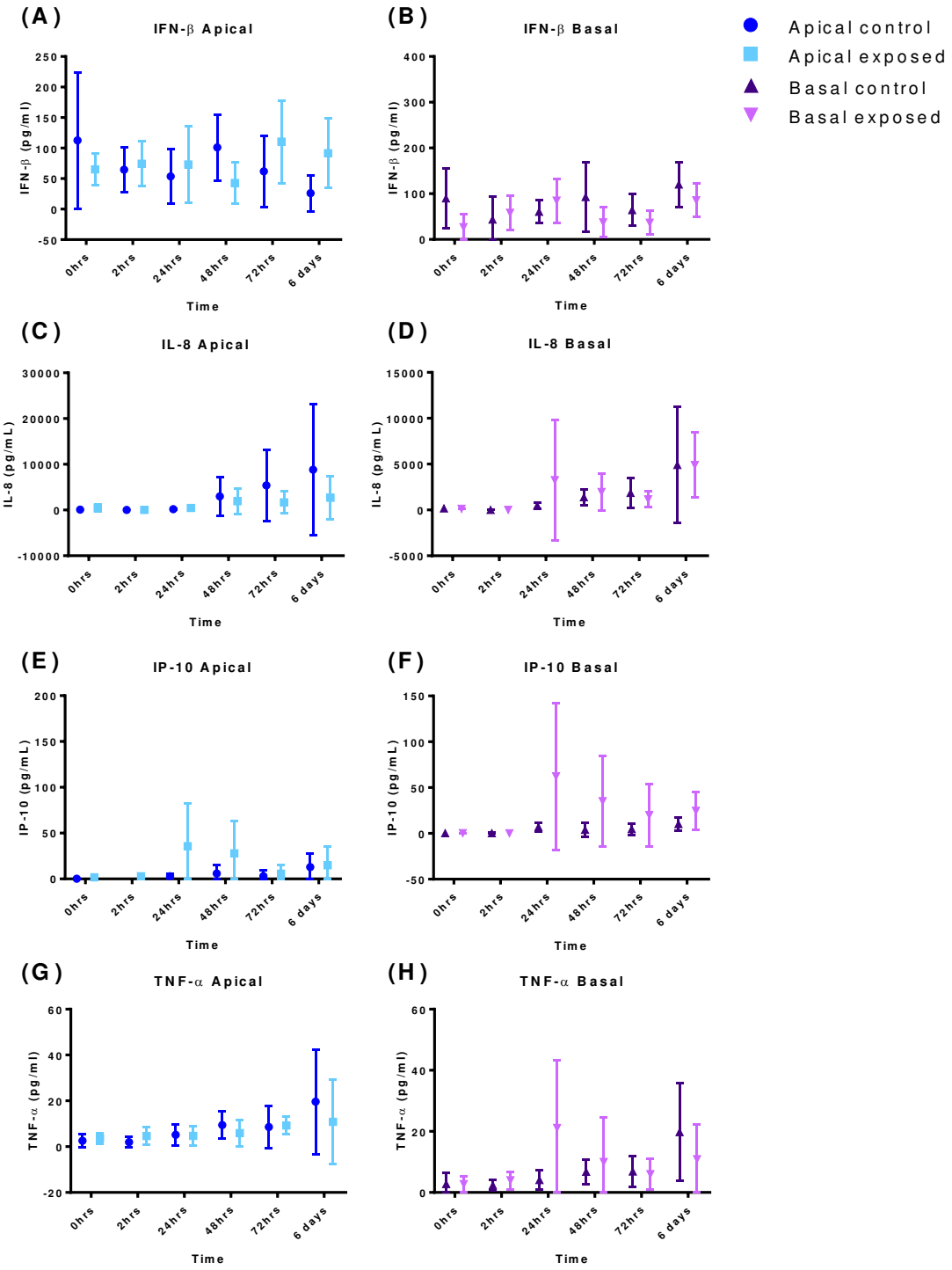


Figure 2-17 TLR 4 agonist exposure. Wash and media was collected over a 6 day period and analysed for cytokine release (n=3). (A and B) Exposure to the TLR4 agonist caused no difference in the cytokine release of IFN-β and IL-8 compared to control. (C) IP-10 showed an increase for both apical and basolateral exposed at 24hrs and 48hrs. (D) TNF-α also showed an increase at 2hrs but only for the basolateral exposed. There was no significance seen.

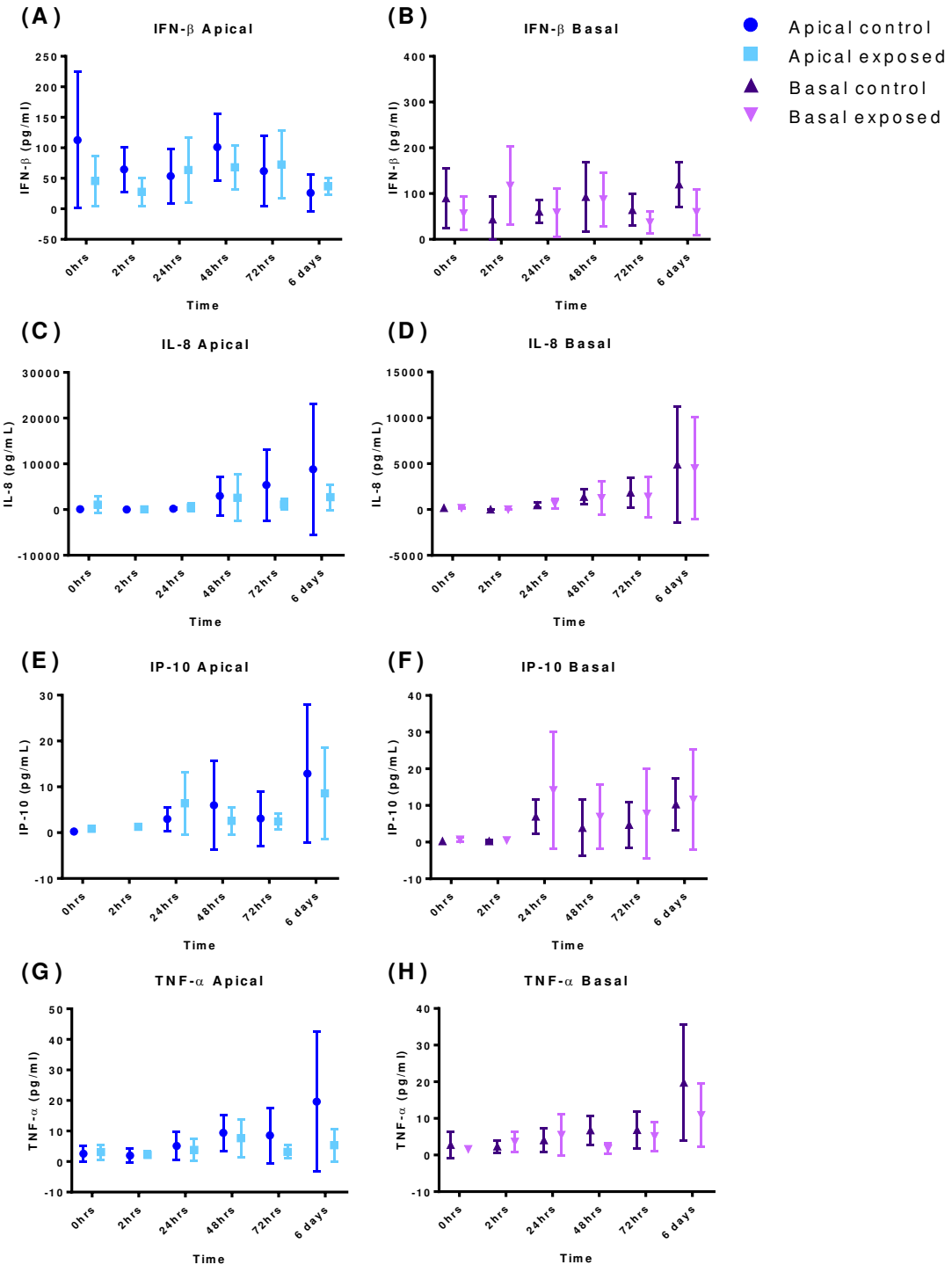


Figure 2-18 TLR 7 agonist exposure. Wash and media was collected over a 6 day period and analysed for cytokine release (n=3). (A, B and D) After exposure to TLR7 agonist there was no significant difference seen in cytokine expression from IFN-β, IL-8 and TNF-α. (C) There was a slight increase of IP-10 in the basolateral exposed at 24hrs, although not significant.

2.6.3 Discussion

Airway epithelial cells are exposed to multiple TLR agonists in normal *in vivo* settings and previous submerged cultures have been unable to accurately measure the effect on cytokine secretion. Using the model of ALI epithelial cells were apically exposed to TLR agonists for 2 hours, with apical wash and the basolateral media then collected and analysed for cytokine release. For all four cytokines tested we were able to see that there was release of each specific cytokine in both the apical and basolateral chambers with detectable amounts using AlphaLISA. There was however no change between those cultures exposed to a TLR agonist and control. The first variable that might have been an issue causing no difference between the control and exposed was the sampling technique. To collect cytokines secreted from the apical surface, PBS was added, and the cells washed at every time point. This can cause disruption to the apical surface with time points not being cumulative. The same occurs in the basolateral chamber where the whole media was changed at every time point and replaced. Future experiments could look at the sampling technique better suited to measure cytokine secretion. This study I was limited to cell number and cell viability which impacted the way the sampling was done.

From previous studies we should have seen an increase in IL-8 when epithelial cells are exposed to TLR agonists because Bailey and Romberger et al report that this exposure initiates an inflammatory cascade resulting in production of inflammatory cytokines like IL-8. They saw expression of IL-8 and also IL-6(193). Along with them, Tengroth and Millrud et al saw that TLR3 agonist activates IFN-β in the nasal mucosa with TLR3 and TLR7 known to respond to viral stimulation with increased secretion of IL-6 and GM-CSF(194). Further afield then the airway epithelium Esalani and Movahedan reports that epithelial wounding in the cornea induces expression of functional TLR4 and appears to contribute to wound repair by enhancing migration and proliferation(195). Understating TLR and airway epithelium relationships is very complex and connected with one study showing that stimulation of the airway epithelial cells with a specific TLR agonist effects gene expression of the other TLRs(196).

TLR expression on the airway epithelium should be explored in much more detail to fully understand and although not shown to be effective in these experiments, growing nasal epithelial cells at ALI can be used as an effective model. However, without a reliable way to sample the cytokine release from the ALI cultures we can conclude that this is not the appropriate method for this hypothesis.

2.7 Conclusions

These data highlight the challenges in working with primary cell models and the need for careful characterisation and selection of systems to answer specific research questions. Stewart suggests that all model systems should be validated to ensure that the most suitable model is being used(156). The validation of the ALI system has highlighted its strength and weaknesses in airway epithelium and wound healing research. This is was represented by the TLR and cytokine Luminex screening and single cell experiments not being suitable at this stage in the ALI cultures, but also the promising results from the wound healing model. It showed that well-differentiated cultures of primary nasal epithelial cells grown at the air-liquid interface are suitable for studying wound healing and that measuring the rate of wound healing gives a reliable outcome variable. The ALI culture must have the presence of basal, goblet and ciliated cells with a stable TEER before wounding of the culture. The next step, in this thesis, was to use this model to study factors influencing wound healing, including host disease status and environmental exposures.

Chapter 3 Impact of atopy and asthma on wound healing in the respiratory epithelium

3.1 Introduction

This chapter will look at the differences in wound healing between different airway disease groups such as asthma and atopy.

3.1.1 Asthmatic and atopic cells in culture

Epithelial cells are the first line of defence in the airways and are made up of a complex arrangement of cells with the ability to remodel its structure and repair its integrity after epithelial damage(197, 198). The epithelium is comprised of goblet, basal and ciliated cells which are essential in establishing mucociliary clearance, produce anti-microbial peptides, chemokines and cytokines and act as a protective barrier. The relationship between these cells acting as one to form a barrier defence can be studied using the method of air-liquid interface. Growing nasal epithelial cells at the air-liquid interface allows for the differentiation of pseudostratified epithelial culture to be developed *in vitro*.

The previous chapter of this thesis looked at the characterizing of ALI cultures from healthy nasal epithelial cells for development of a wound healing model. From this study we identified a reliable method of measuring the rate of wound closure from 18 healthy adult donors. This chapter will look at extending this research further and looking more into different airway diseases, specifically asthma. Asthma is a chronic inflammatory disorder with symptoms of coughing, wheezing and chest tightness and reported in 10.8% of all Australians(199). People with asthma are reported to have abnormal airways with increased EGFR (200-204), altered expression of adhesion proteins E-cadherin and Zonula occluden-1(205) elevated numbers of basal cells marked by cytokeratin-5 (78, 144), with IL-13 playing a distinct role in allergic asthma(206). Atopy is closely related to asthma and defined as the predisposition to develop allergic hypersensitivity reactions and produce IgE in response to allergens. It affects up to half of the population in the western world, with only 20% going on to develop asthma (207). The relationship of atopy and the epithelium has not been extensively explored.

Hypothesis

That asthmatics (atopic or non-atopic) will have a reduced rate of wound healing.

Aims

1. Determine the wound healing rate of nasal epithelial cells grown at ALI from non-atopic asthmatic, atopic and atopic asthmatic subjects.
2. To determine the difference in cytokine secretion, and gene expression from wounded ALI cultures from healthy, non-atopic asthma, atopy and atopic asthmatic subjects.

3.2 Methods

3.2.1 Wound healing

3.2.1.1 Differentiation media

Media was prepared and used as per previous chapter following manufactures instructions. Modified PC-ALI media (PC-ALI altered) was prepared and used at the concentration of 10x base media supplement at 50% and 100X maintenance supplement at 100%. Pen/Strep and hydrocortisone remained at the recommended concentration, with hydrocortisone only removed prior to wounding.

3.2.1.2 Wound healing

Wound healing experimental methods established in chapter 2 were used for ALI cultures from non-atopic asthmatic, atopic and atopic asthmatic subjects.

3.2.2 IL-13

3.2.2.1 IL-13 Exposure

IL-13 (10ng/ml) (208) was added to the basolateral media 7 days prior to wounding and replenished every two days when media was changed for 11 subjects (Healthy = 2, Non-atopic asthmatic = 5, Atopic = 2 and Atopic asthmatic = 2).

3.2.2.2 IL-13 Inhibition

ALI cultures were established from 7 subjects (healthy = 4, atopic = 3) and differentiated in PC-ALI altered media. Human IL-13 monoclonal antibody (10ng/mL) (Clone 31606, R & D Systems) was

added to the basolateral media 7 days prior to wounding and replenished every two days when media was changed.

3.2.3 EGF

3.2.3.1 EGF Exposure

ALI cultures were established from 7 subjects (healthy = 4, atopic = 3) and differentiated in PC-ALI altered media. EGF (25ng/ml, StemCell) (209) was added to the basolateral media 7 days prior to wounding and replenished every two days when media was changed.

3.2.3.2 EGFR inhibition

To determine the effect EGF plays on wound healing the EGFR inhibitor, Erlotinib HCl (2ug/ml)(OSI-744, Shelleckchem)(210), was added to the basolateral media 7 days prior to wounding. Media with inhibitor was replenished every two days when media was changed.

3.2.4 Cytokine

3.2.4.1 Luminex Screening

Luminex screening of apical wash was conducted as in chapter 2 with ALI culture apical wash samples from healthy, non-atopic asthmatic and atopic.

3.2.4.2 AlphaLISA

As established in chapter 2, supernatants of epithelial ALI cells were collected by washing the apical surface of the ALI culture with PBS at pre-wounding, 0 and 48 hours. Washes were tested for cytokine detection using AlphaLISA kit (Perkin Elmer) following manufacturer's recommendations. Cytokines tested in this chapter are listed in Table 3-1.

Table 3-1 Cytokines in AlphaLISA assay

Cytokine		Assay	Detection Limit (pg/ml)	Catalogue number
GM-CSF	Granulocyte-macrophage colony-stimulating factor	AlphaLISA	1.6-10,000	AL216C
IP-10	CXCL10, Interferon gamma-induced protein 10	AlphaLISA	3.3 – 300,000	AL326C
IL-13	Interleukin 13	AlphaLISA	3-100,000	AL240C

3.2.5 Gene Expression

3.2.5.1 Gene expression

Whole Well

RNA was extracted using RNeasy mini kit (Qiagen, USA, Cat 74106), reverse transcribes to cDNA using Quantitect real time kit (Qiagen, USA, Cat 205311). qPCR was performed using pre-designed TaqMan primer/probe combinations Table 3-2 and β-Actin as control gene, in duplicated wells (LifeTech, USA) on ABI 7900HT (Applied-Biosystem, USA). Primer sequences are available on the manufacturer's website. Data was normalized with β-Actin.

Single Cell

Wounded ALI cell cultures were again scraped with a P10 pipette tip along the leading edge of and already wounded culture to collect cells. Cells were resuspended in 10µl of PBS and placed in a well of 1% low melt agarose. This allows the cells to separate and to stick to the agarose for easy selection. With a fine glass pipette and under the microscope, single cells were picked and collected and immediately snap frozen in lysis solution. Other cells from the same well were also picked to act as a control, with approximately 18 cells collected from 10 subjects. RNA extraction and cDNA synthesis were conducted using the SuperScript® III CellsDirect™ cDNA Synthesis Kit (Life Technologies, USA) with mRNA expression examined for the genes describes in Table 3-2.

Table 3-2 Gene assays used in the TaqMan assay to analyse gene expression.

Gene	Target	Supplier	Assay ID
FOXJ1	Cilia cells	ThermoFisher Scientific	Hs00230964_m1
p63	Basal cell	ThermoFisher Scientific	Hs00978340_m1
MUC5AC	Secretory cells	ThermoFisher Scientific	Hs00873651_mH
FOXA3	Secretory cells	ThermoFisher Scientific	Hs00270130_m1
SPDEF	Secretory cells	ThermoFisher Scientific	Hs01026050_m1
TFF3	Goblet cell	ThermoFisher Scientific	Hs00902278_m1
CLDN3	Tight junction	ThermoFisher Scientific	Hs00265816_s1
FN1	Fibronectin	ThermoFisher Scientific	Hs00365058_m1
IL-13	Interleukin 13	ThermoFisher Scientific	Hs00174379_m1
IL-13Rα1	Interleukin 13 receptor	ThermoFisher Scientific	Hs00609817_m1
EGFR	Epidermal growth factor receptor	ThermoFisher Scientific	Hs01099990_m1
Human ACTB (Beta Actin) Endogenous Control	Housekeeping control	ThermoFisher Scientific	Cat# 4326315E

3.3 Results

3.3.1 Cell culture survival

3.3.1.1 Success of ALI cultures

Table 3-3 Success of ALI cultures. Nasal epithelial cells were grown at ALI from 75 adult subjects. From this, 97 primary cultures were attempted at ALI , 76 were successfully established (78%), 12 died after lift (12%) and 9 died before lift (10%). From 36 healthy cultures 28 (78%) were successful, with 6 (16%) dying after lift and 2 (5%) before lift. The non-atopic asthmatics had 14 (88%) successful cultures from 16, 1 (6%) dying after and before lift. Atopic subjects had 24 (86%) successful cultures from 28, and with 2 (7%) dying both after and before lift. Lastly the atopic asthmatic had 11 (61%) successful cultures from 18, with 3 (17%) dying after lift and 4 (22%) dying before lift.

ALI culture success	Healthy	Non-atopic asthmatic	Atopic	Atopic asthmatic	Total
Total	37	16	26	18	97
Successful ALI culture	29	14	22	11	76
Died after lift	6	1	2	3	12
Died before lift	2	1	2	4	9

3.3.2 Rate of wound closure

Table 3-4 Subjects used for wound healing experiments. From the 76 ALI cultures that grew successfully only 71 were used in the wound healing assay detailed below in figure X.

Subject	Disease Status	Sex	Steroid	Days to lift	Days from lift	TEER (Ω.cm²)		Hours	Wound width (μm²), %[1]			Rate of wound closure (%/hour)	R squared	Media
						Control	Wounded		Initial	Final				
1	Healthy	Female	No	4	45	1369	1072	25	586748	4820	0.82%	4.49	0.93	B-ALI
2	Healthy	Male	No	6	51	247	280	31	461552	1790	0.39%	3.36	0.95	B-ALI
3	Healthy	Male	No	9	35	445	445	16	457662	1626	0.36%	6.75	0.94	B-ALI
4	Healthy	Female	No	3	58	940	1270	46	462954	270058	58.33%	0.73	0.67	B-ALI
5	Healthy	Male	No	4	47	1765	1699	27	335258	2804	0.84%	3.95	0.98	B-ALI
5	Healthy	Male	No	3	72	0	0	29	655548	8504	1.30%	3.45	0.99	PC-ALI
6	Healthy	Female	No	2	37	280	280	32	550584	3532	0.64%	3.58	0.96	B-ALI
6	Healthy	Female	No	3	49	253	174	22	1313127	34923	2.66%	4.66	1.00	PC-ALI altered
7	Healthy	Male	No	2	44	280	280	21	307284	8772	2.85%	5.23	0.99	B-ALI
8	Healthy	Female	No	2	49	544	544	31	522444	4378	0.84%	3.47	0.97	B-ALI
9	Healthy	Female	No	3	56	1699	1699	32	432494	3562	0.82%	2.91	0.90	B-ALI
10	Healthy	Female	No	1	59	940	181	33	395260	874	0.22%	3.09	0.98	B-ALI
11	Healthy	Male	No	2	40	1402	1435	43	411136	23218	5.65%	1.91	0.96	B-ALI
12	Healthy	Male	No	5	42	16	16	20	354688	3992	1.13%	6.20	0.98	B-ALI
13	Healthy	Female	No	4	42	198	148	50	1010808	213974	21.17%	1.14	0.83	B-ALI
14	Healthy	Female	No	2	27	82	36	21	652452	10466	1.60%	5.09	0.99	PC-ALI
15	Healthy	Male	No	2	33	478	478	18	674236	2592	0.38%	7.43	0.95	PC-ALI
16	Healthy	Female	No	2	33	141	445	24	1008040	470	0.05%	5.06	0.96	PC-ALI
17	Healthy	Female	No	2	40	141	125	24	754790	1642	0.22%	5.02	0.99	PC-ALI
18	Healthy	-	No	4	31	477	331	15	300616	1399	0.47%	8.11	0.99	PC-ALI
19	Healthy	Female	No	4	27	544	125	39	223369	2292	1.03%	2.99	1.00	PC-ALI
26	Healthy	Female	No	5	47	215	143	48*	1185092	329180	27.78%	1.46	0.98	PC-ALI altered
27	Healthy	Male	No	5	47	405	354	48*	1105966	265158	23.98%	1.44	0.98	PC-ALI altered
28	Healthy	Female	No	5	47	293	224	27	1211443	49555	4.09%	3.39	0.96	PC-ALI altered

[1]Final wound area expressed as a percent with the initial wound area normalized to 100%

*Experiment discontinued at time point. Wound failed to close.

Table 3-5 Non-Atopic Asthmatic Subjects

Subject	Disease Status	Sex	Steroid	Days to lift	Days from lift	TEER (Ω.cm2)		Hours	Wound width (μm^2), %[1]			Rate of wound closure (%)	R squared	Media
						Control	Wounded		Initial	Final				
28	Non-atopic asthmatic	Female	Yes	7	35	1831	2227	19	465336	2452	0.5%	6.292	0.9702	BALI
29	Non-atopic asthmatic	Female	Yes	2	30	280	280	24	607590	4324	0.7%	4.686	0.9841	BALI
30	Non-atopic asthmatic	-	No	3	58	610	148	31	568006	1440	0.3%	3.307	0.9726	BALI
31	Non-atopic asthmatic	Female	Yes	6	70	280	478	20	512358	3014	0.6%	5.154	0.9855	BALI
32	Non-atopic asthmatic	Male	Yes	6	42	313	313	25	538682	5030	0.9%	5.264	0.94	BALI
33	Non-atopic asthmatic	Male	Yes	2	37	346	346	28	408234	2572	0.6%	3.545	0.9744	BALI
33	Non-atopic asthmatic	Male	No	2	28	346	676	37	894806	2550	0.3%	3.339	0.9984	PC
34	Non-atopic asthmatic	Female	No	4	52	2722	2656	26	481670	4030	0.8%	3.703	0.9314	BALI
35	Non-atopic asthmatic	Male	Yes	7	31	214	59	26	794968	3904	0.5%	4.678	0.952	PC
36	Non-atopic asthmatic	Male	Yes	2	44	184	158	24	651034	7840	1.2%	5.251	0.9847	PC
37	Non-atopic asthmatic	Male	Yes	2	46	313	313	30	837408	944	0.1%	4.101	0.9842	PC
38	Non-atopic asthmatic	Male	No	4	31	131	242	30	306826	5491	1.8%	3.658	0.9657	PC
39	Non-atopic asthmatic	-	No	4	29	367	245	50*	326175	198989	61.0%	1.884	0.9691	PC
40	Non-atopic asthmatic	Male	Yes	4	29	319	27	36	355096	172873	48.7%	3.038	0.994	PC

[1]Final wound area expressed as a percent with the initial wound area normalized to 100%

*Experiment discontinued at time point. Wound failed to close.

Table 3-6 Atopic Subjects

Subject	Disease Status	Sex	Steroid	Days to lift	Days from lift	TEER (Ω.cm²)		Hours	Wound width (μm²), %[1]			Rate of wound closure (%/hour)	R squared	Media
						Control	Wounded		Initial	Final				
42	Atopic	Female	No	3	46	82	16	48	521352	321478	61.66%	0.83	0.96	B-ALI
42	Atopic	Female	No	6	65	478	445	26	776570	1552	0.20%	4.68	0.98	PC-ALI
43	Atopic	Female	No	3	58	346	346	45	699870	1034	0.15%	2.21	0.93	B-ALI
44	Atopic	Female	No	7	49	280	313	40	544878	940	0.17%	2.47	0.98	B-ALI
44	Atopic	Female	No	9	38	82	66	24	1016084	4496	0.44%	4.76	0.99	PC-ALI
45	Atopic	Male	No	4	45	181	412	45	835982	24190	2.89%	2.13	0.99	B-ALI
46	Atopic	Female	No	8	25	1666	1732	18	565202	3978	0.70%	6.77	0.95	B-ALI
47	Atopic	Male	Yes	4	39	0	0	67	747090	379758	50.83%	1.68	0.90	B-ALI
48	Atopic	Female	No	5	43	0	0	25	623914	4330	0.69%	4.47	0.96	B-ALI
48	Atopic	Female	No	5	56	105	148	36	900784	2308	0.26%	3.75	0.99	PC-ALI
49	Atopic	Female	No	3	65	874	0	50	619614	137904	22.26%	1.60	0.98	B-ALI
49	Atopic	Female	No	5	53	478	115	23	619748	2462	0.40%	5.03	0.99	PC-ALI
50	Atopic	Female	No	3	63	214	82	36	608410	147804	24.29%	1.65	0.73	B-ALI
50	Atopic	Female	No	2	61	346	66	48	684462	436398	63.76%	0.53	0.86	PC-ALI
51	Atopic	Female	No	3	67	82	0	49	633404	484398	76.48%	0.26	0.28	B-ALI
51	Atopic	Female	No	4	91	0	0	50	714808	328448	45.95%	0.97	0.93	PC-ALI
52	Atopic	Female	No	4	31	354	205	27	324663	1041	0.32%	4.36	0.99	PC-ALI
52	Atopic	Female	No	3	49	346	268	18	1359114	94079	6.92%	5.71	0.95	PC-ALI altered
53	Atopic	Female	No	4	27	184	249	34	328429	1039	0.32%	3.22	1.00	PC-ALI
54	Atopic	Female	No	1	30	250	372	34	169000	559	0.33%	3.35	0.99	PC-ALI
54	Atopic	Female	No	3	49	294	311	24	854624	28593	3.35%	5.27	0.97	PC-ALI altered
55	Atopic	-	No	3	49	304	270	45	1041035	29670	2.85%	2.09	0.99	PC-ALI altered

[1]Final wound area expressed as a percent with the initial wound area normalized to 100%

*Experiment discontinued at time point. Wound failed to close.

Table 3-7 Atopic Asthmatic Subjects

Subject	Disease Status	Sex	Steroid	Days to lift	Days from lift	TEER (Ω.cm2)		Hours	Wound width (μm²), %[1]			Rate of wound closure (%)	R squared	Media
						Control	Wounded		Initial	Final				
55	Atopic asthmatic	Female	Yes	1	45	148	148	21	766218	2188	0.3%	5.27	0.9851	BALI
56	Atopic asthmatic	Female	No	5	43	148	66	44	668058	45114	6.8%	1.786	0.9571	BALI
56	Atopic asthmatic	Female	No	5	49	82	148	33	1036174	2456	0.2%	4.199	0.9512	PC
57	Atopic asthmatic	-	Yes	3	31	108	82	31	822360	5068	0.6%	3.635	0.9916	PC
58	Atopic asthmatic	Female	No	3	33	676	247	28	993454	2126	0.2%	3.932	0.9933	PC
59	Atopic asthmatic	Male	Yes	3	42	207	148	24	977598	16444	1.7%	4.845	0.9928	PC
60	Atopic asthmatic	Male	Yes	3	21	808	128	25	842734	3062	0.4%	4.191	0.9943	PC
61	Atopic asthmatic	?	No	3	28	132	214	24	545620	7658	1.4%	4.681	0.9995	PC
62	Atopic asthmatic	Female	Yes	4	29	203	155	29	260203	113425	43.6%	3.791	0.9927	PC
63	Atopic asthmatic	Female	No	4	29	160	109	50*	257118	17723	6.9%	3.047	0.9506	PC
64	Atopic asthmatic	Female	Yes	4	27	188	236	41	343048	6124	1.8%	2.56	0.9726	PC

[1]Final wound area expressed as a percent with the initial wound area normalized to 100%

*Experiment discontinued at time point. Wound failed to close.

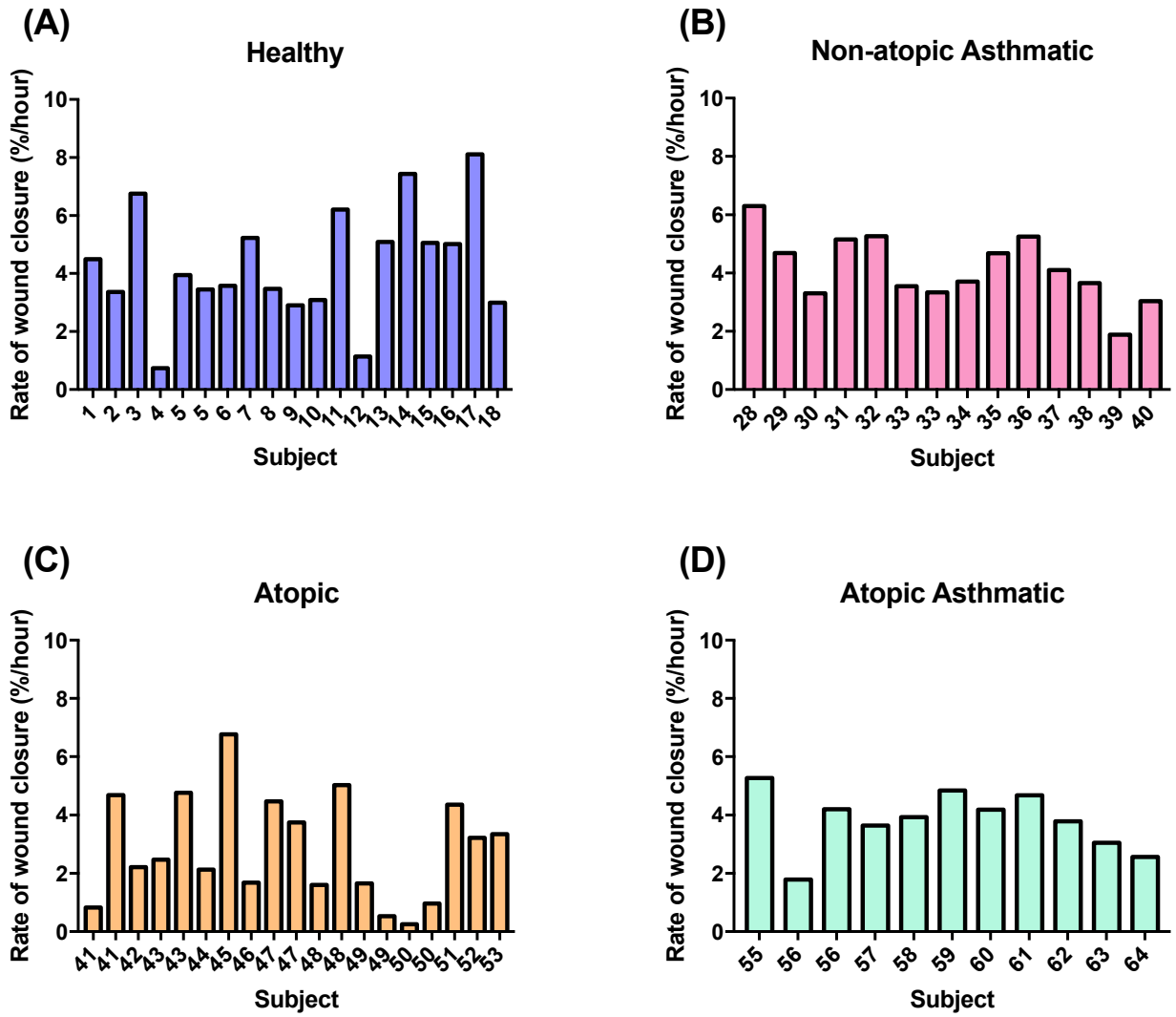


Figure 3-1 Comparison of wound healing rates between healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects. 19 healthy, 14 non-atopic asthmatic, 19 atopic and 11 atopic asthmatic ALI cultures were wounded, and their rate of wound closure was calculated. All but two healthy cultures had a wound healing rate of above 2%/hour, with all but one of the non-atopic asthmatics falling below 2%/hour. 12 of the atopic cultures had a wound healing rate above 2%/hour with the remaining seven below Only one atopic asthmatic was below 2%/hour with 10 above. Some difference is observed between rates of the same subject grown in different media (such as subject 48).

3.3.2.1 Rate of wound closure from all cultures

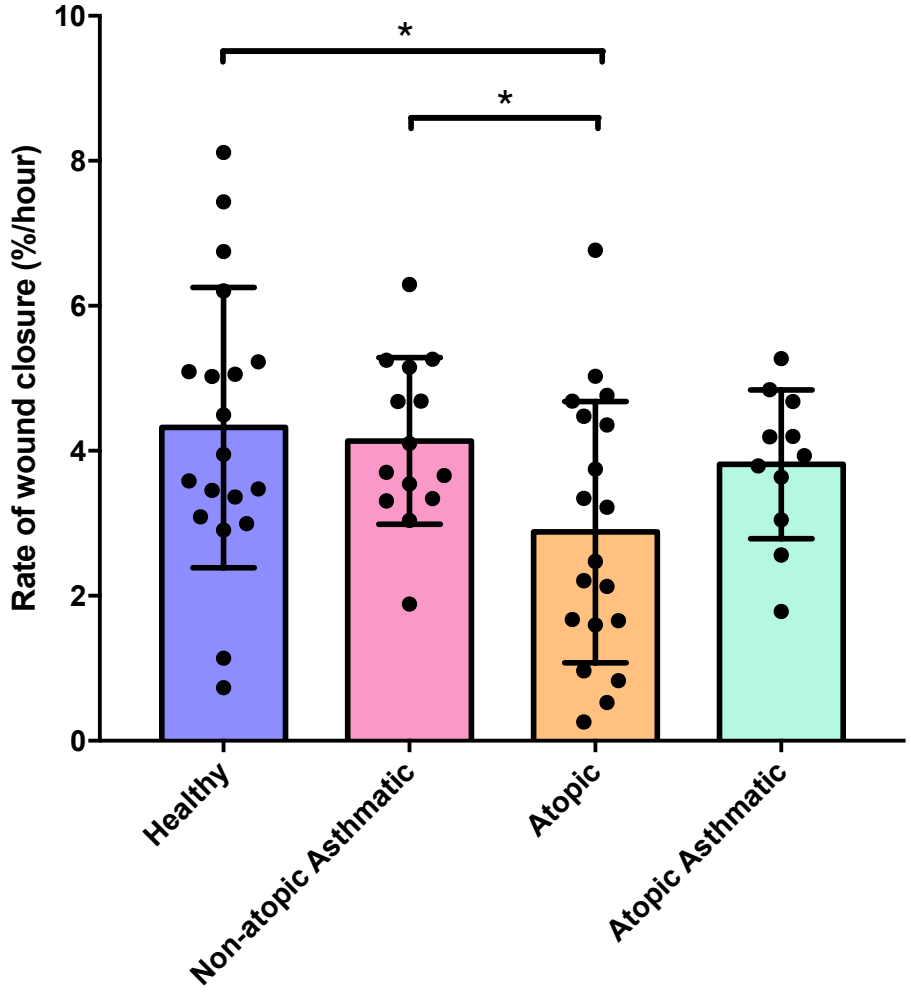


Figure 3-2 Rate of wound closure of ALI cultures from healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects. Significant difference is seen in the rate of wound closure between healthy (Mean = 4.3 ±1.9, p-value = 0.02, n=19) and non-atopic asthmatics (mean = 4.1 ±1.1, p-value = 0.03, n=14) from the wound healing rate of atopic subjects (mean = 2.9 ±1.8, n=19)(Mann-Whitney test). No significant difference was seen between any group and atopic asthmatics (mean = 3.8 ±1, n=11).

3.3.2.2 Rate of wound closure

Table 3-8 Wound closure of slow healing healthy subjects at ALI.

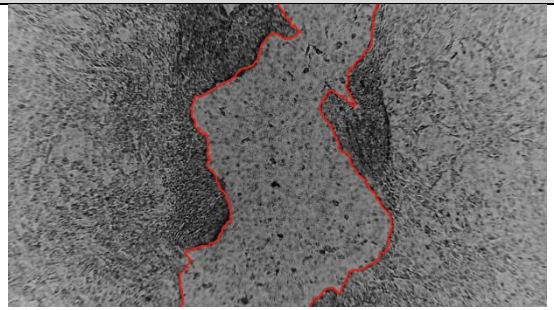
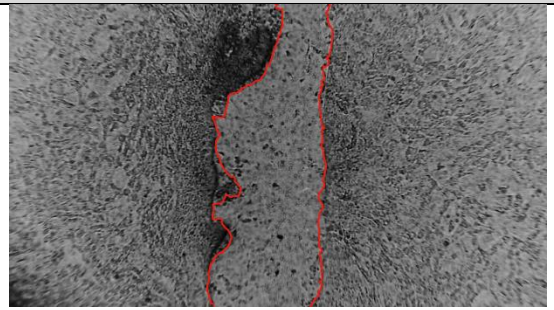
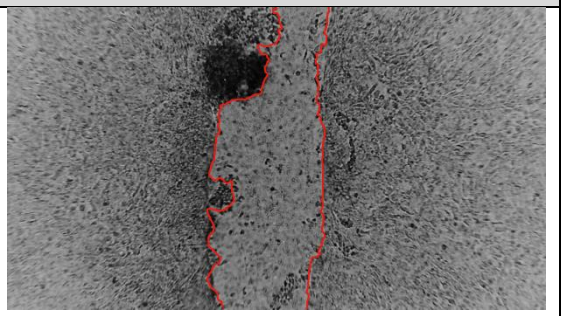
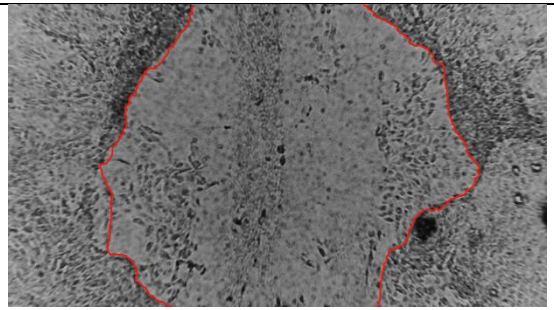
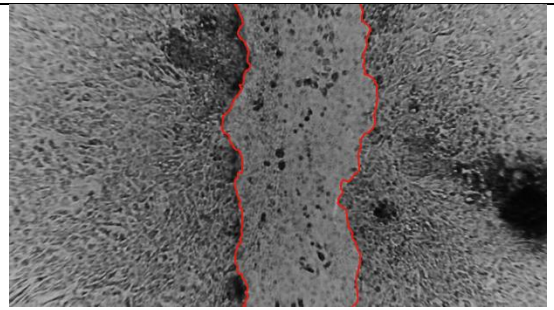
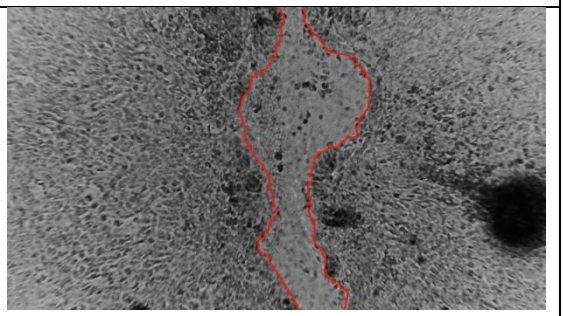
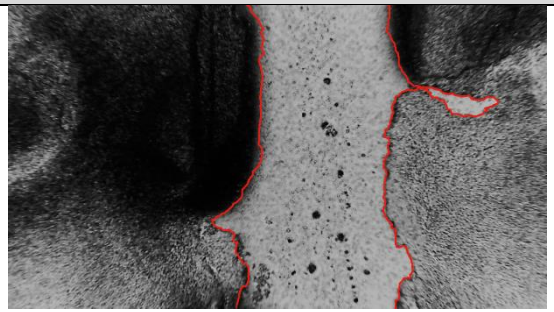
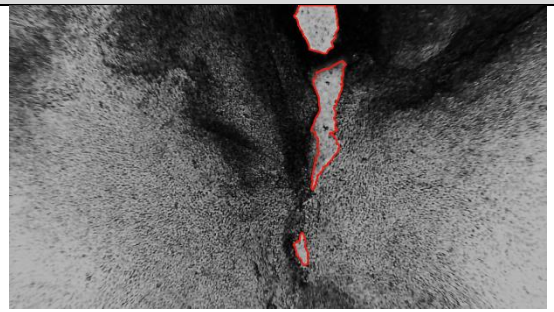
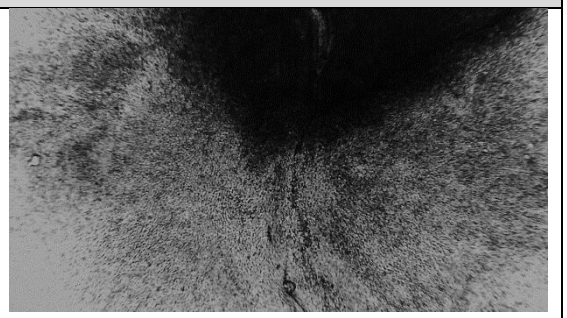
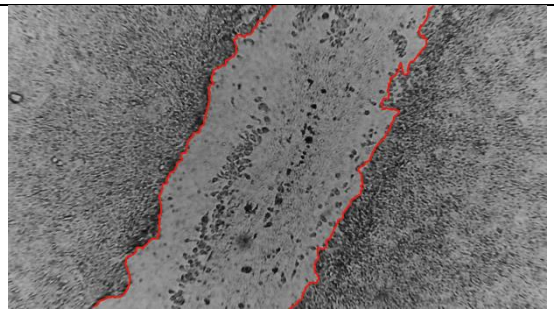
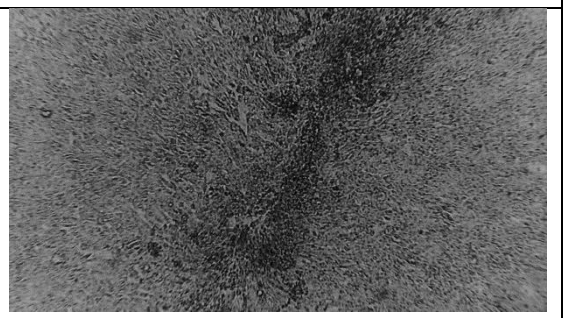
	Subject	0 hours	24 hours	48 hours
Healthy	4	 Area (μm²): 1009008	 Area (μm²): 408338	 Area (μm²): 213794
	12	 Area (μm²): 462954	 Area (μm²): 289310	 Area (μm²): 270058

Table 3-9 Wound closure of fast healing atopic subjects at ALI.

	Subject	0 hours	12 hours	24 hours
Atopic	45	 Area (μm^2): 565202	 Area (μm^2): 41398	 Area (μm^2): 0
	48	 Area (μm^2): 623914		 Area (μm^2): 0

3.3.2.3 Atopic status effecting wound repair

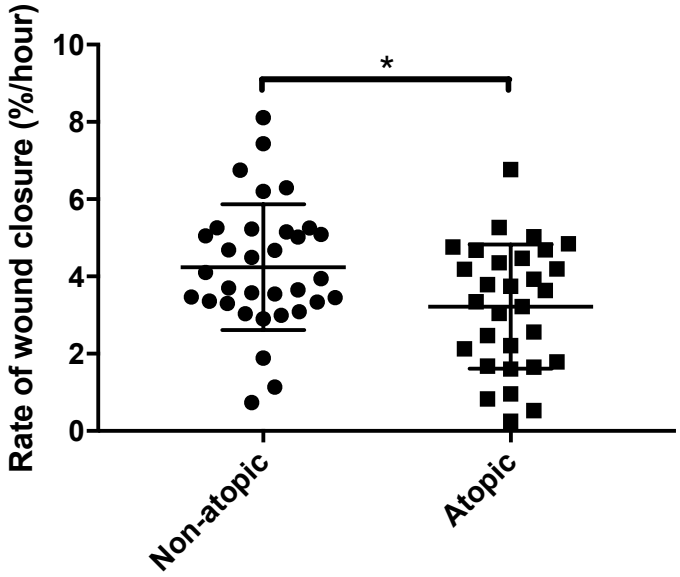


Figure 3-3 Effect of atopic status on rate of wound closure. Non-atopic subjects (n=33) has a significantly higher rate of wound closure than atopic subjects (n=30) (p-value: 0.03) in a Mann-Whitney test.

3.3.2.4 TEER

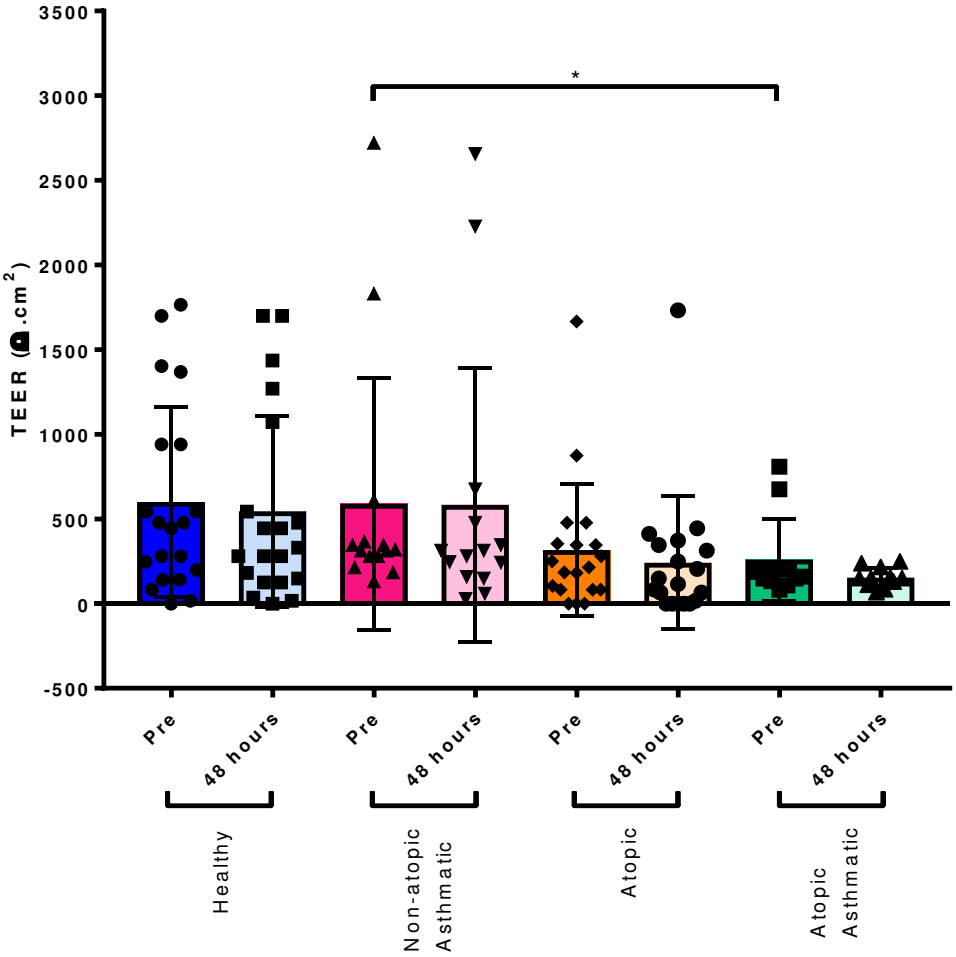


Figure 3-4 TEER values of ALI cultures from healthy (n=19), non-atopic asthmatic (n=14), atopic (n=19) and atopic asthmatic (n=11) subjects before wounding and at 48 hours at wound closure. In a Kruskal-Wallis test there was significance seen with a p-value = 0.01. The TEER was significantly higher between non-atopic asthmatics (mean: 590 ± 744) and atopic asthmatics (mean: 260 ± 243) (p-value = 0.02) pre-wounding, but not between any other group in a Mann-Whitney test.

3.3.3 Age of culture

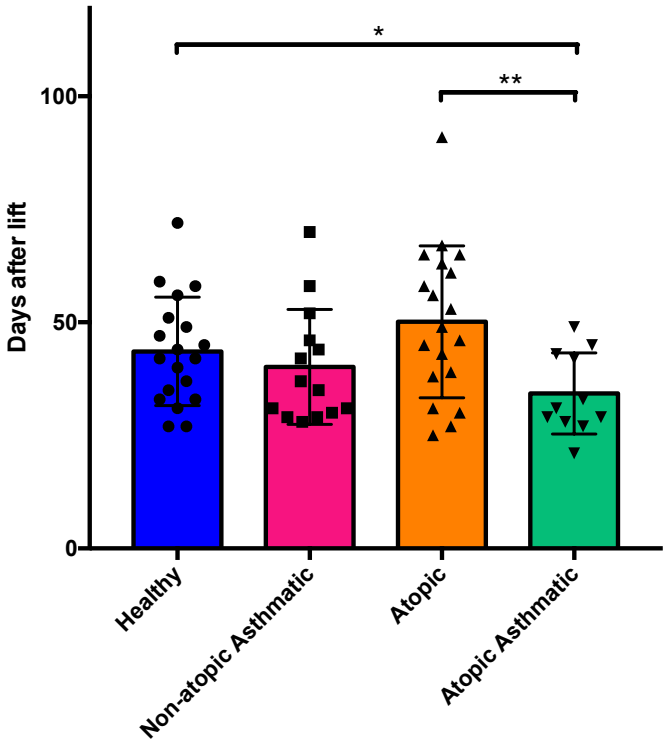


Figure 3-5 The age of ALI cultures at time of wounding for healthy (n=19), non-atopic asthmatic (n=14), atopic (n=19) and atopic asthmatic (n=11) subjects. In a Kruskal-Wallis test there was significance seen with a p-value = 0.03. Significant difference was seen between age of healthy (mean: 43 ± 12, p-value = 0.04), atopic (mean: 50 ± 17, p-value = 0.01) and the age of atopic asthmatics (mean: 34 ± 9) in a Mann-Whitney test.

3.3.4 Topical steroid use

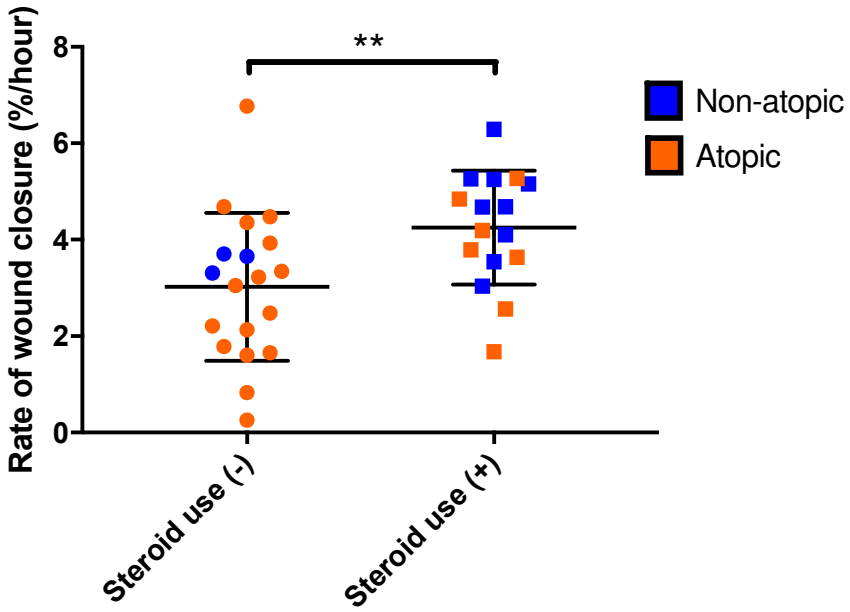


Figure 3-6 Rate of wound closure between ALI cultures of cells from subjects with no topical steroid use (n=19) and those subjects with reported topical steroid use (n=16). There was significant difference between the two with subjects that have used steroids healing at a faster rate (p-value = 0.008, Mann-Whitney test). More non-atopic subjects had previous use of steroids with majority from the non-atopic asthmatic groups. Only a small number of atopic subjects (asthmatic or not) had used steroids. The highest atopic subject with steroid treatment used Nasonex.

3.3.4.1 IL-13 Secretion

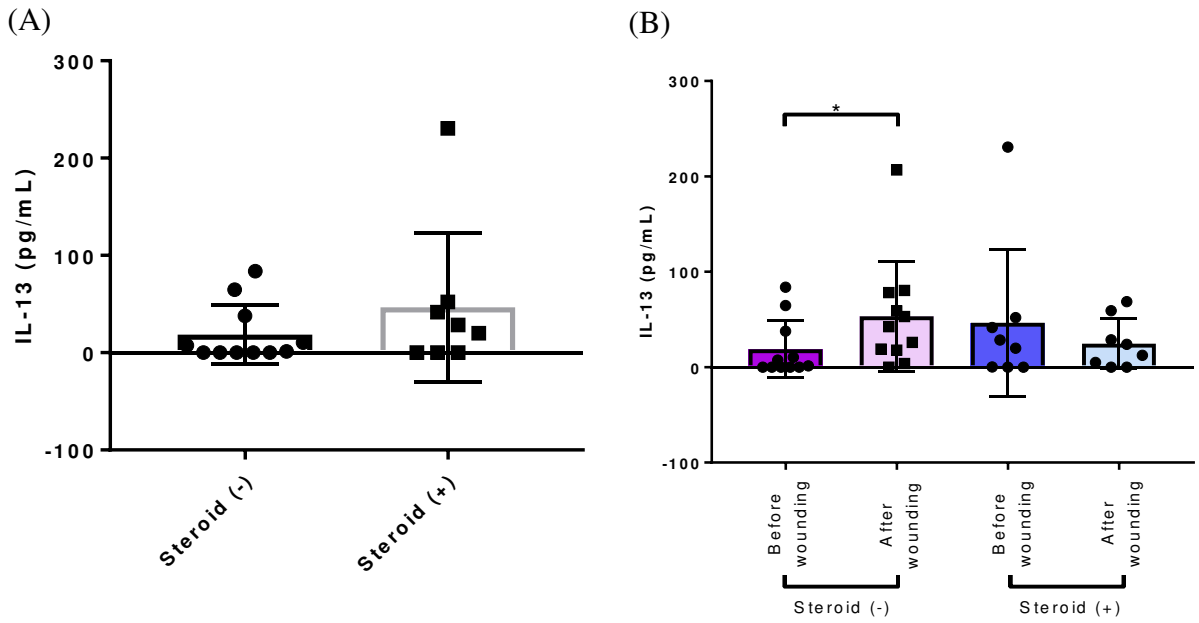


Figure 3-7 IL-13 secretion from subjects (A) with topical steroid treatment (n=11) and without topical steroid treatment (n=8) and (B) before and after wounding.

3.3.5 Altered media

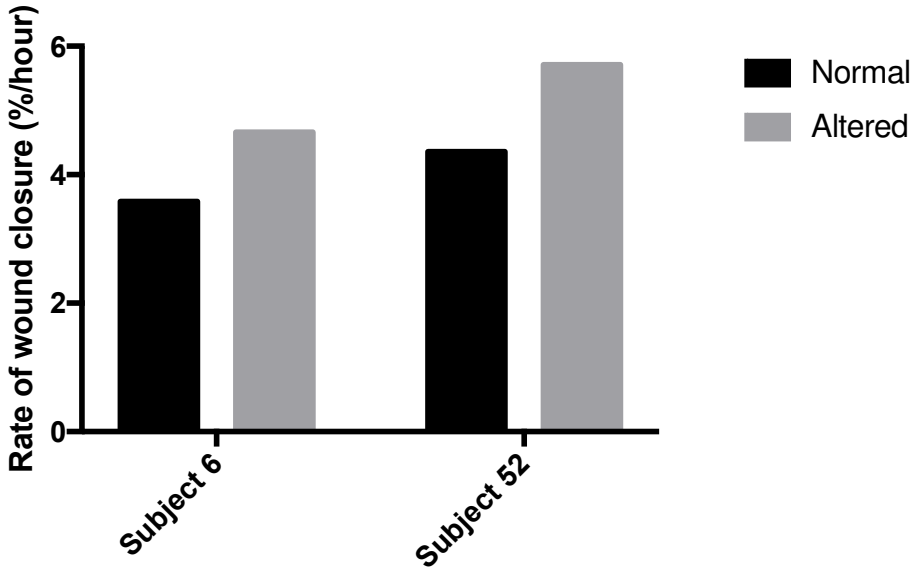


Figure 3-8 Rate of wound closure from two subjects (healthy = 1, atopic = 1) differentiated in normal media (B-ALI and PC-ALI, mean: 4 ± 0.5) and altered media (PC-ALI altered, mean: 5.2 ± 0.7).

3.3.6 IL-13

3.3.6.1 IL-13 Exposure

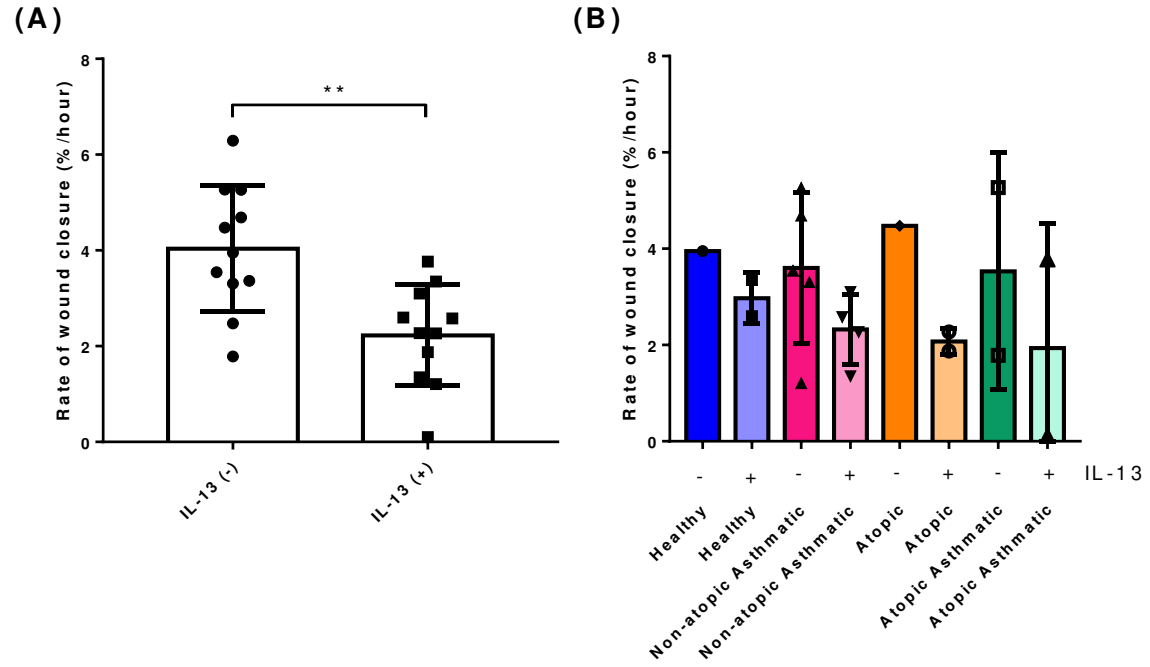


Figure 3-9 Effect of IL-13 exposure 7 days prior to wounding. (A) IL-13 caused a significant decrease (p-value = 0.01, Mann-Whitney test) in the rate of wound closure when added to the ALI culture 7 days prior to wounding. Cultures without IL-13 (n=11) had a mean of 4.04 ± 1.3%/hour and cultures exposed to IL-13 (n=11) had a mean of 2.22 ± 1.05%/hour. (B) All groups showed a decreased in the rate of wound healing when IL-13 was added 7 days prior to wounding, however not significant because of the low sample numbers.

3.3.6.2 IL-13 Inhibition

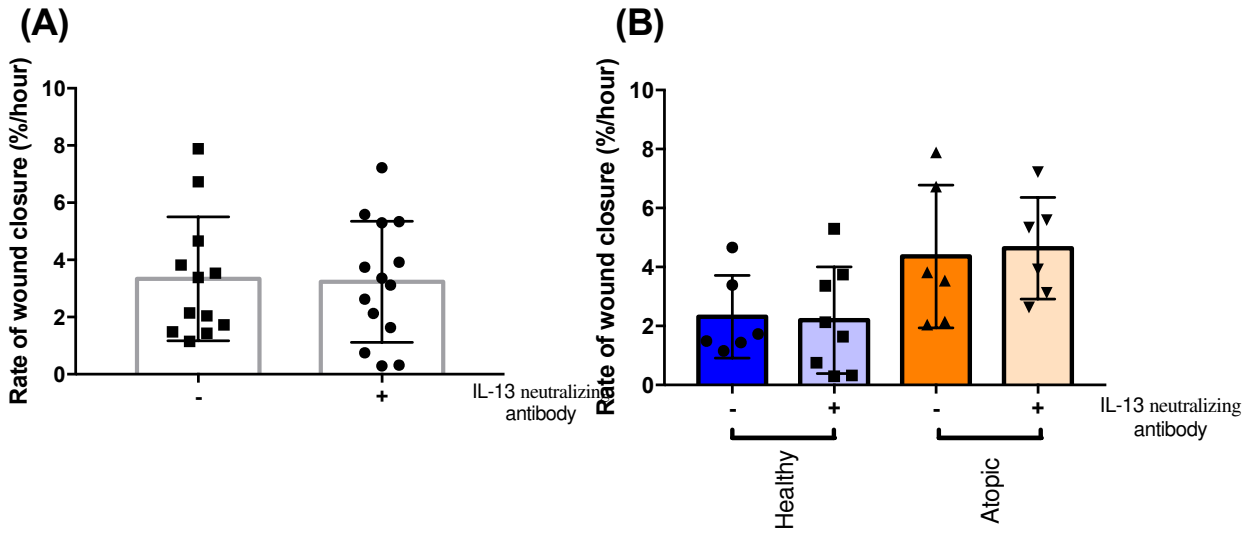


Figure 3-10 Exposure to IL-13 neutralizing antibody 7 days prior to wounding in ALI cultures with PC-ALI altered media. (A) No significant difference was seen between cultures exposed to IL-13 neutralizing antibody (mean = 3.2 ± 2.1, n=12) and control cultures without (mean = 3.3 ± 2.2, n=14)(IL-13 neutralizing antibody (-) = Control, IL-13 neutralizing antibody (+) = inhibition of IL-13 in culture). (B) The same was observed between healthy subjects (IL-13 neutralizing antibody (-) mean = 2.3 ± 1.4; IL-13 neutralizing antibody (+) mean = 2.2 ± 1.8) and atopic subjects (IL-13 a neutralizing antibody (-) mean = 4.4 ± 2.4; IL-13 neutralizing antibody (+) mean = 4.6 ± 1.7), but with the atopic subjects healing at a faster rate than healthy subjects, with or without IL-13 neutralizing antibody in PC-ALI altered media.

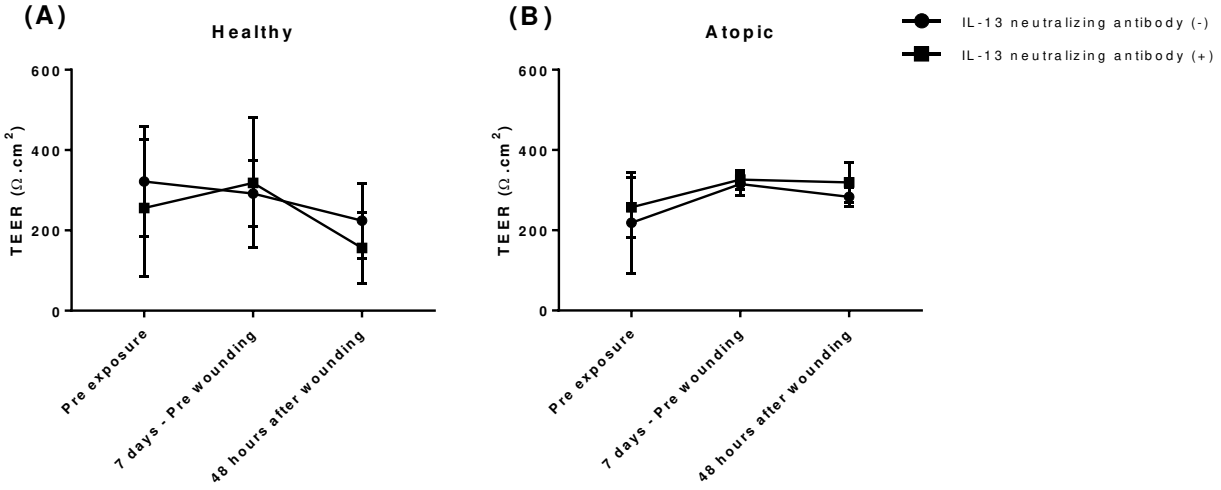


Figure 3-11 TEER values of IL-13 neutralizing antibody exposed cultures. (A) Healthy subjects (n=4) saw a decrease in the TEER over the course of wound closure, with IL-13 neutralizing antibody exposed cultures having lower TEER than the unexposed cultures. (B) The same occurred in atopic subjects (n=3), but the TEER did not decrease as significantly and the IL-13 neutralizing antibody exposed cultures did show some increase at 48 hours at wound closure.

3.3.6.3 *IL-13 Cytokine release*

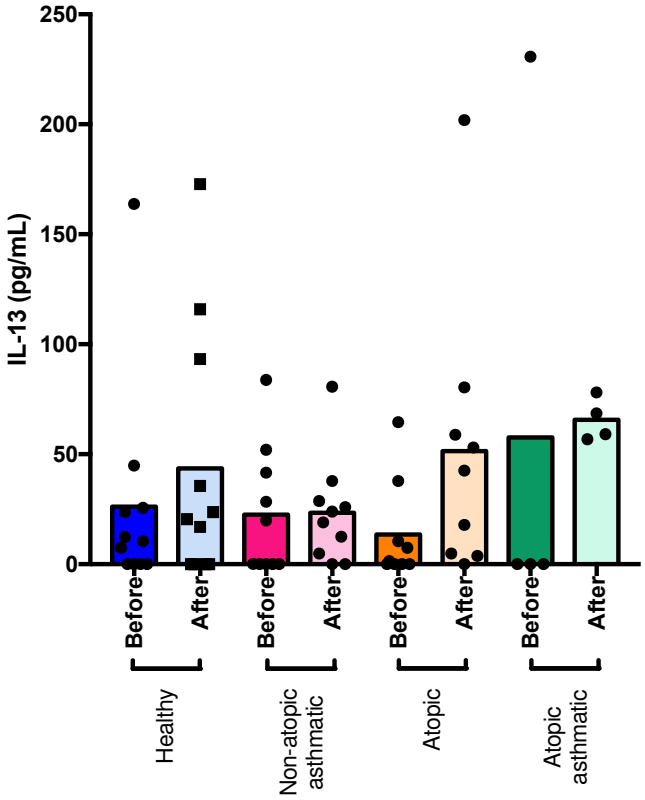


Figure 3-12 IL-13 release from apical wash samples. No significant difference was seen in a Kruskal-Wallis test (p-value = 0.18) either before or after wounding or between groups. Healthy n=11, Non-atopic asthmatic n=10, Atopic n=9 and Atopic asthmatic n=4.

3.3.6.4 *IL-13 effecting wound healing*

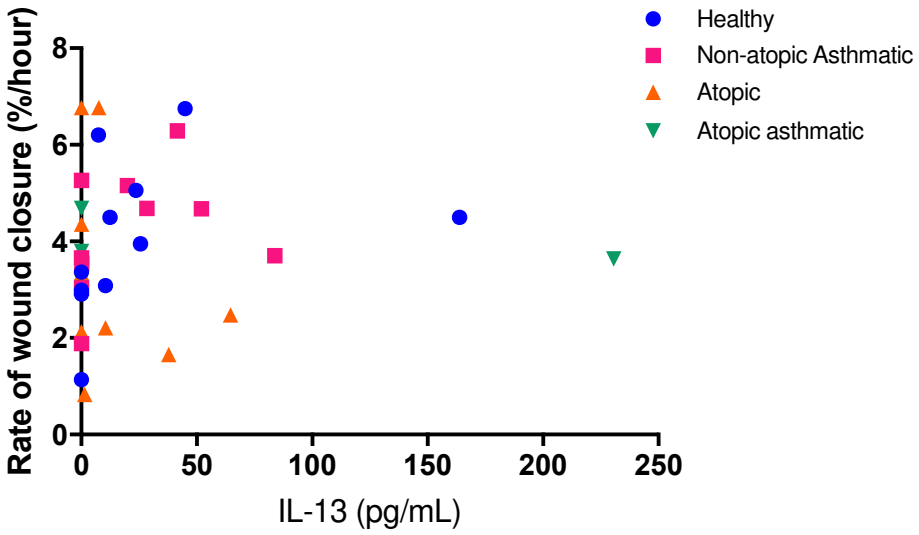


Figure 3-13 Effect of IL-13 on the rate of wound healing. .No correlation was seen between the amount of IL-13 detected in the apical wash and the rate of wound closure. IL-13 is not limiting the wound healing. Healthy n=11, Non-atopic asthmatic n=10, Atopic n=9 and Atopic asthmatic n=4.

3.3.6.5 *IL-13 Receptor Gene Expression*

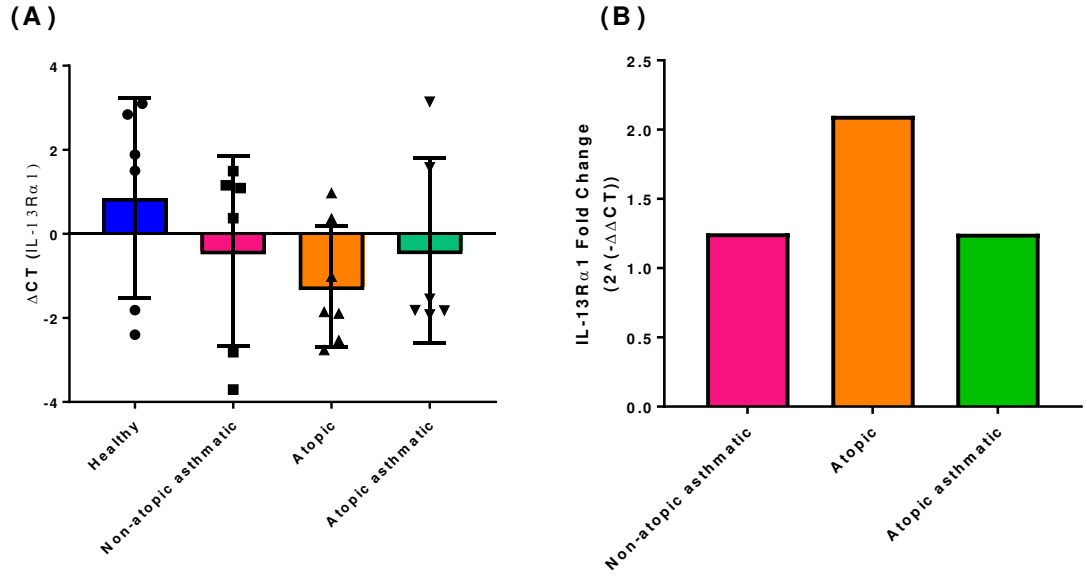


Figure 3-14 Gene expression of IL-13Rα1 in ALI cultures from healthy (n=6), non-atopic asthmatic (n=6), atopic (n=7) and atopic asthmatic (n=6) subjects. A 1-2.5 fold increase of IL-13Rα1 is present in non-atopic asthmatic, atopic and atopic asthmatic subjects when compared to healthy control.

3.3.7 Epidermal growth factor

3.3.7.1 EGF Exposure

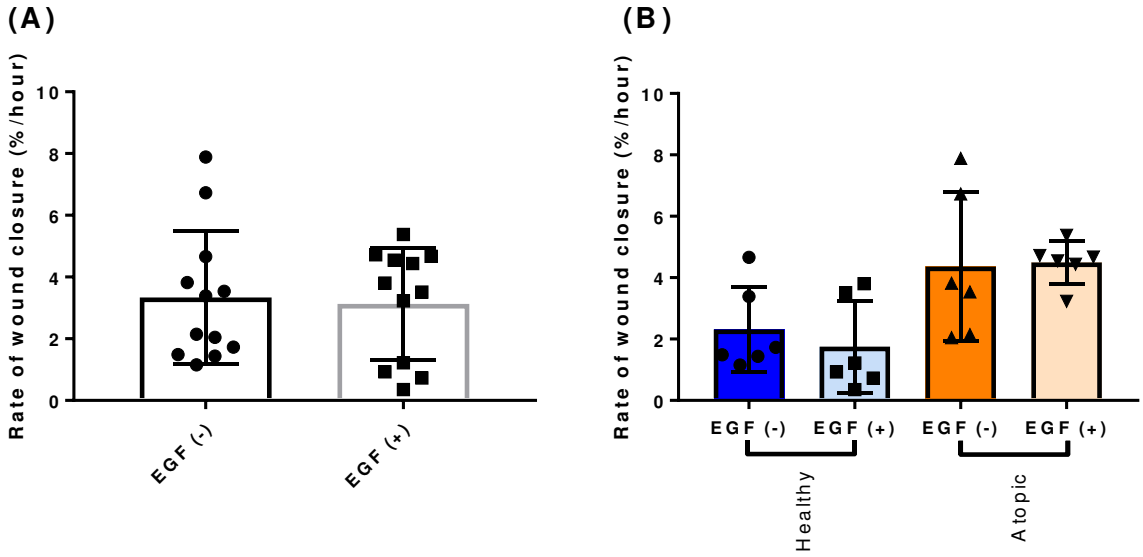


Figure 3-15 Exposure to EGF 7 days prior to wounding in PC-ALI altered media. (A) No significant difference was seen between cultures exposed to EGF (mean = 3.4 ± 1.8, n=12) and control cultures without EGF (mean = 3.2 ± 1.7, n=12). (B) The same was observed between healthy subjects (EGF (-) mean = 2.74 ± 1.6; EGF (+) mean = 2.23 ± 1.7, n=6) and atopic subjects (EGF (-) mean = 4.36 ± 2; EGF (+) mean = 4.5 ± 0.5, n=6), but with the atopic subjects healing at a faster rate than healthy subjects, with or without EGF, in PC-ALI altered media.

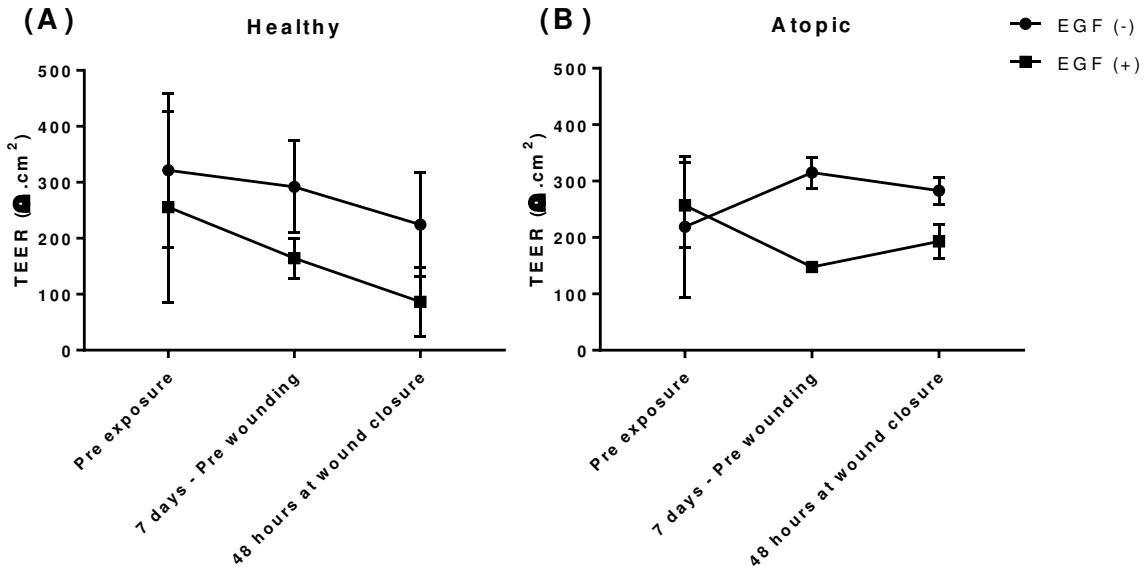


Figure 3-16 TEER values of EGF exposure cultures. (A) Healthy subjects (n=4) saw a decrease in the TEER over the course of wound closure, with EGF exposed cultures having lower TEER than the unexposed cultures. (B) The same occurred in atopic subjects (n=3), but the TEER did not decrease as significantly and the EGF exposed cultures did show some increase at 48 hours at wound closure.

Table 3-10 Morphological changes in the healthy subjects ALI cultures after EGF exposure. No change was observed at 4x magnification in all healthy cultures. Only representative data from two subjects is shown.

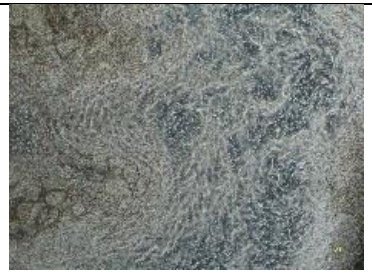
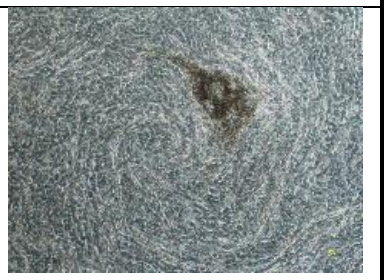
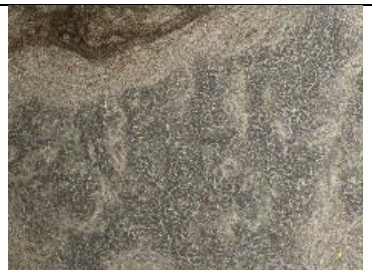
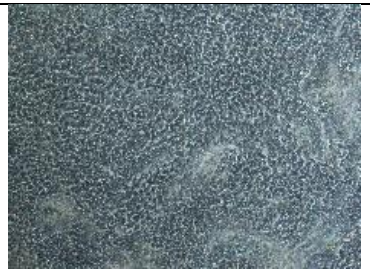
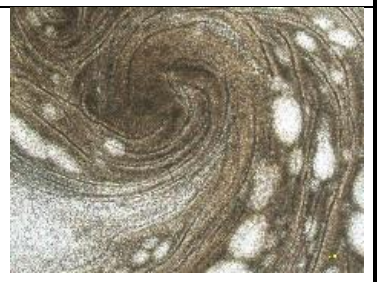
Healthy				
	Subject		Subject	
	EGF (-) (CA)	EGF (+)	EGF (-) (SRE)	EGF (+)
2 days				
4 days				
7 days				

Table 3-11 Morphological changes in the atopic subjects ALI cultures after EGF exposure. Significantly more mucus was present in atopic cultures exposed to EGF than those not exposed. A "creasing" of the mucus layer can be observed forming at two days exposure but becoming thicker and more evident at 7 days exposure.

Atopic				
	Subject		Subject	
	EGF (-) (SRE)	EGF (+)	EGF (-) (SRE)	EGF (+)
2 days				
4 days				
7 days				

3.3.7.2 EGF Inhibition

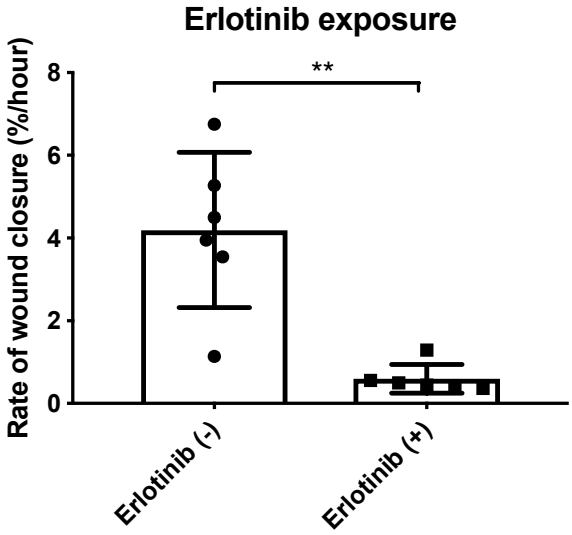


Figure 3-17 Exposure to EGF receptor inhibitor 7 days prior to wounding. Erlotinib caused an inhibition of wound healing across all groups (n=6). There was significant decrease (p-value = 0.0049, paired t-test) in cultures exposed to erlotinib 7 days prior to wounding. Cultures without erlotinib had a mean of 4.191 ± 1.88%/hour and cultures exposed to erlotinib had a mean of 0.6 ± 0.35%/hour.

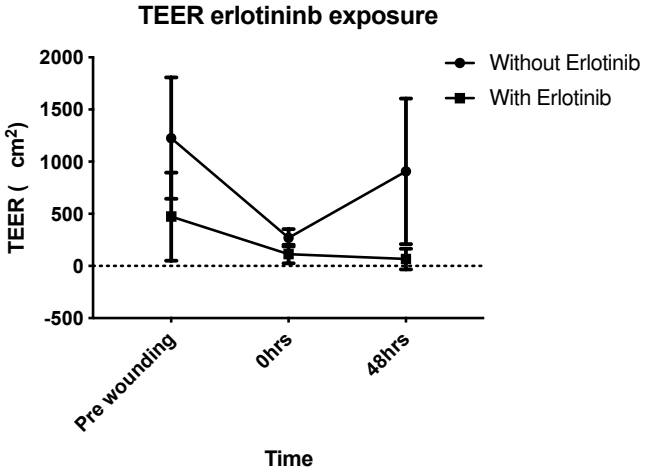
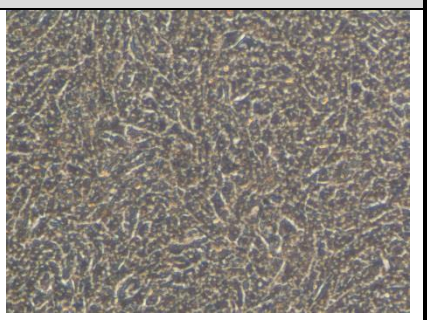
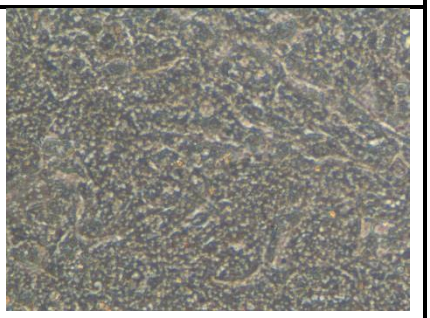
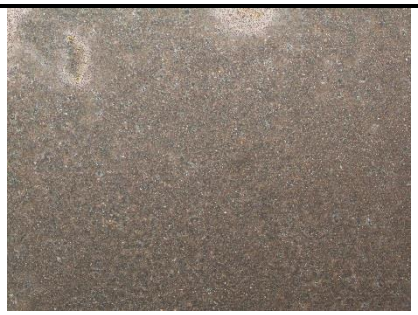
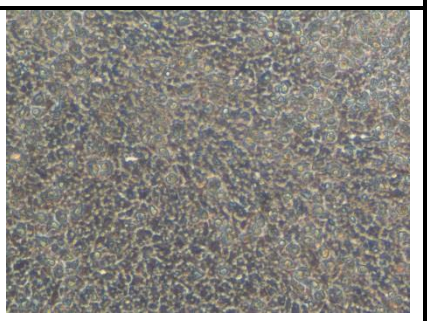


Figure 3-18 TEER measurements of ALI cultures exposed to erlotinib. Exposure to erlotinib for 7 days caused a decrease in the TEER continued to stay low for 48 hours (n=6). This is due to the wound being unable to heal.

Table 3-12 Effect of erlotinib to an ALI culture. Representative images from subject 3 show exposure to erlotinib for 7 days changed the morphology of the cells. Visually slight changes occurred, with the cells becoming smaller and the cultures producing less mucus.

Subject 3	4x magnification	20x magnification
Pre-exposure		
Day 5		
Day 7		

3.3.7.3 EGF gene expression

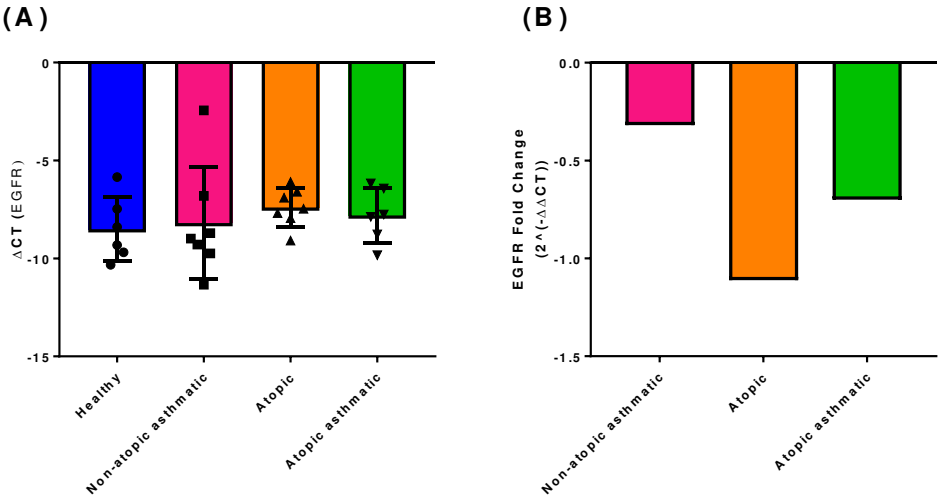


Figure 3-19 Gene expression of EGF receptor in ALI cultures from healthy (n=6), non-atopic asthmatic (n=7), atopic (n=7) and atopic asthmatic subjects (n=6). A 0.2-1 fold decrease of EGFR is present in non-atopic asthmatic, atopic and atopic asthmatic subjects when compared to healthy subjects.

3.3.8 Cytokine

3.3.8.1 Luminex Screening Comparison

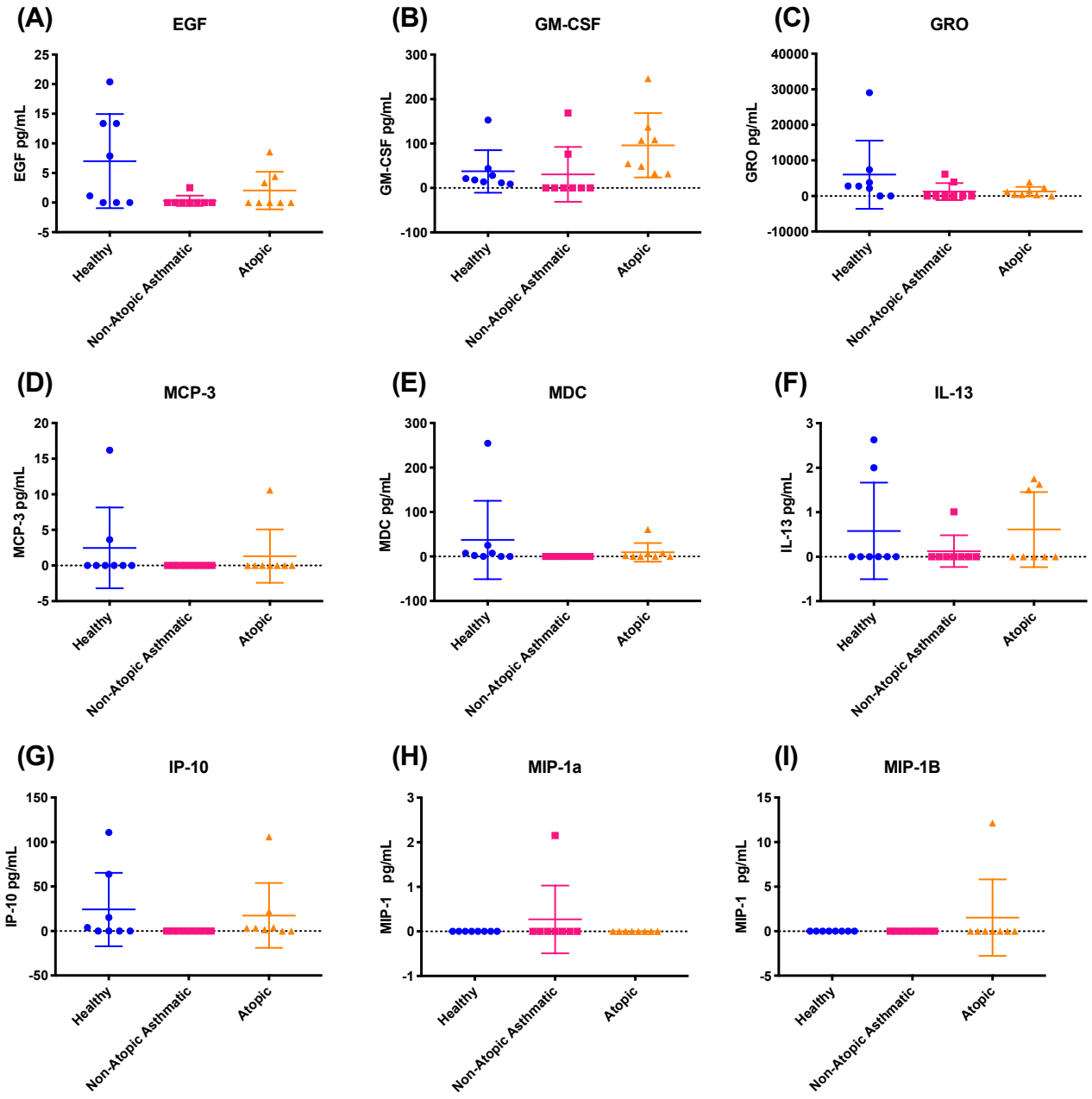


Figure 3-20 Luminex screening of apical wash of ALI cultures from healthy (n=8), asthmatic (n=2) and atopic subjects (n=7) for 9 cytokines. (A) EGF was detectable with a range of 1-20 pg/ml, with the higher values coming from healthy subjects and lower from the atopic and asthma subjects. (B) GM-CSF was detectable with a range of 10-250 pg/ml, with the atopic subject having the highest concentration followed by non-atopic asthmatic and healthy. (D) GRO was detectable with a range of 20-30,000 pg/ml, with all three having high concentrations with the highest from healthy subjects. (D) MCP-3 was detectable with a range of 4-20 pg/ml, with healthy and atopic subjects have secretion but none from the asthmatic subject. (E) MDC was detectable with a range from 2-

250 pg/ml, with the highest reading from the healthy subjects. (F) IL-13 had low detection with a range of 1-2 pg/ml for all. (G) IP-10 was detectable with a range of 1-11- pg/ml, with secretion from both health and atopic but none from the asthmatic subject. (H) MIP-1α had low detection at 2 pg/ml from only the asthmatic subject and (I) MIP-1β was detectable from only one atopic subject. Because of the low subject number for screening, no significant differences were seen across all concentrations. No atopic asthmatics were included in this experiment. Values of 0 pg/mL were below the level of detection.

3.3.8.2 AlphaLISA

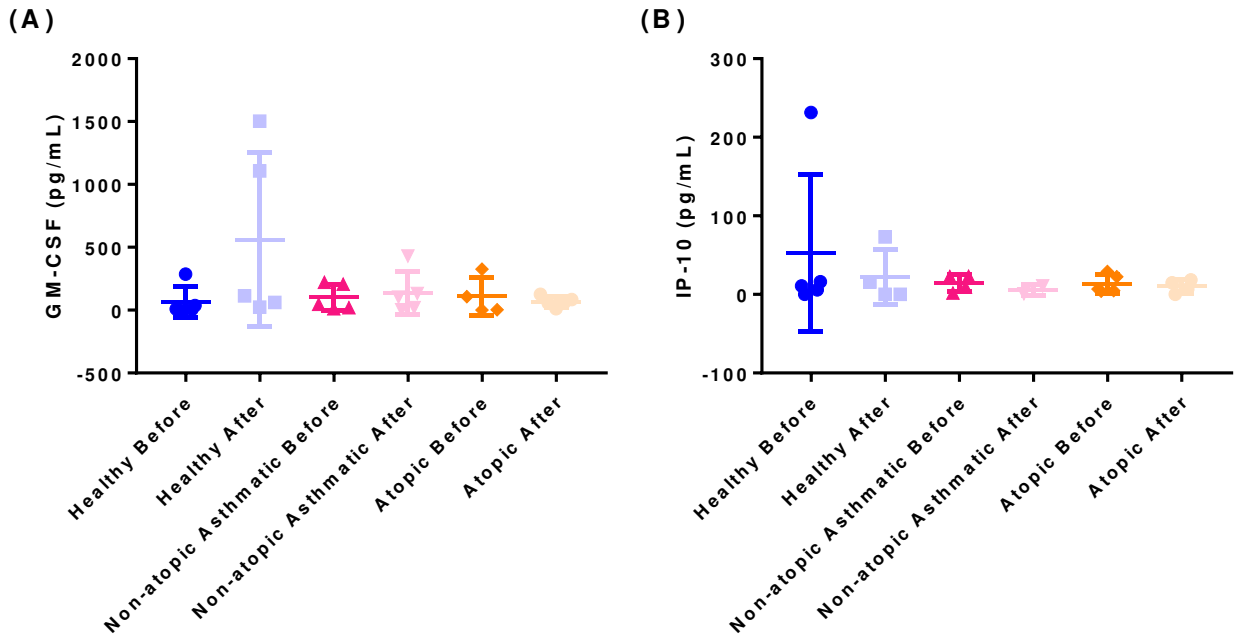


Figure 3-21 Cytokine concentration before and after wound closure. Apical washes were taken at pre wound, and 48 hours when wound was closed. Samples were analysed for (A) IP-10 and (B) GM-CSF (healthy n=5, non-atopic asthmatic n=5, atopic n=5). No significant difference was seen between before or after wounding for the two cytokines measured (n=15). (A) An increase was seen in the GM-CSF release from healthy subjects after wound closure. No difference was seen for non-atopic asthmatics or atopic subjects. (B) High IP-10 was detected in healthy cultures before wounding and decreasing after wound closure. No difference was seen in the other groups with very low levels of secretion. No atopic asthmatics were included in this experiment.

3.3.9 PCR

3.3.9.1 Gene Expression

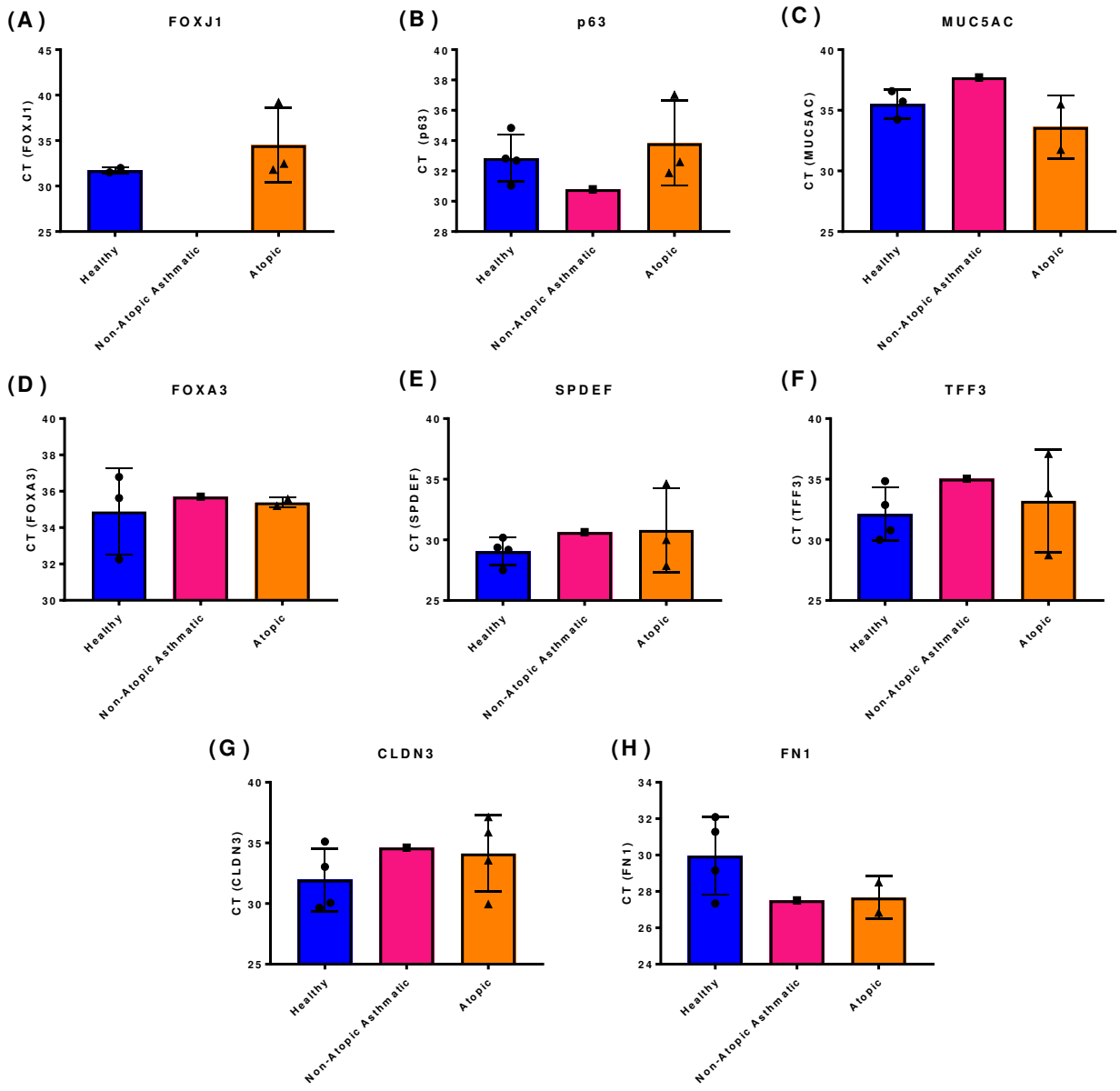


Figure 3-22 CT values from ALI whole well cultures comparing healthy (n=4), non-atopic asthmatic (n=1) and atopic (n=4) subjects. (A) No FOXJ1 was expressed in non-atopic asthmatic subjects but high gene expression was identified in the atopic subject. Lower levels were expressed in healthy subjects. (B) High levels of p63 were expressed in all three groups. (C) Low levels of MUC5AC were expressed in non-atopic asthmatics, with higher levels in healthy and even higher in atopic subjects. (D) Similar expressions levels of FOXA3 were seen from all three groups. (E) High levels of SPDEF was expressed from all three groups. (F) Similar levels of TFF3 was expressed from healthy and atopic subjects with lower levels in non-atopic asthmatics. (G) High

levels of CLDN3 expression was seen in healthy subjects with lower levels in non-atopic asthmatics and spread for atopic subjects. (H) High levels of FN1 were expressed in all three groups.

3.3.9.2 Single cell gene expression

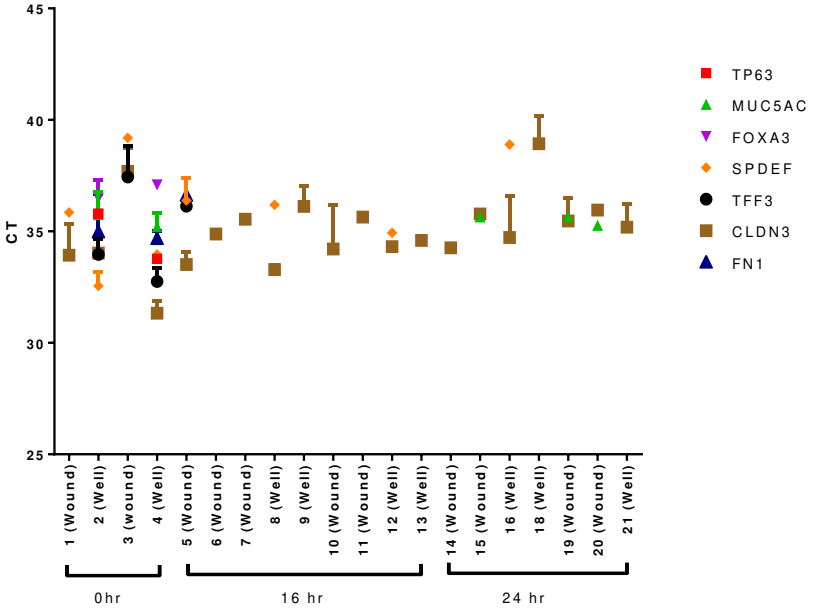


Figure 3-23 Single cell gene expression was measured from cells collected from subject 34. 20 cells were successfully harvested from an ALI culture and analysed against a panel of 7 genetic markers. All cells showed the presence of tight junctions (CLDN3), with cell 2 and 4 expressing basal, goblet and fibronectin markers as well, suggesting more than one cell was collected at harvest. Cells 3, 5, 8, 12, 15, 16, 19 and 20 expressed markers for goblets cells. No FOXJ1 (cilia) was identified in any cell.

3.4 Discussion

The airway epithelium is complex barrier that involves diverse mechanisms to maintain and restore its integrity. Damage to the airway epithelium is a risk factor for infections through by the penetration of microbes or viruses beyond the epithelial surface. By establishing an effective wound healing model using airway epithelial cells grown at ALI we can evaluate different groups of respiratory diseases. In this chapter, epithelial cells were harvested from healthy, non-atopic asthmatic, atopic and atopic asthmatics and grown at the ALI. The wound healing assay developed in chapter 2 was applied to these groups to determine the effect on wound healing.

3.4.1 Survival

Growing epithelial cells at ALI is a tricky method that takes the right concentration of growth factors, antibiotics and steroids. Media can be purchased with these supplements as part of a ready-to-go kit. Even though there is a reliable media used in ALI differentiation there are many factors that can affect the survival of nasal epithelial cell at ALI. This can range from the number of cells harvested from the nose, the health of the cells at passaging, the freeze/thawing of the cells and the seeding on transwell inserts. These are all steps along the way to a successful ALI culture that may have an effect on the survival. Although there are some unsuccessful cultures across all groups (Table 3-3) there was a higher percentage of successful cultures for healthy, non-atopic asthmatics, atopic and atopic asthmatic subjects, than unsuccessful. The ALI success rate is high when using the standard culture conditions mentioned above. However, there are some cultures that fail to differentiate, as seen in Table 3-3. Intrinsic mechanisms might play a role into why this occurs for some cultures and not others, even when the same procedures are followed. These were not explored in this model development study but in future studies could be considered. A more comprehensive look at the history of each subject and better documentation as to any nasal medication used in the days before collection should be included.

3.4.2 Rate of wound closure

To progress on from the discoveries and model established in chapter 2, we recruited subjects with 3 disease groups and were analysed to see their wound healing rates. We saw again that healthy subjects had a wound healing rate above 2%/hour with 9 of the cultures identified as fast healing (>4%/hour) and seven as medium healing (2-4%/hour). Non-atopic asthmatic subjects on average healed at 4.1%/hour, which was similar to the healthy subjects. All except one non-atopic asthmatic

subject healed faster than 2%/hour which was a surprising result. This is discussed further as to why this might be the case in section 3.4.3. Seven atopic cultures fell below the 2%/hour rate of wound closure with some even failing to heal the wound at all within 48 hours. Six cultures were identified as fast healers with a rate of wound closure above 4%/hour. This included subject 46, who reported to be atopic and also have childhood asthma, but not as an adult. The ALI culture from this subject showed huge amount of mucus that caused difficulty when wounding and capturing of the wound healing process. It is suspected that while the microscope can capture the healing process, the mucus blob was just falling into the wounded area, obstructing the view, identified by the black sections in the images in Table 3-9. The TEER value did confirm that the wound had healed, and tight junctions reformed at 48 hours, but delay could have still occurred during the wound closure. Subject 45 also showed fast healing with high TEER values before and after healing. Lastly, one atopic asthmatic culture was below 2%/hour with the other 10 above that threshold. For all groups there were some clear outliers in their rate of wound closure. The groups in this study are self-reported and although each subject answered a questionnaire there still might be failings in the response. Groups like atopy fall into a grey area where some subjects have knowledge of their atopic status because they have a high to severe condition that needs medical treatment ranging from antihistamine tablets to nasal sprays. There are also subjects that might be atopic but only being mild with reaction to certain stimulants. If these subjects have not been exposed to that stimulus they might only report themselves as healthy and non-atopic.

Wounding of the ALI culture has identified atopic subjects as having a decreased capability to repair and remodel the epithelium after injury. This was a new discovery, as it was previously thought that only asthmatic cells had reduced repair capabilities(146, 211). However, these studies were only performed in submerged epithelial cultures that do not correctly model the airway epithelium. Differences already reported to identify a defect in the subjects with atopy (whether asthmatic or not) include a study by Amin that reported the degree of epithelial damage was significantly higher in their atopic group (212). In the past the majority of studies have compared atopic and non-atopic asthma, or asthmatic to non-asthmatic. These have been focused on the inflammatory and immune cells at the submucosal level and not the airway epithelium. There is ongoing debate as to whether atopic and non-atopic asthma are immunopathogicaly distinct or if they are just driven by similar mechanisms. The answer to this was hoped to be identified in this thesis by analysing the apical cytokine secretion and genetic markers from single cells.

3.4.2.1 TEER

The TEER was measured before wounding and at 48 hours when wound was closed. We saw no significant difference between the TEER value pre-wounding and at 48 hours after wounding for each group. The atopic and atopic asthmatic cultures had lower mean TEER values than healthy and non-atopic asthmatic cultures. Atopic asthmatics also had significantly lower TEER values than non-atopic asthmatic cultures. For cultures that failed to close, the TEER value is low, as expected, because of the hole still present in the culture. This allows for the current to pass freely between the probes. A high TEER value is achieved because of the resistance and tight junctions present in a fully differentiated ALI cultures and low TEER indicating low quality of the culture, with Chen et al reporting that is could potentially be from a leaky epithelium(213). Further examination of these group groups saw no difference in the age of wound closure or the initial wound size that would help to explain this variation in TEER. Atopic subjects have been reported in literature to have impaired tight junctions in the epidermis effecting their barrier function(214), but no studies have yet looked at this in airway epithelial cells.

3.4.2.2 Age of culture

There was significant difference in the days to achieve a fully differentiated ALI culture between healthy and atopic asthmatic subjects and between atopic and atopic asthmatic subjects. All took longer than the 21 days recommended by the manufactures of both medias. 21 days is also the benchmark used in numerous papers as the time of full differentiation(159-161). As identified in chapter 2, ALI culture does take longer than 21 days and each culture should be visually monitored for presence of cilia and mucus. Both of these features can be observed by eye at 10x magnification.

3.4.3 Previous topical steroid use and its effect on ALI cultures

It was hypothesised that asthmatic subjects, atopic or non-atopic, would have delayed wound healing compared to healthy subjects, but in this wound healing model this was not seen. The unexpected result of non-atopic asthmatic subjects healing at a rate above 2%/hour with all of those cultures successfully healing in under 40 hours lead to a deeper analysis of these subjects and their background. Each subject answered questions about their asthma medication and usage at time of harvesting. The wound healing rate of subjects that reported to use steroid medication had a significantly faster rate of wound closure then those that did not report steroid use. This was independent of group, with the non-atopic asthmatic, atopic and atopic asthmatic subjects not

separated for this comparison. Healthy subjects were excluded from this comparison because all had zero relevant medication use reported. Steroid medications that were reported included Prednisone, Seretide, Symbicort and Alvesco for asthmatic subjects, and Flixonase and Nasonex for atopic subjects. No studies have looked at the effect of corticosteroid use (in our case for asthma treatment) and the effect it can have on cells in culture. It has always been thought that the passaging of cells in culture would remove any historical reference and "wash-out" the cells from previous steroid exposure. Also, of interest is that cells were collected from the nose and most asthma corticosteroid treatment is delivered through the lungs. All this was previously thought as assumed knowledge, but this study opens up more questions about a steroid responsive, Th-2 driven defect in wound healing at ALI.

3.4.4 Altered media

Cell growth and morphology differences early in the culture did not have a significant effect on the rate of wound healing between B-ALI and PC-ALI for healthy, non-atopic asthmatics and atopic asthmatics, but did show an increase in atopic subjects. The fast healing of the wounded cultures (<40 hours) made experiments of wound healing enhancement difficult with a small window of time to monitor the effect. Limiting the effectiveness of the media by reducing the supplements caused the ALI cultures to be less optimal, allowing for EGF exposure and IL-13 inhibition, be studied more closely. In the PC-ALI media additives included 10X base supplement and 100X maintenance supplement, added into the base media before feeding of the cells. The formula of these supplements is proprietary information and we could not knowingly exclude parts of the media, just alter the concentration of these supplements in the base media. Media concentration tested by others in the lab included altering the 10X base supplement to 50% and 10% and the 100X maintenance supplement to 50% and 10% also. The wound healing assay was used as the control model to test the appropriate concentration. From the 5 subjects tested (healthy = 2, atopic = 3), the concentration of 50% 10X base supplement with 100% 100X maintenance supplement saw healthy subjects heal at 30 hours and atopic subjects not healing at 48 hours. Once this media concentration was determined we could supplement the media to identify increases in wound healing rate. By examining the relationships both IL-13 and EGF have on the airway epithelium and the role they play in wound healing, we can start to understand the mechanisms involved.

3.4.5 IL-13

IL-13 is an inflammatory cytokine that is released after injury and is a normal mediator of airway epithelial repair. Allahverdian has shown that when IL-13 secretion is neutralised there is significantly reduced epithelial repair and that there is a balancing act lead by IL-13 secretion, with too much causing airway remodelling(189). This remodelling is seen in a study by Malavia where IL-13 exposure was shown to increase MUC5AC and TGF-β_2 and decrease in β-Tubulin IV, with goblet cell hyperplasia the end result(208, 215, 216). Our experiment tested this theory by exposing the ALI culture to IL-13 at an increased level than naturally secreted by the cells at injury. This created a negative feedback effect that saw a significant decrease in the rate of wound healing. This was seen across all groups, including both asthma groups. Natural IL-13 secretion was measured by cytokine assays and there was very little to no IL-13 detected in an apical wash (Figure 3-12). This supports the findings that epithelial cells do not produce IL-13 *in vivo*. IL-13 is secreted at high levels by T helper 2 (Th2) cells, natural killer T (NK T) cells and mast cells. Increased levels from these cells is in response to inflammation, viral infection and allergy(217). The raised levels detected in asthmatic patients normally or after an asthma exacerbation are not produced by the epithelial cells and can be confirmed to not increase due to injury (Figure 3-1). However, epithelial cells do have the appropriated IL-13Ra1 receptor and are affected by IL-13 levels. IL-13 neutralizing antibody was added in the ALI cultures to eliminate any IL-13 present. However, as low levels of IL-13 are secreted by epithelial cells there is very little effect caused by the neutralization. The addition of IL-13 neutralizing antibody saw no increase in the rate of wound closure. Recently, IL-13 inhibition is a way of treating asthma that cannot be controlled by glucocorticoids. Unfortunately, cell availability meant that no asthmatic donors were tested but a similar (and more beneficial) effect might be seen.

3.4.6 EGF

A functional EGF-EGFR pathway is critical for wound healing in the submerged monolayer culture system and in wound healing assays(218). The results from the present study show that this is also critical for healing wounds in ALI culture (Figure 3-17). EGFR is part of a pathway involved in the repair of damaged epithelium(218) and by blocking this pathway with erlotinib we saw a reduction in the proliferation and survival of epithelial cells(219), with a rate of healing <1%/hour and failure to heal within 48 hours. Erlotinib was added to the ALI culture as a positive control to test the effectiveness of the ALI method of growing epithelial cells and to also test the wound healing model. It caused a complete inhibition of the wound healing with all three wounds failing to heal and a lowering of the TEER after a seven-day exposure. The addition of erlotinib to the ALI culture altered

the appearance reinforcing the change in both cell-to-cell interaction and the uniformity of the ALI culture. Erlotinib was again added 7 days prior to wounding to ALI cultures for all four groups, showing a significant decrease in the rate of wound healing. Not only did inhibition of EGFR cause a decrease in wound healing rate and morphological differences in healthy subjects, it also was the case for non-atopic asthmatics, atopic and atopic asthmatics.

To monitor the effects EGF has on the wound repair of the epithelium, additional EGF was added for 7 days prior to wounding. In contrast to what was observed when the EGFR was inhibited, additional EGF saw an increase of mucus production on the apical surface of the cells (Table 3-10). There was no change in the rate of wound closure in cultures exposed to EGF and those without, indicating that additional presence of EGF plays no significant role in enhancing repair of the epithelium after injury. High levels of EGF might already be present in the differentiation media but because this is proprietary information we would not confirm this.

3.4.7 Cytokine results

Collection of the apical wash from samples before and after wounding was performed to determine the differences in the cytokine secretion at these stages between the groups. Three cytokines were tested in the wound healing assay as identified in chapter 2 as being expressed by the epithelial cells, GM-CSF and IP-10. Also, of interest was to analyse IL-13 and its relation to asthma wound repair. Using the newly developed model of wound healing that identified atopic subjects as having a reduced capability of healing, we also hoped to show some change of key cytokines identified in the epithelium. Slight differences were seen in the diseased groups (non-atopic asthmatic, atopic and atopic asthmatic) with decreased GM-CSF and IP-10 release compared with the healthy controls. This was only very slight and not statistically significant. Again, we saw low secretion of cytokines from the apical surface of the epithelial ALI cultures. These concentrations were similarly reported by Callejas, who also tested GM-CSF (2.5 pg/ml) and IP-10 (20pg/ml)(220). There is also a study by Humbert et al and although they measured mRNA they saw no difference in the amount of GM-CSF and IL-13 between atopic asthmatics and non-atopic asthmatics(221). Our data and the studies highlighted above are contrary to what has previously been shown where there is an increase in pro-inflammatory cytokines such as IL-8(222-224) and chemokines like GM-CSF(222, 225, 226), when compared to control. Although there are a few differences between atopic and non-atopic asthmatics the similarities outweigh these. Although there was no clear distinction between the groups or even

a clear difference in the cytokine release before and after healing, this has not been too surprising. ALI might not be the appropriate model to measure cytokine response.

Atopic asthmatic subjects were not included in these cytokine experiments because of availability of sample at the time of experimentation. No atopic asthmatic subjects were sampled and with non-conclusive results seen from healthy, non-atopic asthmatics and atopic subjects from GM-CSF and IP-10, atopic asthmatics subjects were not tested. Expensive reagents were not wasted in what is predicted to also show low levels of GM-CSF and IP-10 in the apical wash of atopic asthmatic ALI cultures.

3.4.8 Gene Expression

3.4.9 Single cell gene expression

Measuring the expression of specific genes from an ALI culture allows verification of a fully differentiated epithelium with the makers of goblet, basal and ciliated cells present. Although we were unable to compare the expression of gene expression in the whole well and single cell, we were able to see that there was expression of the specific genes tested. The main purpose of this was that ALI cultures are able to measure for gene expression to compare to single cell sampling. Looking at single cell gene expression we see that using the methods available to us in the lab we cannot simply just select one cell. For subject 34 we saw from "one" single cell collection that FoxJ1, MUC5AC, FOXA3, SPDEF and TFF3 were all expressed. All these markers are evidence of multiple cells being selected from the collection with there being a tight connection between the cells with separation once differentiated almost impossible. This same tight connection (thought to be through the formation of tight junction at ALI) is also evident in the wounding of the ALI cultures. By dragging a pipette tip across the culture to create a wound there is a process of pulling and dragging of the cells because of their attachment to each other. This is a new phenomenon only seen in the ALI culture and not in submerged scratch assays.

The feasibility of the single cell PCR does not seem effective at this point for this thesis. Measuring the gene expression of one single cell might seem like a way to effectively use the precious ALI samples, however the number of cells needed for statistically significant data would destroy the ALI culture, because of the collection process. It can be done, but the cost of the Cell-to-CT kit,

microdissection equipment, TaqMan primers, and the optimization needed would be a whole study all together. This data shows that it is feasible, however not pursued further in this thesis.

3.5 Conclusions

The wound healing assay established in chapter 2 was an effective and reliable way to compare the response to injury between healthy, asthmatic and atopic subjects. When epithelial cells were wounded at ALI, atopic subjects were slower to heal compared to healthy, non-atopic asthmatic and atopic asthmatic subjects. No decrease in wound healing rate was seen in asthmatic subjects but there was a higher use of steroid medication among that group, with subjects that used steroid medication having a faster healing rate than those who don't. Two differentiation medias were used in the development of an ALI culture, but no difference was observed in their wound healing rate. Lastly, mechanisms of wound healing were examined through IL-13 and EGF. IL-13 exposure and EGF inhibition reduced wound healing, with the inverse of EGF exposure and IL-13 inhibition having no effect. To follow on from this comprehensive look at different respiratory diseases, viral infection and the effect on wound healing for these four categories will be looked at in the next chapter.

Chapter 4 Respiratory viral infection delays wound healing in healthy and asthmatic airway epithelium

4.1 Introduction

Damage to the epithelial layer disrupting barrier integrity can be caused by inhaled allergens, pollution particles and respiratory viruses. There have been reports by many studies that epithelial loss or damage has been reported in adult asthma and recently in children with mild asthma(175, 202, 227, 228). Normal and rapid re-epithelisation of the airways is required to maintain the integrity of its immune barrier function with epithelial cells implicated in the remodelling process(211). Cells grown at air-liquid interface are physiologically and structurally similar to cells of the upper human airways using the model previously established we can see the effects of damage to an infected epithelium and how these cells repair after injury.

4.1.1 Background of RSV

Wheezing and asthma exacerbation, especially in children or the elderly, are often due to respiratory viruses such as respiratory syncytial virus, with studies showing in 7.7% of asthma exacerbations in children 2-15 years RSV was present and in 15% there was a coinfection with more than one respiratory virus(116, 229-233). RSV does not only increase the severity of an asthma exacerbation it has also been shown to increase the levels of histamine receptors and CC chemokines (eotaxin, RANTES [regulated upon activation, normal T-cell expressed and secreted]) in airway epithelium and fibroblasts(234). Rhinovirus infection increases the airway responsiveness with atopic children more susceptible to the development of severe asthma exacerbation after viral infection(235-237). Although this is only the case for rhinovirus currently, research into RSV predict it might respond similarly.

Many previous studies, including Spann et al, have examined the interactions between airway epithelial cells by using a submerged monolayer of cells grown in culture(102). These submerged monolayers are known to only have basal cells present. A study by Collins and Melero has shown that RSV replicates exclusively in apical ciliated cells in stratified ALI epithelium models and causes less sloughing of the cells at ALI indicating it as less cytopathic in epithelial cells(88, 238). RSV

does not cause significant cytopathic effects *in vitro* but does cause significant pathology in the airway *in vivo*, where replication can continue in the airway for around 8 days (88, 239-241).

4.1.2 Virus and its effect on wound healing

The airway epithelium is exposed to numerous environmental factors with the most damage coming from respiratory pathogens. As identified earlier the repair process is rapid, with migration of cells to cover the damaged area, followed by proliferation and differentiation until the epithelial integrity is restored(242) (243). It has been proposed that damaged and repair to the epithelium leads to airway wall thickening and other pathologic changes such as bronchial hyper-responsiveness and asthma symptoms, it is well documented that virus induced cytotoxicity is involved in these changes(244). This can lead to airway narrowing, bronchial hyper-responsiveness and asthma symptoms with virus induced cytotoxicity well documented for the majority of these agents(245). The model developed in the past two chapter is ideal for measuring the effect RSV has on the airway epithelium and how it can affect wound repair.

4.2 Methods

4.2.1 Wound healing

Wound healing experimental methods established in chapter 2 were used for ALI cultures from healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects. Green RSV was imaged with EVOS FL Cell Imaging System microscope (ThermoFisher Scientific) at 4x magnification with GFP filter.

4.2.2 Virus exposure

Primary nasal epithelial cells grown at ALI were exposed to RSV at a multiplicity of infection (MOI) of 1 and 0.1 plaque-forming units (pfu)/cell. Cells were incubated with virus for 2 hours in PBS at 37^{o}C in the apical chamber, after which they were washed with PBS and TEER recorded. Cultures were maintained in hydrocortisone free media for 6 days before wounding. Apical and basal wash was collected at 6 days to determine viral titre and toxicity of the virus. At the appropriately determined time point of 6 days after infection, cultures were wounded following the methods developed in chapter 2. A subset of ALI cultures were also wounded and then exposed to RSV for 2 hours in PBS at 37^{o}C and monitored for virus interaction during wound closure.

4.2.3 Virus stocks

Green fluorescent tagged RSV-A2 was generated in Hep-2 cells (OptiMEM/2% foetal bovine serum [FBS]; Life Technologies), and concentrated by centrifugation at 50,000x g for 2.5 hours through a 30%/60% (w/v) sucrose cushion. The interface was collected, diluted in OptiMEM and pelleted at 13,000x g for 3 hours. The virus was resuspended in OptiMEM without FBS and stored as stock. The virus was quantified by immune-plaque assay described below.

4.2.4 Viral titre

ALI culture supernatants of infected nasal epithelial cells infected with RSV at MOI 1 and 0.1 were collected using PBS washing and stored at -80^{o}C. Washes were used to infect monolayers of Hep-2 cells in a plaque assay to quantify the RSV. Hep-2 cells were exposed to a 10-fold dilution series of RSV-infected supernatants for 4 hours, then overlayed with 0.8% methyl cellulose/OptiMEM/2% FBS and incubated for 6 days at 37^{o}C. Monolayers were then fixed and using anti-RSV polyclonal

HRP-conjugated (Virostat, Cat# 0604) where RSV-positive plaques were identified. From the RSV-positive plaques the quantity of RSV in cell culture supernatants was calculated as pfu/ml.

4.2.5 LDH Assay

The apical surface was washed with 200uL of PBS. Cytotoxic assay was perform using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Cat # G1780). Standards and solutions were prepared following manufactures instructions. Triplicate samples (5ul) and standards were placed in a 384-well plate with 5ul of CytoTox96 Reagent added on top. Protected from light, the plate was incubated at room temperature for 30 minutes. Stop solution was added (5ul) to each well and plate was read at 490nm within one hour.

4.2.6 Cytokines

Similar to previous chapters, supernatants from all ALI cultures were collected by washing the apical surface of the ALI culture with PBS. Washes were collected in this chapter at pre infection, 6 days post infection/at time of wounding and 48 hours at wound closure . The same sampling was conducted on cultures infected at time of wounding. Washes and media were tested for cytokine detection using AlphaLISA kit (Perkin Elmer) following manufacturer's recommendations. Cytokine tested was IFN-β (AL265, dynamic range 9.6-100,000 pg/ml).

4.3 Results

4.3.1 Infection of RSV

4.3.1.1 Effect on wound healing

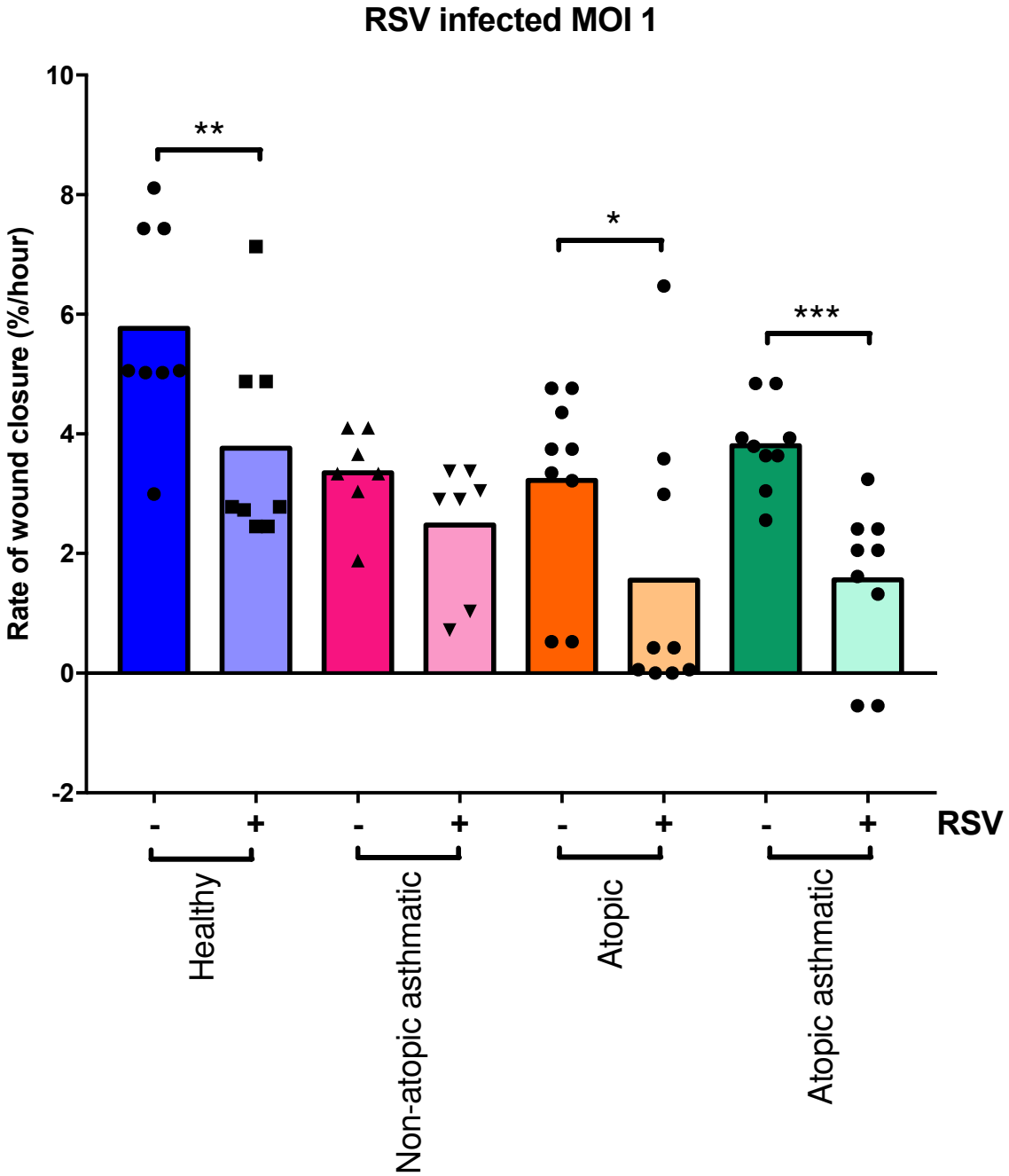


Figure 4-1 The rate of wound closure after RSV infection at MOI 1 (p-value = <0.01, Kruskal-Wallis test). Significant decrease in the rate of wound closure is seen for healthy (p-value = 0.01, n=8), atopic (p-value = 0.02, n=7) and atopic asthmatic (p-value = 0.01, n=9) subjects when

infected at MOI 1. Non-atopic asthmatic (p-value = 0.12, n=7) subjects did show a decrease although not significant.

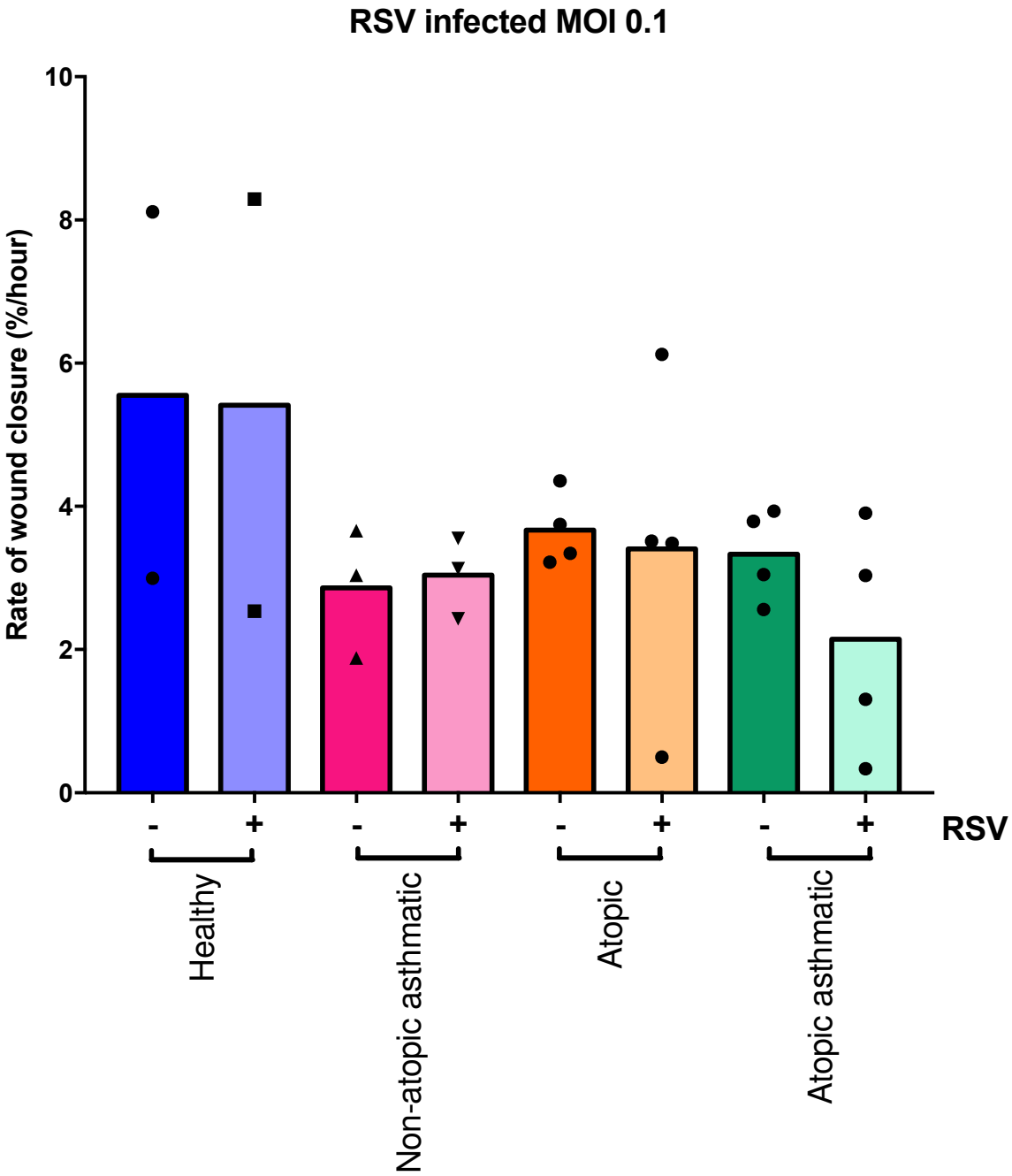


Figure 4-2 The rate of wound closure after RSV infection at MOI 0.1 (p-value = 0.78, Kruskal-Wallis test). No significant difference in the rate of wound closure was seen for healthy (n=8), non-atopic asthmatic (n=7), atopic (n=9) and atopic asthmatic (n=9) subjects when infected at MOI 0.1.

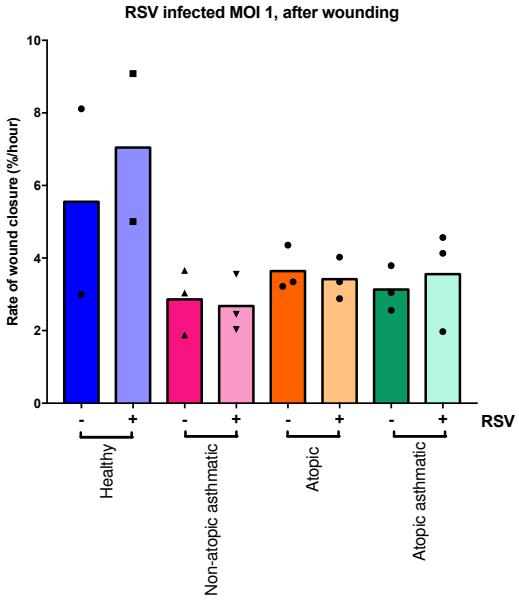


Figure 4-3 The rate of wound closure after RSV infection at MOI 1 after wounding (p-value = 0.35, Kruskal-Wallis test). No significant difference in the rate of wound closure was seen for healthy (n=8), non-atopic asthmatic (n=7), atopic (n=9) and atopic asthmatic (n=9) subjects when infected at MOI 1.

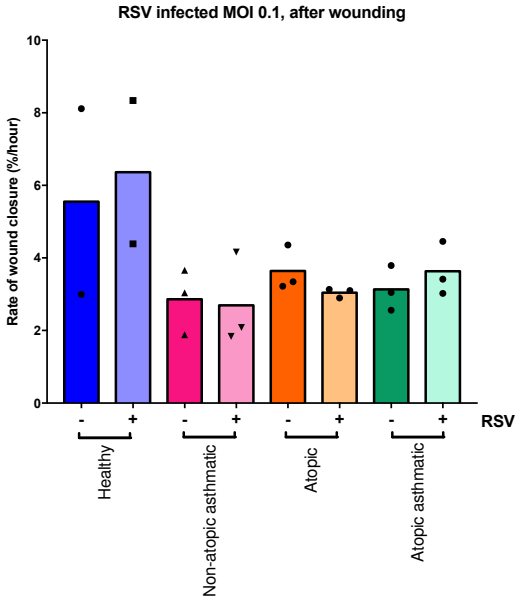


Figure 4-4 The rate of wound closure after RSV infection at MOI 0.1 after wounding (p-value = 0.34, Kruskal-Wallis test). No significant difference in the rate of wound closure was seen for healthy (n=8), non-atopic asthmatic (n=7), atopic (n=9) and atopic asthmatic (n=9) subjects when infected at MOI 0.1.

4.3.1.2 RSV Infection images

Table 4-1 Representative pictures of RSV infected ALI cultures at MOI 1 for healthy and non-atopic asthmatic subjects.

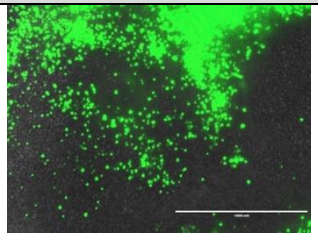
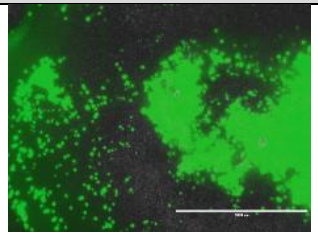
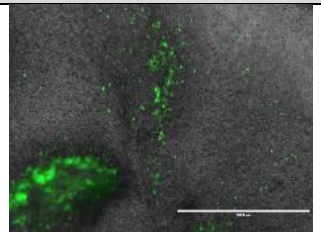
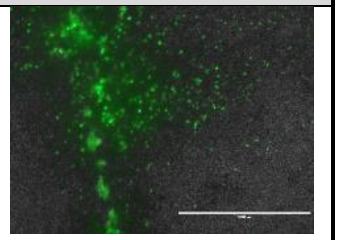
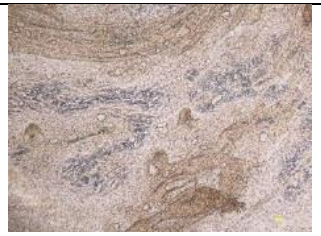
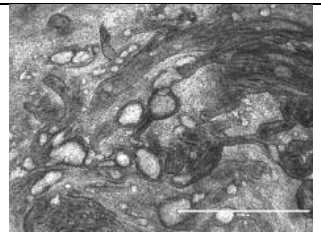
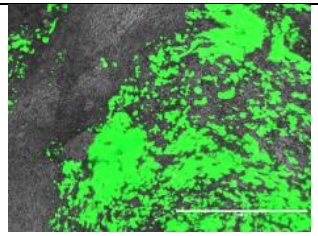
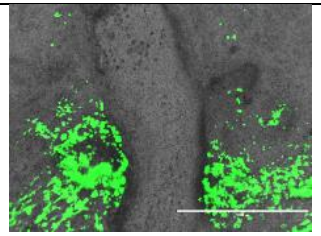
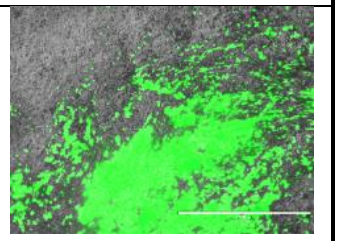
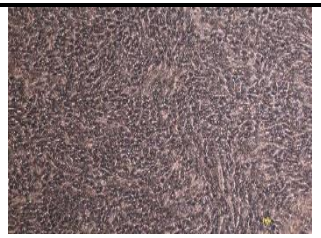
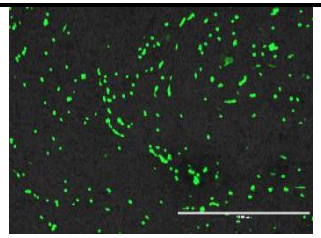
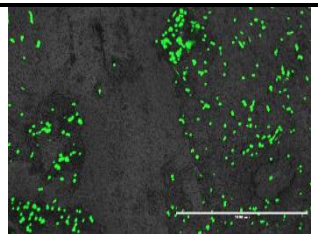
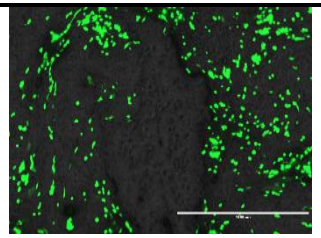
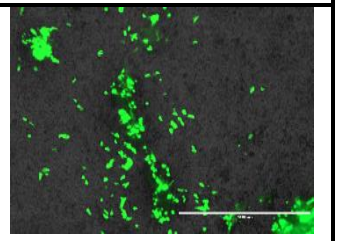
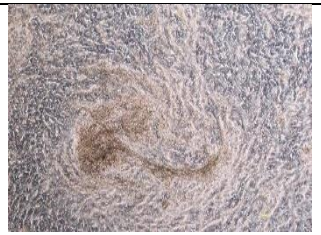
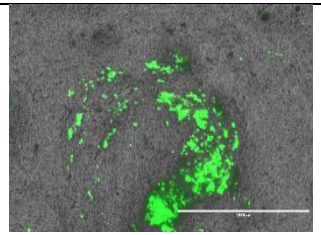
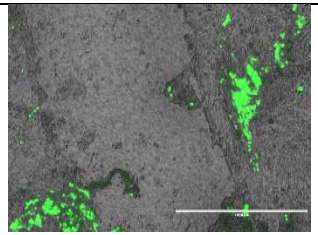
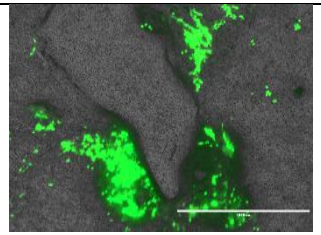
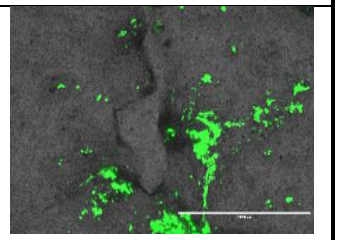
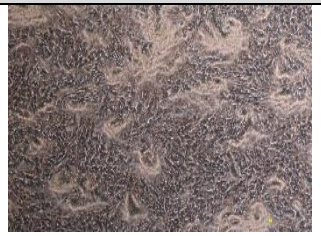
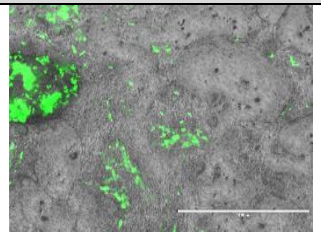
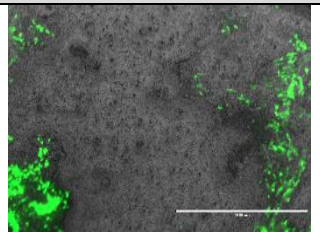
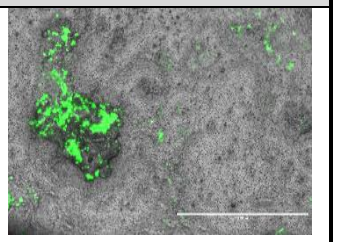
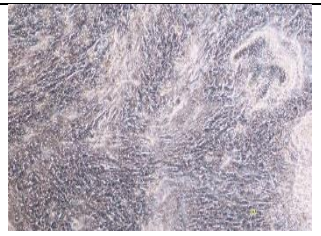
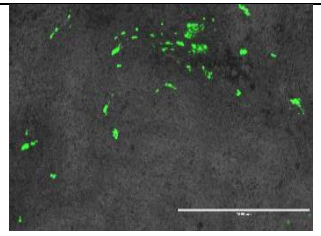
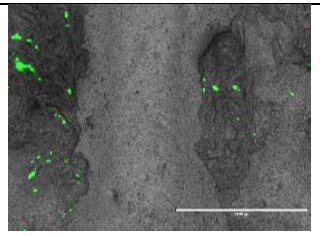
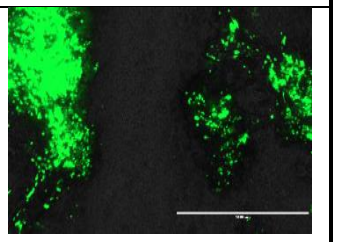
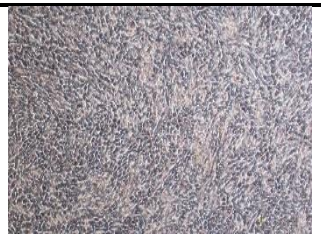
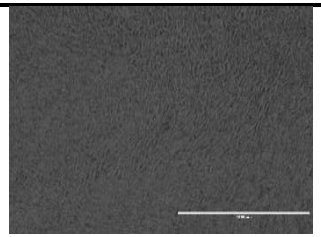
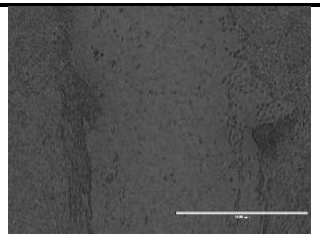
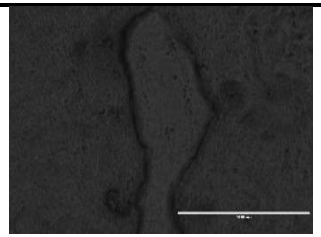
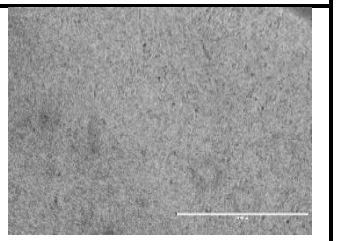
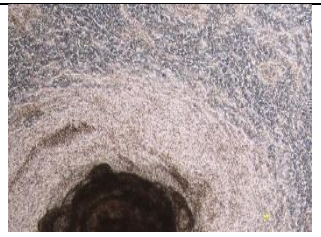
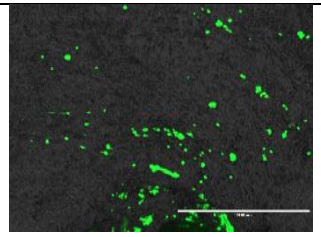
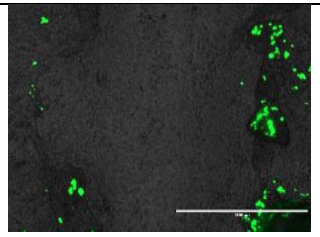
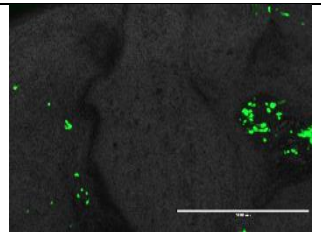
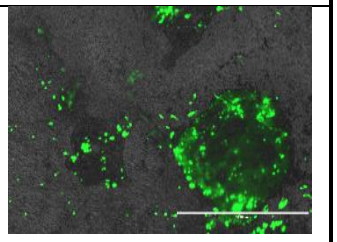
		Pre Infection	6 days post infection	At wounding	24 hours after wounding	48 hours after wounding
Healthy	Subject 16					
	Subject 15					
Non-atopic Asthmatic	Subject 31					
	Subject 35					

Table 4-2 Representative pictures of RSV infected ALI cultures at MOI 1 for atopic and atopic asthmatic subjects.

		Pre Infection	6 days post infection	At wounding	24 hours after wounding	48 hours after wounding
Atopic	Subject 47				NA*	
	Subject 45				NA*	
Atopic Asthmatic	Subject 55					
	Subject 54					

*Images lost due to image formatting.

4.3.2 Virus titre on ALI cultures

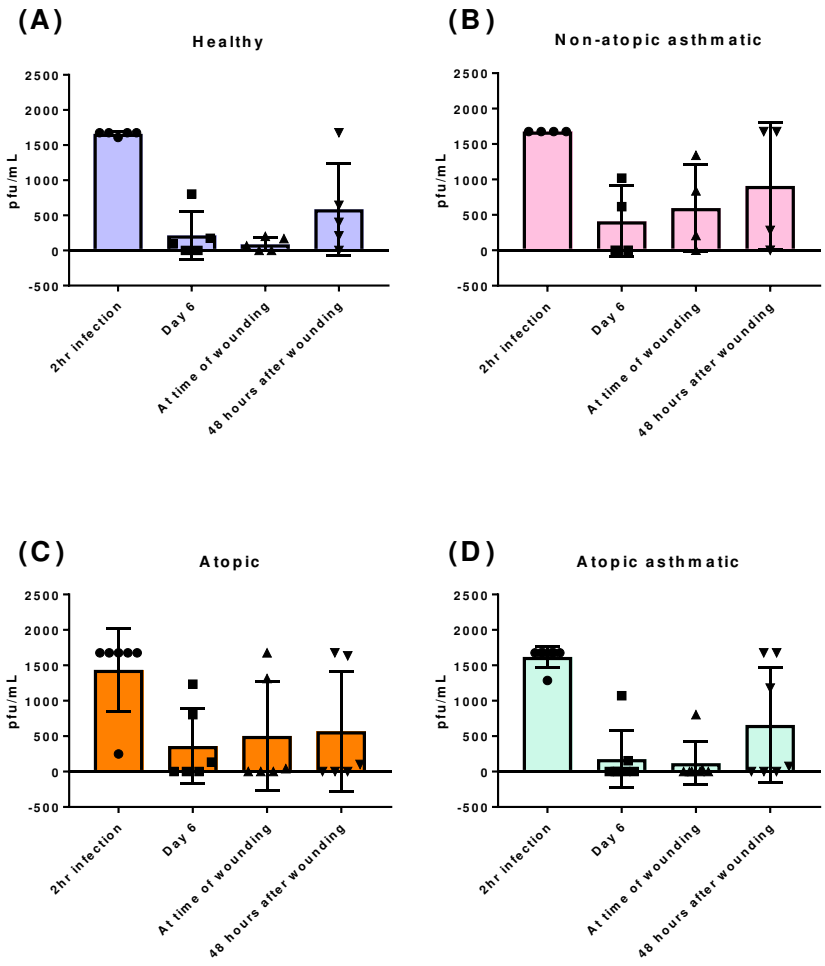


Figure 4-5 RSV titre from the apical wash of ALI cultures. High RSV was present after 2 hours RSV incubation with consistent viral titres for all phenotypes after 6 days. Slight variation is seen at wounding and 48 hours after wounding, but these are not significant because of low sample number, healthy (n=5), non-atopic asthmatic (n=4), atopic (n=6) and atopic asthmatic (n=7).

4.3.3 TEER

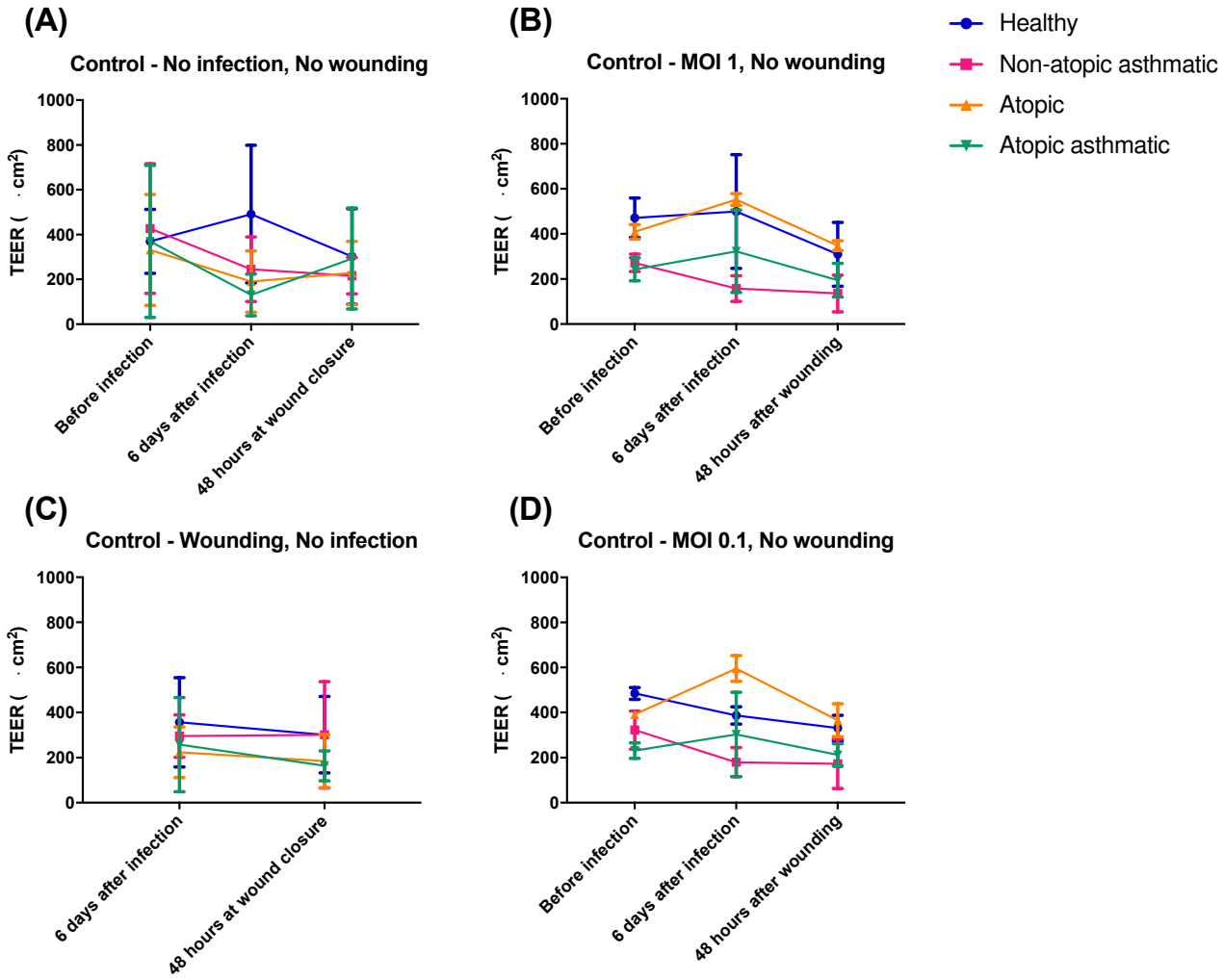


Figure 4-6 Control TEER values of RSV infected and uninfected cultures, both wounded and not wounded. There was no significant difference seen for all conditions (A-D) in the TEER values. Slight variations are seen at 6 days for (A,B and D) but all returning to original TEER by 48 hours after wounding. (A) healthy n=5, non-atopic asthmatic n=5, atopic n=6 and atopic asthmatics n=6; (B), (C) and (D) healthy n=2, non-atopic asthmatic n=3, atopic n=3 and atopic asthmatics n=3

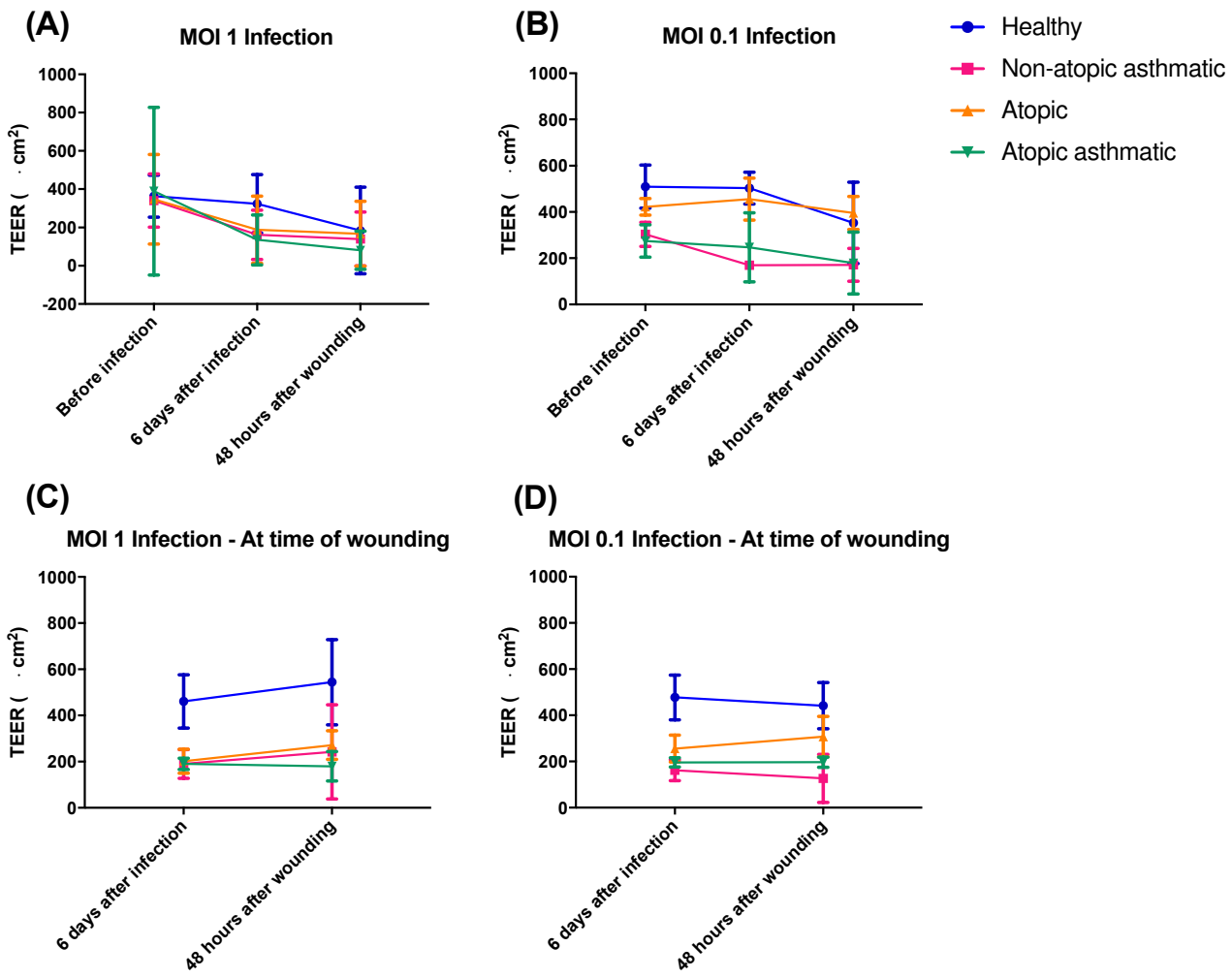


Figure 4-7 Effect of RSV infection on TEER values from wounded cultures. No significant difference was seen in all conditions (A-D) with all phenotypes showing similar TEER values before infection, at 6 days after infection, and at 48 hours after wounding. (A) healthy n=5, non-atopic asthmatic n=5, atopic n=6 and atopic asthmatics n=6; (B), (C) and (D) healthy n=2, non-atopic asthmatic n=3, atopic n=3 and atopic asthmatics n=3

4.3.4 LDH Assay

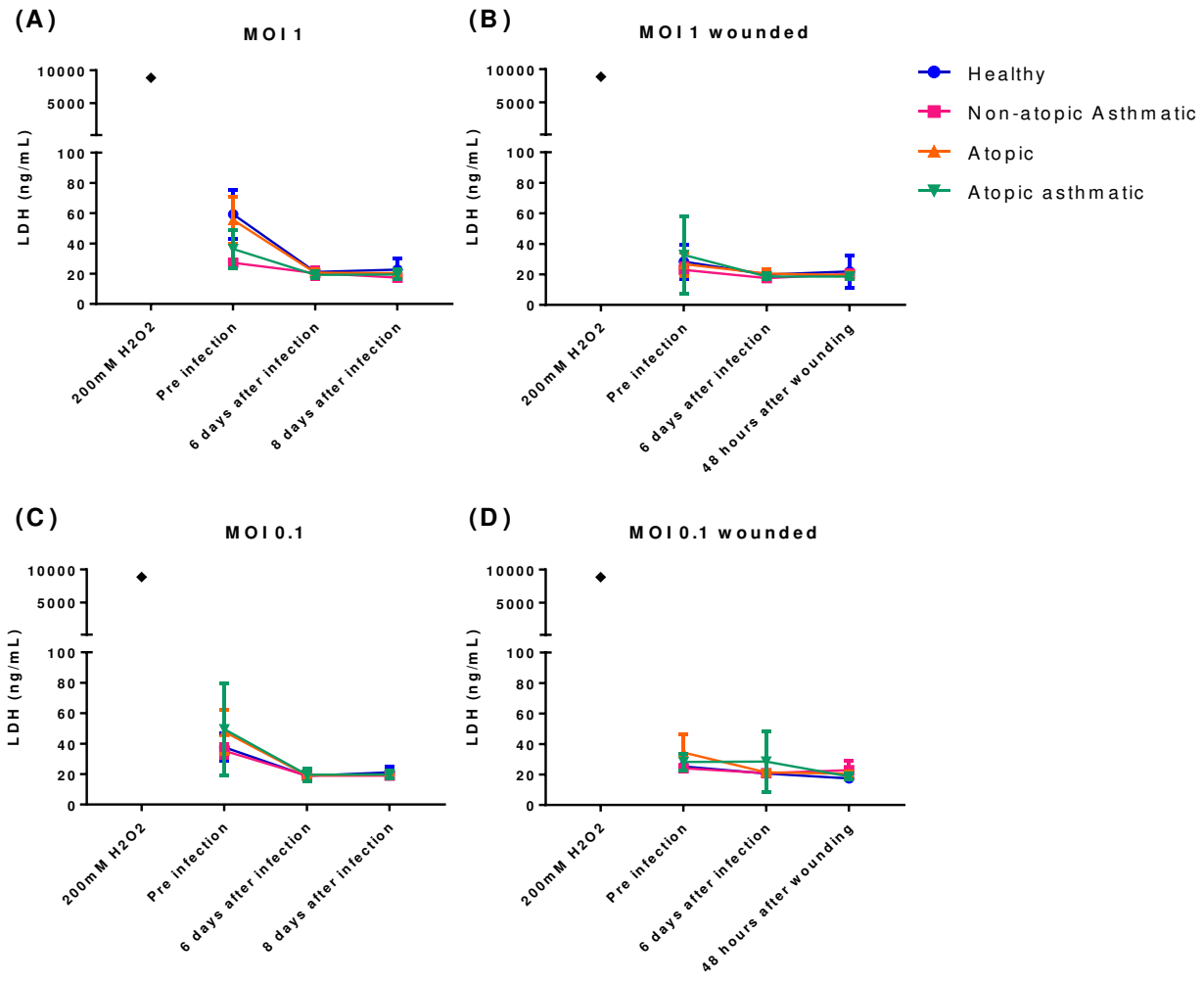


Figure 4-8 LDH release from the apical surface after RSV infection and wounding. 200mM H2O2 signifies 100% cell death after 2 hours of treatment. Low levels of LDH was detected from the apical surface of the cultures with all phenotypes showing similar levels of detection at all time points. (A and C) Cultures infected with RSV at MOI 1 and 0.1 did have higher levels of LDH pre infection, with levels dropping after 6 days, but such low levels of LDH not significant. (A) healthy n=5, non-atopic asthmatic n=5, atopic n=6 and atopic asthmatics n=7; (B), (C) and (D) healthy n=2, non-atopic asthmatic n=3, atopic n=3 and atopic asthmatics n=4

4.3.5 Cytokines

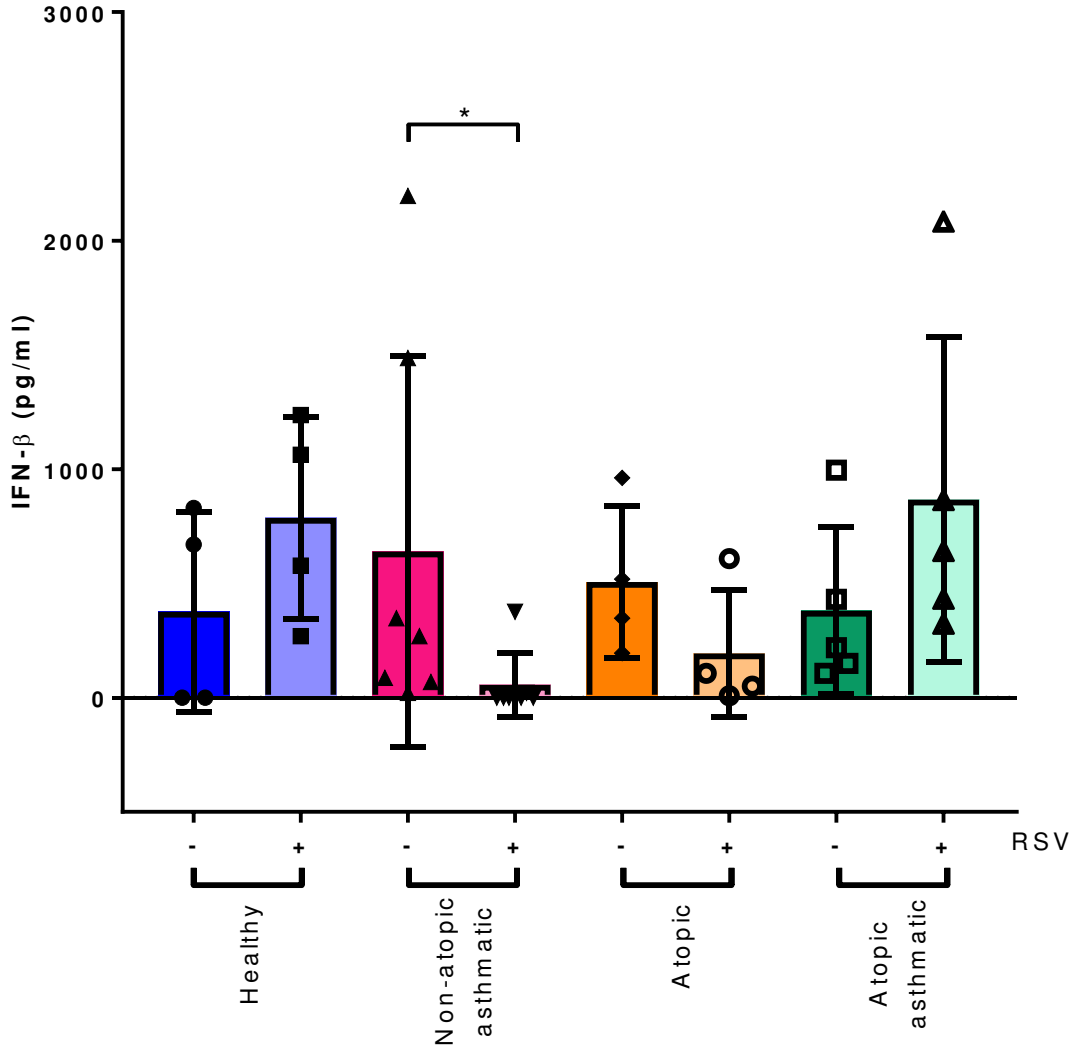


Figure 4-9 IFN-β secretion from the apical wash of ALI cultures before RSV infection (RSV -) and at 6 days after infection (RSV +). Only non-atopic asthmatics (p-value=0.01, n=7) showed significant difference in a Kruskal-Wallis test (p-value = 0.02). Minimal increase is seen from healthy (n=4) and atopic asthmatic subjects (n=5), with minimal decreases from atopic subjects (n=4).

4.3.5.1 *Previous topical steroid use and its effect*

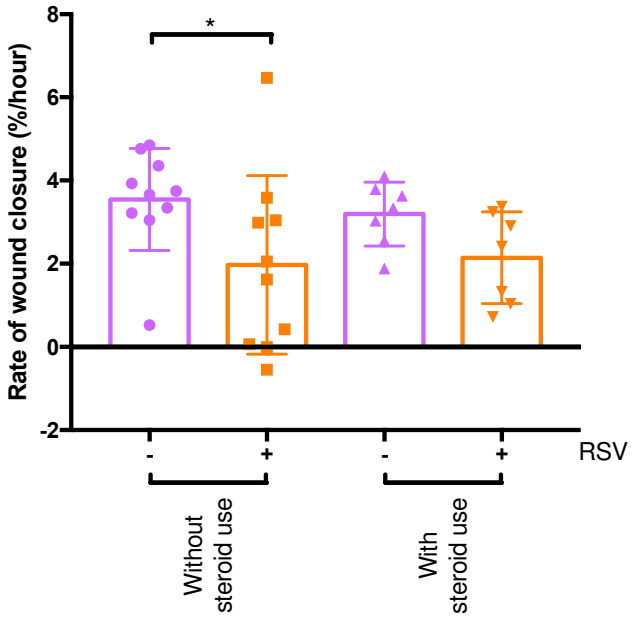


Figure 4-10 Previous topical steroid effect on rate of wound closure after RSV infection at MOI 1. Rate of wound closure was significantly reduced for cultures from subjects that had no steroid use (p-value = 0.02, n=10)), with no significant reduction in cultures from subjects that have used steroids (p-value = 0.07, n=7) in a Mann-Whitney test.

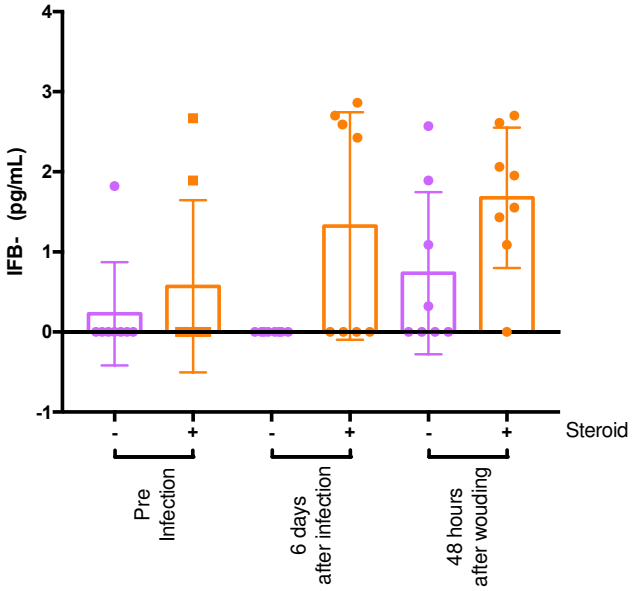


Figure 4-11: IFN-β secretion from the apical surface from subjects identified to have used topical steroid medication previously. There was significant difference identified in a Kruskal-Wallis test (p-value = 0.01, n=8), however no significant difference was identified between IFN-β secretion at time points with and without steroid.

4.4 Discussion

This chapter looked at the effect viral infection had on epithelial wound repair, RSV replication number, epithelial cell apoptosis and IFN-β production. It was identified that the airway epithelium of adult subjects is susceptible to RSV infection and at a MOI 1 there was a significant effect on the capacity for the epithelium to repair. We demonstrated significant decrease in the rate of wound closure in healthy, atopic and atopic asthmatic subjects after RSV infection. Non-atopic asthmatics, however, did not have a significantly lower capacity to repair the wound when infected with RSV, but did show a decrease. There was no significant or observed difference in viral replication, apoptotic release and production level of IFN-β in response to RSV when wounded, across all subject groups. Although there was no significant difference in viral titres between groups, there was varying degrees of infection from each individual subject. There was also no difference in the TEER values of an RSV infected culture before wounding and after wound was closed.

4.4.1 Virus infection

The RSV infection model established in this thesis mirrors several characteristics of RSV infection *in vitro* and is an excellent substitute for human infection *in vivo*. The mechanisms of RSV pathogenesis in epithelial cells and the effect on wound healing can be elucidated using this ALI wound healing model. Table 4-1 and Table 4-2 shows the complete snapshot of the RSV infection over a period of time for all phenotypes and identifies a clear relationship between viral load, identified by green fluorescent tagged RSV, and the subject disease status. In this study MOI 1 is used to consistently induce clinical disease effects seen in viral infection, and MOI 0.1 is used to more accurately represent the real life exposure(102). Time course experiments have already been done by other studies with 6 days post infection being the key time for high viral titre with minimal cells death(122). This balance is key for establishing a wound healing model in RSV infected cultures as any delay in wound healing should not be the result of a dying culture, but more the RSV infection effect. One key aspect of this study was to only use the A2 strain of RSV. Minimising and reducing complexity by including many viral strains would remove the focus from the epithelium and how it responds to infection. Different RSV strains would play different roles and use different mechanisms in the infection of epithelial cells and wound healing capabilities. RSV-A2 was chosen for this thesis because of its higher prevalence than RSV-B and also produces a higher viral load(246, 247). RSV-A seemed like the most appropriate place for preliminary data of RSV infection with future ALI wound healing experiments now being possible with RSV-B. This also opens up the potential for not

only RSV infection research but to also look at other potential pathogens and how they might affect epithelial wound healing.

Determining the optimal time to wound the cultures after RSV infection came down to when RSV was shown as an active infection and still replicating, but to not causing damage to the cells. This was measured by the cell cytotoxicity, and at 6 days we saw medium virus titre with baseline levels of cell death. Studies by Villeneuve show that at 6 days the RSV titre was plateauing with cultures still forming a polarized layer(122). At 6 days post infection RSV can be shed exclusively from the apical surface at titres consistent with those in airway aspirates from hospitalized infants and at this time point it is reported that there is the formation of syncytia(122). In Table 4-1 and Table 4-2 we can see that only at day 6 post infection the fusion neighbouring infected cells creating a multi-nucleated enlarged cells at ALI. This is observed by the green fluorescence of RSV in neighbouring cells across sections of the culture. This occurrence by RSV, identified in its name, can cause caspase-dependent cell death and loss of epithelial integrity(248). From the epithelial perspective, cells infected with virus utilize virus-associated molecular patterns (VAMPS) to trigger an inflammatory response. More in-depth analysis of genetic markers such as the action of HMGB1 in RSV infection and how it can identify inflammatory pathways at the molecular level that may be amenable to therapeutic interventions should be looked at as a progression from this study(249).

4.4.2 Virus infection causing reduction in wound repair

Exposure to RSV 6 days prior to wounding caused all phenotypes to have a reduced capacity to heal the wound with all ALI cultures taking longer to heal. Each culture was infected at MOI 1 and 0.1 with green fluorescent tagged RSV used to easily show an infection on the epithelial cells. Without fluorescent tagging more complex experiments would be needed to determine that an infection is occurring in the ALI cultures. As observed in Table 4-1 and Table 4-2, there was large amounts of RSV detected in the ALI cultures across all phenotypes with varying levels of green seen between individual donors. These images do represent an infection but are not a clear indicator of wound healing inhibition, with subjects with high levels of infection not always in correlation to reduced wound repair. RSV infection delayed epithelial cell wound repair in all phenotypes, with the inhibitory effect significantly greater in epithelial cells from healthy, atopic or atopic asthmatic subjects. Only one group, the asthmatic subjects, did not have a significant reduction and this was a surprising and unlikely result (Figure 4-1). Very few studies have investigated the effects of RSV on epithelial cell proliferation and repair, even though RSV plays a significant role in triggering asthma

exacerbations. RSV infection may delay epithelial wound healing by affecting epithelial cell proliferation or interfere with the epithelial wound healing mechanisms.

One mechanism that could explain the difference in the rate of wound healing between asthmatics and the other phenotypes could be the transformation of the epithelium in asthmatic subjects. One characteristic of asthma is the loss of columnar epithelial cells which can lead to loss of its integrity and density. This change in pseudostratified epithelium could also cause less ciliated cells which is the most common site for RSV infection. Less ciliated cells results in a reduced RSV infection, causing less of an effect on would healing. There changes to the epithelial layer also effect the barrier function of the culture. Lopez-Souza et al has shown that differentiated bronchial epithelial cells grown at ALI develop tight junctions that are considerably resistant to RV infection(250). This could potentially be the same for RSV infection in the airway epithelium. Data from this study suggest that the barrier function does not deteriorate during RSV infection because the TEER remains high and at levels no different than before infection.

Other studies have looked at viral infection in asthmatics and atopic subjects, but this was for human rhinovirus and parainfluenza virus such as a study from Moskwa(251). They found that there was an immune response to virus infection and that it may not be deficient in asthmatics but is modified by atopic status. These results echo what is seen in this study with both atopic groups having a more significant reduction in wound healing than the non-atopic asthmatics. The viral titre was not a defining feature of the phenotypes with the same levels of RSV detected across all groups. Another study by Xatzipsalti modelled an atopic environment and saw an increase in RV proliferation and increased RV-induced toxicity(252). Ours are the only data available to functionally demonstrate the differential effects of infection in epithelial cells from healthy, non-atopic asthmatic, atopic and atopic asthmatics in wound repair. We saw a similar effect with atopic subjects having a much slower rate of wound healing after RSV exposure than subjects what were non-atopic asthmatic.

From the images of green RSV infection (Table 4-1 and Table 4-2) we see that some have a reduction in the wound closure rate but do not seem to show any green fluorescence or viral infection. A possible explanation for this could be that the epithelial cultures are fighting the infection with that effect causing a decrease in the cells capability to repair the wound. This should be tested by using mock (RSV inactive) RSV on the apical surface to mimic an infection and to understand the effect of

non-infectious particles have on the wound healing. PBS was added to the apical surface of the cells to stimulate a mock infection, but as we saw in chapter 2 that even PBS can initiate a response. There was no significant effect seen in the secretion of IFN-β when compared to the PBS only treated cultures. Stress mediators could also be a measurement for the health of the ALI culture to couple the TEER data. It is fair to assume that when epithelial cells were infected with RSV there is some degree of stress on the cells to fight the infection. Viral infection is known to induce the peroxidation of lipids in epithelial cells through oxidative stress, which was recently associated with non-apoptotic cell death(253, 254).

Lastly, as mentioned above, it is important to note is that different RSV strains are not equally capable of (killing/infecting) epithelial cells and different RSV strains result in variations in asthma exacerbations severity(255). ALI cultures may express particular cell surface receptors previously not discovered in submerged cultures, favourable for attachment and fusion of a clinical isolate compared to high passage laboratory isolate such as RSV-A2. Even knowing this, RSV isolates are still an important place to initiate experiments in model development.

4.4.3 Cell death during viral infection and wound repair

Damage to the epithelium during RSV infection could have a detrimental effect on the barrier function of the epithelium. This chapter examined the reduction in rate of wound closure and if this was due to infection, not cell death. It has been reported by Adam and Sacco that RSV may lead to more profound damage that is independent of the causative mechanisms. This alone could disrupt the repair mechanisms causing the reduction in wound repair seen in this study. Apoptosis is an important immune response to viral infection that limits virus spread and allows the non-inflammatory removal of infected cells. RSV did not induce cell death 6 days after infection and injury, with no increase in LDH released from the apical surface. This was verified by the measurement of TEER during the 6-8 days of infection and also during wound healing, where there was no variation in the polarization of the epithelium layer. This indicates that the epithelium maintained its integrity throughout infection.

Previous studies have examined how cell apoptosis and necrosis can promote inflammation and that the reduction of cell death in the airway epithelium can perpetuate prolonged infection. Not only

that, it can decrease the capacity for the epithelium to repair after wounding(256-258). However, we saw no increase in the LDH detected across all subject groups with no distinguishable difference in cell death. Spann et al reported asthmatic and wheezing subjects may have a defect in caspase-3/7 activation and apoptotic cell death, which resulted in a higher viral load(102). They suggest an association with asthma and atopy, and its effects on wound healing. However, we did not see a higher viral load associated with atopy, or the effect of injury, and cannot conclude that caspase-3/7 activation would play a role in this model. This is not surprising as the model used in Spann et al study used submerged epithelial cells, and the ALI cultures used in this our study have more complex mechanisms and receptors for the virus to interact with. Discrepancies can be seen across the literature between findings in submerged cultures and differentiated cultures. Knowing this we can still expand on research performed using different culturing of epithelial cells. Studies of cell death in ALI cultures are limited, particularly in response to viral infection. One study did investigate ALI cultured bronchial epithelial cells infected with influenza and demonstrated elevated cell death and apoptosis in response to infection(259), however just as comparisons between RSV stains should be taken into consideration so should comparisons between pathogens. No current studies have looked at the effect of cell death by RSV infection during wound healing.

Along with further investigation into the apical sampling for cytokine secretion identified in previous chapters, the timing and sampling to measure cell death needs further thought. Removal of apical LDH at specific timepoints results in a non-cumulative measurement of the LDH released and could cause results to be negative with little detected. Basolateral media might be a better source for LDH measurement, but a similar phenomenon might occur at feeding timepoints. Increasing cell number and cultures dedicated to measuring cell death should be considered in the future to examine the epithelial cell death from viral infection after injury. Other assays for measuring cell death could be considered in future studies to look at differentiating between necrosis and apoptosis such as the how RSV can suppress apoptosis by function of the 2 non-structural proteins NS1 and NS2(260).

4.4.4 Inflammatory cytokine production during infection and repair

The antiviral response of the epithelium is complex with much debate about the epithelial cells from healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects and their ability to produce IFN in response to RSV infection. There was no significant difference seen in the IFN-β secretion from all donors before infection and at 6 days post infection. Small sample numbers with no clear

correlation saw no conclusions from measuring the IFN-β in ALI cultures. IFN-β has previously been reported to have decreased secretion in asthmatic and wheezy children compared to healthy as identified in Spann et al paper(102). A critical component of the innate immune response is IFN-β and studies have reported a reduction from both epithelial cells and macrophage cells(54, 261-265). However, several studies have found that no difference or high expression in asthmatics when there is defective IFN-β production(250), with the opposite also found, with impaired IFN response by asthmatic individuals associated with increase viral susceptibility(54, 264). In our study we saw that asthma was not associated with elevated susceptibility to RSV infection.

One avenue to explore further and will benefit the knowledge of RSV infection is IRF-7. As in Moskwa et al, IRF-7 expression could correlate with IFN-β expression in response to RSV in asthmatic patients. Both TRF-3 and IRF-7 activate transcription of the genes for IFN-β, IFN-α1 and RANTES, with mRNA expression also of interest(251). Although there was indistinguishable levels of IFN-β detected in the apical wash from all subjects a representative way of measuring IFN-β would be to look at mRNA. Previous studies showing RV infection containing lower IFN-β mRNA in non-atopic subjects when no IFN-β protein was detected(251).

Mechanisms of RSV and the effect on IFN-β have been established, with IFN-β being a potential in treatment. IFN-β can be inhaled and a potential treatment for virus-induced deteriorations of asthma with Kicic showing that IFN-β was lower after RV infection in asthmatics(266). IFN-β could be added to decrease infection and increase capacity for repair with treatment repairing the response to injury. IFN-β treatment only has short term therapeutic potential and should be taken into consideration in more severe cases. Lastly, the method of cytokine analysis should look at in a more specific study with the addition of other cytokines in the inflammatory process. These would include IP-10 and RANTES which have also been included in the Spann paper(102).

4.5 Conclusions

In conclusion, airway epithelium repair is significantly affected by RSV infection and this effect is less prominent in non-atopic asthmatic subjects. We have provided strong evidence that epithelial cells from adult subjects are all effected by RSV infection and this is the first study to look at the effect RSV has on healing and also the effect on an already wounded epithelium. Further research is

needed to confirm that this effect would occur in other viruses or pathogens and to clarify the significance of this occurrence in the pathogenesis of respiratory virus infections in asthmatic patients.

This PhD thesis did not extend to the investigation of different programmed cell death, strains of virus, other respiratory pathogens and viral interaction. This main objective was to establish an effect to the wound healing model that was clinically relevant and clinically supported. The question of what happens during an RSV infection to these process can now be studied in more depth building from the results generated in this study.

Chapter 5 Final Discussion

The final discussion is divided into smaller sections to easily interpret the results and develops further on the ideas identified in each chapter.

5.1.1 Method Evaluation

The first objective in this study was to establish an ALI wound healing model that was reproducible and effective. This model was characterised to verify that it was a true representation of the airway epithelium *in vivo*. Firstly, a pseudostratified epithelium was established using the ALI method of growing cell on a transwell insert for at least 3 weeks. This was a slow process with large amounts of cells needed for each experiment. Validation experiments at the beginning of this study determined the capabilities of the culture achieving the most realistic data output for these cells and ALI culture. This included, staining cultures for presence of basal, goblet, ciliated cells, and tight junctions with the conclusion that at least 7 weeks of culturing is needed to achieve a differentiated culture. Levels of secretion from the apical and basolateral chamber from numerous inflammatory cytokines both before and after TLR agonist exposure were also measured. Gene expression was compared from a range of makers for basal, goblet and ciliated cells with tight junctions and fibronectin also included. Lastly, and the most effective measurement was in detecting the rate of wound closure. After establishment of a differentiated ALI culture, the epithelial layer was mechanically wounded, and the rate calculated. This method was repeatable and informative as to how epithelial cells respond after injury. The establishment of this model was the basis of this study and used in all following chapters.

5.1.2 Benefits of ALI

Establishing epithelial cells in culture at ALI is beneficial, primarily because it allows replication of an *in vivo* airway. Throughout this thesis the ALI method of culturing epithelial cells *in vitro* has represented a physiological model of wound healing. Previously methods of cell culture have not generated data directly applicable to the human system, with submerged culture unable to undergo mucociliary differentiation resulting in numerous receptors and cell types being absent from the culture. Using cells from healthy, asthmatic and atopic subjects, this thesis could recapitulate *in vivo* disease characteristics and the effect on wound repair, all from a robust *in vitro* model. By differentiating the epithelial cells into basal, goblet, and ciliated cell, with tight junction's present, we could study disease mechanisms, the selectively targeting of cell types during RSV infection, and mimic the wound repair of an injured epithelium. Establishing these in a robust and repeatable model allows for further research into specific mechanisms and potential treatments.

5.1.3 Wound healing

The wound healing progression previously reported comprises of 4 main stages: dedifferentiation, migration, proliferation and redifferentiation. Two of these stages are seen in the wound closure of epithelial cells at ALI. Migration can be seen via images taken every hour and the reduction of wound area and redifferentiation is measured by TEER values returning to the pre-wounded value. Because of the fast healing of most cultures (<40 hours) we were unable to measure dedifferentiation and proliferation from the wound healing model alone. Collecting apical wash before and after wounding was proposed to identify key cytokines that were either increased or decreased during wound repair. Unfortunately, there was no difference seen between cytokine secretion in the apical wash, with very low levels of EGF, MCP-3, MDC, IL-13 and IP-10. Higher levels of GM-CSF and GRO were detected which correlates with previous data in corneal epithelial cells showing the role GM-CSF plays in wound repair(267) and the importance of GRO has after repair(268). In a more sensitive testing of the apical secretion of GM-CSF and IP-10, no difference was seen both between groups and before and after wound healing, except healthy subjects differing slightly in secretion levels before and after repair. A key explanation as to the low levels detected could come from the washing of the apical surface. The process of washing the apical surface of the cells causes the activation and production of specific cytokines, even with no external exposure, except the sample collection with PBS. This effect can be seen occurring in Figure 2-14, with the gradual increase of IL-8, IP-10 and TNF-α after multiple washings. Experiments to combat this require dedicated wells for sampling at each time point with no well resampled. This approach is not feasible when using primary cells which are limited, but also the extended time to establish an ALI culture would make these simple experiments extremely expensive. Another aspect to explore and identify wound healing mechanisms would be to isolate each stage of wound repair and detect the gene and cytokines present. This would involve the stopping of ALI wounded cultures at time increments (4, 8, 12, 16, 20 hours etc) after initial wounding. It is believed that at different stages of the repair process cytokines play specific roles in the healing, such as an increase of IL-8 at cell migration and proliferation, with IL-8 decreasing and the MMPs increasing at pseudostratification and surface airway epithelium differentiation(269). By isolating each stage, the wound healing mechanisms might be more easily identified than just capturing the cytokines released after the process is complete.

The downside of this would be that the repair process seen in these ALI epithelial cultures is occurring at such a fast rate (<40 hours) and any change might be hard to measure. It would also involve large number of cells at initial sampling, which might not be realistically achievable in primary cells.

Another way to evaluate the dedifferentiation in a healing culture was by the collection of cells from along the leading edge of the wound over time of repair and measure the genetic markers in cells during the processes. Single cells were isolated and tested to determine the type of cell (basal, goblet, ciliated) and markers for fibronectin and tight junctions. This study identified that it was possible to isolate individual cells from an ALI wounded culture and determine the genes expressed. However, for any comparable data to be generated a large number of single cells would have to be collected at different locations within the well and at each time point. The cost of this and the potential to disturb the ALI culture meant that this was not possible for this study but is very possible in studies dedicated to studying single cells of the airway epithelium.

5.1.4 Air-Liquid Interface culture development

5.1.4.1 Differentiation media

The process of differentiating epithelial cells at the air-liquid interface is extremely complex with many vital steps. One of these is the use of differentiation media to establish a pseudostratified epithelium *in vitro*. Two media types were used in this thesis, manufactured by Lonza (B-ALI) and StemCell (PC-ALI), with both claiming to achieve a fully differentiated epithelium after 3 weeks at the air-liquid interface. Both types of media have been widely used for many diverse studies. These have included for B-ALI (Lonza), the establishment of an ALI culture system(158), respiratory viral infection(122), drug transport studies(270), TLR agonist exposure(192) and barrier studies(23). PC-ALI however is relatively new on the market compared to B-ALI media, so studies using this media have only been published in recent years. It is widely becoming the media of choice for differentiation of epithelial cells, firstly because of the greater survival rate of cells in culture and secondly because it is more affordable. Studies have used PC-ALI media to study extracellular matrix components(271), airway inflammatory response(272) and bacterial infection(273).

This thesis examined the differences in both medias when used for differentiation in a wound healing model with no significant difference seen across all phenotypes for the rate of wound closure. Not only is it reassuring that all wound healing experiments can be compared, it is critical for comparison between all previous research performed using B-ALI media. Early and important discoveries from epithelial cells at ALI using B-ALI media can be carried forward and pursued in PC-ALI media.

Between the two types of media from Lonza and StemCell growing epithelial cells in culture results in changes in epithelial cell morphology in submerged culture (Figure 2-1). Lonza cells grew in an even monolayer of cells with even spacing, but StemCell cells grow as clusters of densely packed cells. When cryopreserved at passage 2 there were significantly greater number of cells when StemCell media was used. This carried over to the ALI cultures with a higher success rate of atopic asthmatics in the PC-ALI media and higher percentage of unsuccessful cultures in cells differentiated in B-ALI media. Future studies should look more closely at the mechanisms and make up of each media involving the identification of additives and their concentrations. As this information is proprietary, and Lonza no longer produce B-ALI, this is a difficult task.

5.1.4.2 ALI cultures

As reported in the literature review of this thesis, there are many varying results in the time to best use ALI culture. For this thesis were strict guidelines of cell culture morphology. There must be presence of cilia, no holes present in culture and a stable TEER reading. TEER values were not required to be above a certain threshold, as this was a comparable output between subject groups.

Typically, primary epithelial cells do not survive in the laboratory after 2-3 passages, due to cell senescence but the process of differentiating cells at ALI allows for the cells to be cultured and survive for weeks to months. Because of this extended time epithelial cells spend in culture at the ALI, studies in the future should look at the effect of an ALI culture at different ages to see the effect long term culturing can have on the barrier function and also the distribution of each cell type. After establishment of the ALI cultures we can better compare results between respiratory diseases and environmental exposures. This thesis has also built a foundation for research into other sections of the upper airways such as the trachea with development of the ALI presenting a more realistic and complex epithelium when comparing to submerged cultures.. A unique aspect to this study was the use of nasal epithelial cells instead of the usual bronchial epithelial cells often harvested for airway research. Nasal epithelial cells have both positives, from easy collections sites resulting in higher collection numbers, to the negatives of less reported studies using nasal epithelial cells. Our study used nasal epithelial cells derived from adults to establish a reliable and repeatable model of the airway epithelium in a large cohort. This can then be translated into both bronchial epithelial cells and children's samples to measure wound healing changes and is desirable with prior studies demonstrating epithelial cells from infants can be used to study early-life antiviral response(122, 131, 274, 275)

5.1.5 Asthma and atopy

Previous wound healing research has focused on severe respiratory diseases such as asthma and COPD, and mostly this has been in submerged cultures. The benefit of this study was firstly to understand wound healing in an ALI culture but to also see the effect of asthma and atopy, with limited studies comparing these two groups. Atopy can affect the upper epithelium and can be a predecessor of asthma for children with a link identified through gene expression, IgE levels and many longitudinal studies.

Through the model of wound repair atopic subjects have shown a significantly lower rate of wound closure and this discovery has identified the need for further investigation into the mechanisms involved in wound healing between all subject groups.

5.1.5.1 Wound repair

Establishment of a reliable and repeatable model to measure the wound healing in a 3D pseudostratified epithelium was the main focus of this study. Successfully, this was achieved using two types of differentiation media with cells from healthy, asthmatic and atopic subjects. This study was able to identify that atopic subjects had a reduced rate of wound closure compared to other disease groups. This surprising result allowed further investigation into the mechanisms involved and that may be different in atopic epithelial cells. Also, of interest was the rate of wound closure from asthmatic subjects and how they were not significantly reduced when compared to healthy subjects. Previously, studies have reported that asthmatic cells have a reduced healing capacity after injury which contributes to airway remodelling and increases in asthma exacerbations. These studies however, used epithelial cells in a submerged culture. As noted earlier, submerged cells only contain basal cells and are a poor representation of the *in vivo* airway epithelium. ALI cultures are differentiated into basal, goblet and ciliated cells and are a much better representation of the *in vivo* epithelium. Presence of specific receptors to each cell types add further complexities to study.

The healing of a wound after injury occurred in less than 40 hours for all groups except for the atopic subjects. Excluding them, the healthy and asthmatic subjects showed fast and simple healing after injury. Speculation in to the cause of this accelerated healing could be from the small environment these cells are going in (Transwell insert) and also the small size of the wound. Creating a space in

such small wells may just be causing the cells to fill the space and closing the wound without signals from the cells that normally indicate a wound has occurred. Bigger wounds in much larger transwell inserts may show a slower rate of wound closure and allow time for the cells on the leading edge of the wound to signal surrounding cells of an injury. This may be an explanation as to why little difference was seen in cytokine release and gene expression in this wound healing model before and after wounding across all sample groups. It does, however, still leave the question as to why the atopic subject showed such a delay in the repair after wounding. Structural changes in the cells from atopic subjects could be the answer to its delay and is explored further over the next few pages.

5.1.5.2 Atopy

Disruption to the epithelium is caused by exposure to the external environment with the airways the key interface. There is much debate as to whether atopic and non-atopic asthma are immunopathologically two distinct entities or if they just both driven by similar mechanisms. The answers to this are not currently clear. Previously atopic and non-atopic has been classified based on presence or absence of clinical symptoms precipitated by one or more common aeroallergens. This is supported by the presence of allergen-specific antibodies, evidenced by skin prick +/- serological tests(276). The majority of the studies comparing atopic and non-atopic asthma have been focussed on the inflammatory or immune cells at the submucosal level rather than the resident cells in the mucosal epithelium, with Amin et al stating that epithelial damage was significantly higher in the atopic asthma group(212). Many areas have been researched comparing asthma and atopy in past studies, identifying differences in cellular development with basal cells having reduced desmosome lengths in asthmatics and goblet cell hyperplasia with an increased secretion of mucus in atopic asthmatics(277).

The identification of reduced healing capability for atopic donors to heal after injury has made us reflect on the reason for this. Determining the status of asthma in a subject is much easier as the symptoms often occur at childhood or are severe enough to seek doctor diagnosis. The case is not the same for atopic status. As identified in the questionnaire, atopic subjects were classified as having hay fever with treatment either by antihistamine tables or nasal sprays. Most were self-diagnosing, with very few saying the allergies were confirmed by a skin prick test. We do not propose that any of these are false positives, however we do believe that because respiratory related allergies aren't as severe, there is a chance of non-atopic subjects also could fall into the atopic phenotype. Given the

knowledge we now have thanks to this study, there should be more emphasis placed on the atopic status of subjects. Previously, skin prick tests have been a reliable way to identify allergies to specific grasses, animals and dust, but the test is limited. A more appropriate test would look be to look at IgE levels. This can be done using a simple blood test IgEQAA, which has high sensitivity and specificity and should be considered for future research in this area.

This thesis had planned to look at the intrinsic details of these two phenotype groups however limitations arose with media availability, cell numbers and subject availability. We were able to identify some key differences between atopic and atopic asthmatic subjects. Firstly, was that in culture atopic asthmatic cells were harder to grow and cultures were less successful, however once an ALI culture was established atopic asthmatic subjects had a faster rate of wound closure. Other differences between the two phenotypes were that the IL-13 receptor was highly expressed in atopic subjects, with the EGFR higher in atopic asthmatics. Levels detected for each cytokine were very similar between the two with no difference observed. RSV did play a role in effecting the rate of wound closure with atopic asthmatics having a more significant reduction in wound repair after infection, also with less fluorescence seen during the 6 day infection. Detection of IFN-β during infection and after wounding saw a decrease in atopic subjects, but an increase for atopic asthmatic subjects. These observations do highlight that there are differences in the airway epithelium between atopic subjects with or without asthma. More mechanistic research on a cellular level is needed to fully understand the differences between these two phenotypes. Identification of this would allow for better and more specified treatment for atopic subjects.

5.1.5.3 IL-13

IL-13 mediates physiological changes induced by allergic inflammation in many tissues, including the airway epithelium. In this study we saw that increased exposure to IL-13 caused inhibition of wound healing to all subject groups. We were also able to determine that epithelial cells produce very small amounts of IL-13 indicating external cell types as the source such as macrophages or T-helper 2 cells. Low levels detected in AlphaLISA assays and inhibition of IL-13 caused no difference in wound repair as confirmed in this study. To elucidate the role IL-13 plays in epithelial wound healing the next logical step should be to look at co-culturing with macrophages. Co-culturing is needed to replicate the innate immune system where macrophages are the main producer of IL-13 which can inhibit the wound healing in all cultures. Once IL-13 is better understood in epithelial wound healing it has potential for asthma treatments through IL-13 monoclonal antibodies. Inhibition

of IL-13 can restore lung function and reduce asthma exacerbation, with the potential to elevate the occurrence of damage in the epithelium. Studies have also shown it can block disease progression through airway epithelial dysfunction(189). The model established and tested in this study can act as an effective model to further investigate IL-13 treatment for asthma.

5.1.5.4 EGF

The importance of EGF in a differentiating culture is key to the development of all cell types within an ALI culture. Inhibiting EGF caused dramatic effects to cell morphology, reduced the TEER value, and caused no wound repair. It does appear that over saturation of EGF is not detrimental to the culture but also does not have any added benefits. Treatments in cancer are currently available that block the EGF receptor but very few studies have looked at the enhancement of EGF and the effects in wound repair. We saw in this study that addition of EGF alone did not have an enhanced effect on the rate of wound closure regardless of subject group. The EGF receptor is not exclusive to EGF with TNF-α also binding to the receptor. TNF-α is a major regulator of immunity and inflammation as well as cell differentiation and death. TNF-α may exert both EGFR-dependent and -independent pathways and potentially act as an alternative to EGF treatment alone with saturation of EGF showing no added benefit to wound repair. As there is unknown concentration of EGF in the media and we cannot conclusively determine the direct effect EGF has on the wound healing in culture. Concentrations of each supplement and additive is needed before future studies of EGF and its effect on wound healing is essential to properly determine its effects.

5.1.5.5 Mechanisms in atopic epithelial repair

Identification of the specific mechanisms involved in atopic wound healing and what is causing the dysregulation of repair should be examined in future studies. One hypothesis includes that the tight junctions in ALI cultures from atopic subjects might be less effective and causing a delay in the wound repair. As identified in the literature review IL-13 and TNF-α both effect the tight junctions of the airway epithelium, but only IL-13 has been tested in this thesis. The measurements of TNF-α in the ALI cultures might help in identifying the role tight junctions play. Although TNF-α is not produced by the epithelium, higher levels of TNF-α in the airway enhance proliferation through the EGF receptor and modulates the cytoskeleton(278). TNF-α can bind to the EGF receptor in place of EGF and treatment with TNF-α has been shown to regulated wound healing and could potential increase repair in the epithelium. Also, of interest is the gene expression of ALI cultures and would be essential in determining the characteristics of the epithelium developed in culture. Along with cell

and tight junction markers, fibronectin (FN) was one of the genes tested to see if adult epithelial cultures from both asthmatic and atopic subjects had a reduction when compared to control. Kicic has reported this in paediatric primary epithelial cells, where they show that reduced FN production cause a reduced wound repair capability(146). We saw no difference in the single cell expression or whole well expression between subjects of different phenotypes. Measurement of fibronectin protein in the apical or basal wash might represent the physiological environment more than gene expression alone. By including other cytokines and genetic markers into the wound healing analysis when comparing atopy and asthma we could potentially identify faults in pathways of cell proliferation and migration.

5.1.5.6 Previous use of topical steroids

Many studies have looked at the effect previous use of topical steroids have on cell culture once the cells have already been harvested. They use either primary cells from the lung or cornea and then expose these cells to a steroid and observe the effects(195, 267, 279). No studies have looked into the effect on cells when a subject uses steroids to treat asthma and atopy symptoms before harvesting of the cells, let alone look at the effect on wound healing.

In this study we saw a significant increase in the rate of wound closure from epithelial cells harvested from subjects that have previously used topical steroids. This was independent of disease group with only the asthmatic and atopic subjects compared. Logically, healthy subjects had no reported use of topical steroids. Also, of interest was the effect topical steroid use had on RSV infection. RSV infection at MOI 1 did cause a reduction in the rate of wound closure across all phenotypes. However, subjects that did report using steroids had less of a reduction in rate of wound closure.

From these data we can see the effects topical steroid treatment can play on cells even after treatment has ceased. It should be noted that only one subject used topical nasal steroids and it is not clear how the epithelial cells were exposed to inhaled steroids as these drugs are not taken through the nose. The effect is still clear and possibly from systemic exposure or exposure on exhaling. The harvesting, growing and differentiating or these epithelial cells did not erase the "memory" of steroid treatment, even though this was believed to be the case. A recent informative paper by Ordovas-Montanes et al characterized the cellular ecosystem in allergic chronic rhinosinusitis by the use of genomic, epigenetic and intervention techniques(280). One significant finding from this study was that basal epithelial cells "remember" type 2 inflammatory stimuli to maintain a chronic allergic disease

phenotype. As seen in this thesis, similar properties of long term effects are occurring to the epithelial cells even after harvesting and culturing. This discovery can be translated further with asthma and atopic subjects receiving topical steroid treatments as highlighted above. It has been widely published that glucocorticoid steroids are used in the treatment of asthma because they supress type-2 inflammation(174), and with on-going treatment and exposure may be an explanation to the asthmatic subjects showing no effect in wounding at ALI.

Also of interest is direct use of topical steroids such as dexamethasone, an anti-inflammatory drug/immunosuppressant glucocorticoid, has an immunosuppressant action when used and causing delayed healing(281). The impact can also occur through inhibition of fibroblasts proliferation and decreased collagen production. Because of the aspects of an ALI cultures these cannot be studied in this model alone but can be paired with co-culturing methods or *in vivo* studies. Further down the line the effects of previous steroid usage could affect the interleukin signalling, cytoskeletal remodelling and keratinocyte proliferation. All of which affect the inflammatory and proliferative phases of wound healing.

Knowing that previous exposure has an effect on cells in culture, other ignored treatments may be given a second look such as antibiotics which cause morphological changes in the characteristics of migration in canine corneal epithelial cells in tissue culture (279) or the popular asthma medication salbutamol, with reports of *in vivo* and *in vitro* effects on alveolar epithelial repair in acute lung injury (282). Important to note for this thesis, only nasal cells were used and should not have been exposed to the effects of salbutamol as it administered orally. Other studies report that along with salbutamol, salmeterol has an effect on ciliary beat frequency of cultured human bronchial epithelial cells, *in vitro* (283) and all previous treatments should be recorded and considered when analysing future effects on ALI epithelial cultures.

5.1.6 Co-culture

With establishment of this model, avenues for further research can be pursued. One of these areas of interest is co-culturing of airway epithelial cells with macrophage cells. The breach of the epithelial barrier seen in this wound healing model replicates injury to the epithelium *in vivo*. During injury *in vivo* neutrophils can break the mucosal surface of the epithelium. There is potential to explore the

neutrophil and macrophage transepithelial migration of the epithelium and how this can impact wound repair. A study by Yonker et al has already attempted similar research with a co-culture system of bacteria-induced neutrophil transepithelial migration and seen the effect on the physiological properties of the epithelium(284). This could further be adapted to fibroblasts where a study by Ishikawa et al used collagen gel matrices with lung fibroblasts in the ALI culture and measured the interactions and effect on airway remodelling (285). The culturing of more than just airway epithelial cells at ALI may highlight structural changes otherwise not seen in epithelial cultures.

Co-culturing will also benefit the knowledge of cytokines expressed during wound healing. The limited expression of cytokines from the apical and basolateral surface of the epithelial has posed issues to the analysis of specific cytokines previously reported to be present in the airway. It is not fully explored yet the possibility that ALI cultures may not be the most accurate way to measure these inflammatory cytokines because it is an isolated culture. Co-culturing of epithelial cells and T-helper 2 cells, keratinocytes or mesenchymal cells would better represent the response to injury in the airways. Ashcroft et al already shows that TGF-β1 stimulates migration of keratinocytes with increases in TNF-α, EGF and keratinocyte growth factor(286). This along with mesenchymal cells and macrophages that act as paracrine mediators would allow better representation of the airway environment.

5.1.7 Viral infection

The airway epithelium is constantly exposed to various pathogens and the response of the epithelium to these contaminants is vital in the understanding of the epithelium barrier function. In this thesis the response of the epithelium after RSV infection was measured but also how this viral infection effected the wound repair.

5.1.7.1 Susceptibility to infection

It was hypothesised that there would be a difference in the susceptibility of infection between the different subject groups, however, epithelial cells from asthmatics (atopic or non-atopic) were not more susceptible to RSV infection than healthy cells regardless of MOI. This study demonstrated an elevated viral titre but not an increase in cell death/cytotoxicity in non-atopic asthmatic and atopic

subjects after 6 days. Low levels were detected in the healthy and atopic asthmatic subjects. These results match what is already reported by Spann et al when comparing RSV shed from paediatric cells grown in submerged cultures(102). One key difference is that the effect seems to be dampened in ALI cultures with the removal of RSV occurring much more frequently. Presence of cilia, mucus and tight junctions all play a role in removal of the virus from the ALI culture, and along with manual washing of the cells can cause a much lower shed virus over time. Instead of constant re-infection of the epithelial cells seen in submerged cultures the ALI can function similar to the *in vivo* epithelium and eradicate the RSV infection. This process can only occur at lower dosage of RSV in the culture and may not happen at high MOI.

The ALI cultures also demonstrated a delayed RSV shedding which may be due to the mucus inhibiting the spread of infection to neighbouring cells. A study removing and quantifying the mucus prior to infection of the cells in this model may give explanative results. Previous studies have attempted this by the addition of fluorescent beads and exogenous MUC5B to visualize mucus dynamics(287). One negative identified by removal or interruption of the mucus layer would be the possibility to interfere with the epithelial integrity. The addition of a substance to the cells can have detrimental effects which has previously be seen by the washing of the apical surface with PBS and the effect of IL-8 increasing just from that simple and harmless exposure.

5.1.7.2 Viral infection inhibiting wound repair

Viral infection of the ALI culture caused a significant decrease in the rate of wound closure at MOI 1. This delayed response was across all groups and saw a significant decrease in healthy, atopic and atopic asthmatic subjects. Interestingly, there was not a significant decrease in non-atopic asthmatic subjects at a MOI 1. The relationship between asthma and RSV infection has widely been reported with many studies identify that early life exposure to RSV caused a higher risk of that child developing asthma (288). This study identifies that once asthma is already established, RSV infection has less of an effect than healthy or atopic subjects. Pre exposure to the receptors might play a role in the memory of these cells already having significant exposure to RSV. Further research is needed on asthma cells at the ALI and why this interaction is different. Also, of interest is the significant decrease in atopic subjects, with a study by Trefny et al highlighting that infants with a family history of atopy have more sever RSV infections(289). This idea has not been studied extensively and with the model of RSV infection and wound healing a great way to measure the effect, it is now possible to study further.

One common difference between RSV infection studies is the strain of RSV and if clinically sourced or commercially purchased. This can have a dramatic effect on the damage to the epithelial cells, shedding of the RSV and effect on wound healing. Majority of comparable studies to this one have used RSV A2 as the standard stain to accurately represent an RSV infection *in vitro* (122, 290, 291). This was important for this study as it allowed any data to be compared to previous findings and to most importantly test the model and its effectiveness. One mechanism for RSV infection may relate to the expression of RSV receptor on the epithelial cells. Studies have already demonstrated that cilia is a vital site of initial infection of RSV, but little has been shown in the role of goblet cells, increased mucus, and also epithelial damage. Measuring the amount of cilia, goblet and basal cells in each ALI culture before and after wounding, in relation to the viral titre of RSV could potentially identify the relationship between epithelial cells, repair and RSV infection.

5.1.7.3 IFN response to injury

The response to injury has been the main focus of this thesis with an effect occurring after RSV infection. Using this model there is the potential to identify what mechanisms is defective in the antiviral response and how it effects wound repair. There was no difference seen in the susceptibility to infection associated with asthma or atopy when compared to healthy subjects. This would suggest that there may not be any defect in the antiviral IFN response in asthma or atopy in an ALI culture.

The possibilities of underlying mechanisms are diverse and have not been addressed fully in this thesis. Differential expression of soluble factors, such as interferons, may regulate either susceptibility to infection or the proliferative potential of RSV. The possibility that a viral infection may reprogram epithelial responses towards a "remodelled" phenotype has also been proposed, based on a mouse model of paramyxoviral infection, again highlighting a memory in the epithelial cells(292). The role of type I IFNs have in both healthy and atopic subjects could be of future interest however, IFN-β was not seen to be very significant from the data presented even though past studies have looked at IFN-β pre-treatment as having potential for improving repair of epithelial cells infected with rhinovirus(211). Also, of interest would be to develop the model to have a more realistic understanding of IP-10 release specific to acute virus induced asthma(293, 294) and to better understand variability in immune responses to respiratory virus infections of airway epithelial cells in patients with asthma.

In this thesis we saw that there was IFN-β produced by the apical surface of epithelial cells at ALI but there was no significant difference between IFN-β production between selected groups, and no correlation in reduction in wound healing. There was significant association between asthma and atopy and the IFN-β produced during viral infection in this current wound healing model.

5.1.7.4 Timing of RSV infection and wounding

Wounding of the epithelium occurred at 6 days after infection, as reported by previous studies including Villenave et al, as an optimal time for RSV infection(122). This study did hope to test the timing of an RSV infection over a two week period and see the RSV infection occurs at MOI 1 and 0.1 in an ALI culture. Unfortunately, cell culture contamination and cell unavailability lead to that experiment failing and results in no new data. Further experiments of an RSV infection time course could be beneficial to understand the infection and the effect of both early and late infection on wound healing. It could be hypothesized that wounding when the RSV infection is at the highest (high RSV shedding) would be much more delayed that an early infection of 1-2 days, where minimal change has occurred to the epithelium.

5.1.8 Limitations

Given that the main focus was to describe the methodology, feasibility, and potential use of ALI as a wound healing model, many aspects were not looked at because of cell availability and the extended time ALI cultures take to develop. Some of the most important results from this thesis also came as a surprising shock. This included the rate of wound healing from atopic subjects, effect of media on wound healing and infection at time of wounding. Because of these unexpected results there is no inclusion of the mechanisms causing these results. In hindsight there should have been some key experiments included. These have been highlighted below and could be considered for future studies.

Firstly, the use of nasal epithelial cells over the more widely used bronchial epithelial cells from the trachea. As identified in this thesis, nasal epithelial cells are easier to harvest resulting in a higher number of subject's cells collected and from a more diverse range of respiratory diseases. Nasal epithelial cells do play an important role in the upper airways and all data generated in this study still fills previous scientific gaps. However, a comparison between nasal and bronchial epithelial cells would be a great place to bridge the information from this study to already generated data in many previous studies. This would involve collecting bronchial epithelial cells from a small sample group

of healthy, non-asthmatic, atopic and atopic asthmatic subjects, and comparing the wound healing using the model established in this thesis.

This study has included some mechanistic experiments including gene expression of IL-13 and EGF from ALI cultures. Here we found that across all phenotypes both receptors were present but with no significant difference between the diseased and healthy subjects. More translational studies could be conducted using flow cytometry looking again at these receptors and the difference not only in disease groups but also the changes occurring during wound repair.

Lastly, the time frame of the study was a limiting factor in recruiting patients, collecting samples and the time it takes for differentiation. Some sections of this thesis have been limited by the number of subjects, such as single cell PCR data, Luminex screening data and TLR data. This was initially due to samples available at the time of culture and after collection of more subject cells was deemed not significant to pursue further. Directing the precious cell samples into experiments for wound healing, RSV infection and cytokine secretion was much more valuable.

In future studies, the inclusion of an environmental effect such as exposure to cigarette smoke or the separation of non-smoker and smoker groups for each phenotype would be a logical next step. This would confirm that findings are truly based on disease state rather than representing just the biological effect. Again, one reason for not including this was that this thesis was to focus on the establishment of the wound healing model that can be used in future studies like this.

5.1.9 Future directions

With much of the previous research on airway epithelial wound healing being done on respiratory diseases such as asthma and COPD, there has been little research in the link between these and atopy. There is a clear relationship between asthma and atopy, but does this translate down to the epithelial cell level. This thesis has highlighted the need for a more comprehensive look in to the specific mechanisms of atopic wound repair.

Firstly, by the surprising results of atopic subject have a lower rate of wound closure than asthmatic subjects there needs to be a through and detailed questionnaire before harvesting of cells. This should

include past use of topical steroids, history of atopy, family history of atopy and asthma and any inhaled drugs (including smoking) and where possible confirmation of atopic status though IgE tests. This more detailed background history of each subject would confirm the hypothesis that primary epithelial cells retain a memory well into culturing *in vitro*. With a more detailed subject history epigenetic markers would be of higher significance

Previous research shows TGF-β as a major mediator involved in pro-inflammatory responses and fibrotic tissue remodelling, and although controversial as a treatment for asthma might potential be a therapeutic agent for atopy(295). This highlights the importance of co-culturing airway epithelial cells as the logical next step in modelling the airways and to better represent the mechanisms of wound repair. Once established, there is potential for this model to be adapted in to other respiratory diseases such a chronic obstructive pulmonary disease, cystic fibrosis and also the testing of pharmaceutical agents.

5.2 Conclusion

This study has established and optimized a model for measuring wound healing using primary epithelial cells in ALI cultures. Numerous readouts were used to accurately measure the healing process of the epithelium and determined briefly the mechanisms involved in the process. Immunofluorescent staining using several antibodies evaluated and identified the main cell types including tight junctions in the ALI culture 7 weeks after lifting. Time lapse imaging monitored the wound over time to determine the rate of wound closure, revealing the migration that occurs during injury repair. This wound healing model was applied to different airway diseases including non-atopic asthmatic, atopic and atopic asthmatic subjects with atopic subjects revealed to have a reduced capacity to repair the wound after injury. The atopic status of subjects seems to have an effect on the recovery of a wound after injury, whereas the asthmatic status of a subject does not seem to play much of a role. No difference was observed between atopy and healthy or asthmatic groups for cytokine secretion or gene expression, only with minor differences.

Using the established wound healing model external environmental exposure was simulated by RSV infection and caused a reduction in rate of wound closure for all ALI cultures from healthy, non-atopic asthmatic, atopic and atopic asthmatic subjects. The viral infection showed a major negative

effect to the migration, proliferation but had no effect on the physiological barriers. The significant inhibition in wound repair could potentially be used to investigate pharmaceutical compounds of cellular pathways response to cellular migration.

The wound healing model established and validated in this study should provide as a useful basis for future development of wound healing, viral exposure and epithelial barrier mechanisms, with new focus on steroid responsive, Th-2 driven defects . The analysis tools and results presented by this project will be beneficial and serve as a base in future studies of both airway epithelial cells and asthma.

Chapter 6 Bibliography and List of References

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6.1 Appendix

6.1.1 Media declaration

Lonza

Lonza Australia Pty Ltd
2nd Floor, 541 Blackburn Rd
Mt Waverley, VIC 3149
Australia
Ph. (03) 9550 0883 or 1300 657 508
Fax. (03) 9550 0890
ABN 95 111 167 858

ATTN: Lonza Bioscience Solutions Customers

RE: BioWhittaker™ Classical & Specialty Media & TheraPEAK™ Media Kits

DATE: 14 February 2017

Please be advised, Lonza Walkersville, Inc. has become aware of a sterility issue in a media product specifically developed for one customer. We are investigating to determine the cause of the problem, and the potential impact to other products.

As a precautionary measure, all inventory of media products labeled "For Further Manufacturing", which was produced in the applicable manufacturing area, has been quarantined. Inventory of media products labeled "Research Use Only" has not been quarantined.

Manufacturing activities in the affected manufacturing area have been temporarily suspended, pending the results of the investigation.

We apologize for the inconvenience and we will contact you again with additional information as soon as possible upon completion of the investigation, which we expect during the week of February 19th.

In the meantime, if you have any questions or product concerns, please contact Lonza Scientific Support at:

- Americas: scientific.support@lonza.com / +1 800 521 0390
- Europe and Asia Pacific: scientific.support.eu@lonza.com / +32 87 321 611

Lonza is committed to ensuring all of our products meet the required standards. We appreciate your business and thank you for your patience as we work to resolve this matter.

Sincerely,

Michele Jones
Director Quality Assurance
Lonza Walkersville, Inc.
8830 Biggs Ford Road
Walkersville, MD 21793-0127, USA

6.1.2 Questionnaire

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Do asthmatics respond to respiratory pathogens differently?
Information Statement

Thank you for taking the time to read this information statement. This information statement and consent is 4 pages long. Please make sure you read this information carefully. You can ask us questions about anything in it. You will be given a copy of this information statement to keep.

You are being given this information sheet because we are hoping you may be interested in helping us with our research.

Everyone catches colds and other respiratory viruses. Sometimes the cold stays in the nose and sometimes the virus spreads to the chest. We know very little about why some colds spread to the chest. It has been recently suggested that adults with asthma may get more colds and more may spread to their chest. While you may not have asthma, we want to study both people who have asthma and who don't have asthma. We hope to study this by getting some cells from adult's noses, growing them in the laboratory and giving the respiratory pathogens (bacteria or viruses) to the cells to see what happens.

This study involves collecting cells from your nose with a special curette (a device with a small cup on the end). This study has been approved by the human ethics committees of the University of Queensland in accordance with the National Health and Medical Research Council's guidelines. The following information describes each part of the study. Please read each section carefully.

1. Information about you
We will need to ask you if you have asthma, and if you use any asthma medications. We will also ask you if you smoke cigarettes.

Do you have any questions about providing information about you before your proceed?

2. Collection of cells from your nose
To collect cells from the nose we also use a specially designed curette.
This procedure is not painful and will not cause any problems. The collection is very quick (less than one minute).

We will grow these cells in the laboratory. Once the cells are grown we will see how they respond to pollutants and different bacteria and viruses. We do this by exposing cells to pollutants and/or infecting the cells and seeing how they react.

Do you have any questions about the collection of cells from your nose before you proceed?

Frequently asked questions
1. What are the benefits?
There is no direct benefit for participation in the study. The study is being performed to give us information about how airway cells respond to respiratory infections (both bacterial and viral), to help us better understand how to treat and prevent asthma and asthma exacerbations.

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AUSTRALIA

2. What are the possible risks and/or side-effects?
There is a theoretical risk of bleeding from the collection, which is why we ask whether you are taking blood-thinning medicines or have a bleed disorder. Bleeding is very rarely (1/100,000) seen in people without bleeding disorders.

3. What are the possible discomforts and/or inconveniences?
During sample collection there may be slight discomfort, but it will not be painful or cause any problems. You may notice that your eye waters immediately after the collection. The collection will be very quick (only a minute) and should not cause much inconvenience.

4. Do I have to participate?
No. You can say no to the study and this will not affect how you are treated.

5. What will happen with the results of the study?
We hope to learn about how the cells lining the nose and lungs respond to respiratory viruses and bacteria, and whether there is a difference in the response in cells obtained from individuals with asthma.

We hope to be able to publish the results of our studies so that other researchers and doctors will be able to share in the knowledge we gain. All information we collect about you will remain confidential and no information that can identify you will be published or shared with others. Results may be discussed at conferences but participants will not be identified.

6. Can I withdraw from the study?
If you decide to participate you can withdraw your consent and discontinue participation in the study at any time. Your decision to withdraw from the study will not affect how you are treated. If you do withdraw after your cells have been collected, the cells will be destroyed.

7. What if I am not happy with this study?
This study has been cleared by one of the human ethics committees of the University of Queensland in accordance with the National Health and Medical Research Council's guidelines. You are of course, free to discuss your participation in this study with the Principal Investigator, Professor Peter Sly (contactable on 3069 7383). If you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Officer on 3069 7002.
Please remember that participation in this research is optional. If you choose not be involved or withdraw from the study your routine treatment will not be affected.

Thank you for your time and consideration. Please complete the attached consent form and questions if you wish to participate.

If you have any questions regarding this research study please contact the principal investigator Peter Sly on 3069 7383.

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FORM OF CONSENT

Please note that participation in research studies are voluntary and subjects can withdraw at any time with no impact how they are treated.

I, _____ have read the information statement explaining the study

Do asthmatics respond to respiratory pathogens differently?

I have read and understood the information given to me. Any questions that I have asked have been answered to my satisfaction.

I understand I may withdraw from the study at any stage and withdrawal will not interfere with how I am treated.

I agree that research data gathered from the results of this study may be published, provided that names are not used.

Signature: _____ Date: ____ / ____ / _____

I have explained the above study to the signatories who stated that he/she understood the same.

Investigator Signature: _____ **Date:** ____ / ____ / _____

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Participant Questions

Please note that participation in research studies are voluntary and subjects can withdraw at any time with no impact how they are treated.

Participant Information

Name _____ Age_____ Sex_____

Email Address_____

Mobile _____

Eligibility Criteria

Inclusion Criteria – must be "yes" for this participant to enrol in the study	No	Yes
Adult of either sex, aged between 18 and 65 years	☐	☐
Written informed consent signed according to local regulations	☐	☐

Exclusion Criteria – must be "no" for this participant to enrol in the study	No	Yes
A significant medical disease or condition, other than asthma, that is likely to interfere with collection of clinical samples or study results	☐	☐
Taking blood-thinning medicines or have a known bleeding disorder	☐	☐

Date of sample collection: __ __ / __ __ __ / __ __ __ __

Asthma

	No	Yes
Have you ever had asthma?	☐	☐
Have you had asthma in the past year?	☐	☐
Has a doctor diagnosed your asthma?	☐	☐

If yes, please list your asthma medications

Medication	Prescribed?*	Reason for use	Dose	Route	Frequency

*Was the medication prescribed by a doctor or purchased over the counter at the chemist?

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Rhinitis and Allergies

	No	Yes
Have you ever had "hay fever" or other allergic nasal symptoms (sneezing, itchy, runny nose) as from pollen or animals?	☐	☐
Have you had "hay fever" or other allergic nasal symptoms in the past year?	☐	☐
Did a doctor tell you that you have hay fever or nasal allergy?	☐	☐
Have you had a positive result for an allergy test? (e.g. RAST test, other blood test, skin prick test etc)	☐	☐
Do you have any other allergies? Please list any other relevant allergy, e.g. conjunctivitis, dermatitis, eczema, food allergy, etc.	☐	☐

Do you ever take any medications for rhinitis or allergy? Please list your medications

Medication	Prescribed?*	Reason for use	Dose	Route	Frequency

*Was the medication prescribed by a doctor or purchased over the counter at the chemist?

Smoking

	No	Yes
Do you currently smoke? If yes, please list how often below	☐	☐
Did you smoke in the past? If so please write when you quit below.	☐	☐