Inhibitor of Apoptosis Proteins as Regulatory Factors in the Normal and Inflamed Airways

Thesis submitted by

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For my Mother, Teresa Roscioli

Table of Contents

Abstract

Asthma is potentiated by complex gene-environment interactions, and characterised by inflammation and degenerative changes to the conducting airways. Current therapeutics targeting the inflammation and bronchoconstriction are restricted to prophylactic effects. The airway epithelium is known to participate in the pathogenesis of asthma, and represents a therapeutic target. Airway epithelial cells (AEC) from asthmatics exhibit apoptotic changes, which correlate with disease-associated factors presented by the epithelium. However, the mechanisms which cause the apoptosis are not well defined. In particular, there has been little study of the role of the family of Inhibitor of Apoptosis Proteins (IAPs) in models of AEC apoptosis. The over-arching hypothesis in this thesis is that anomalies in one or more of the IAPs contribute to inflammation-induced AE apoptosis in asthma. Experiments in this thesis explored the role of XIAP, cIAP1 and cIAP2 in asthma, models of asthma-related inflammation, and genetic susceptibility to asthma.

The major methods used in these experiments included cell culture of primary AEC from both asthmatics and controls with/without treatment with IFNγ and TNFα. siRNA knockdown, qPCR, western blotting, immunocytochemistry, functional caspase assays, and genotyping.

The major findings of this study are i) surprisingly, primary AECs do not undergo apoptosis after prolonged exposure to the proinflammatory cytokines IFNγ and TNFα *ex vivo*; ii) rather, IFNγ elicits a proapoptotic state in AECs, evidenced by, partial processing of procaspase-3, the absence of Poly (ADP-ribose) polymerase (PARP) cleavage, an increased Bax:Bcl2 transcript ratio, and the absence of morphological changes associated with apoptosis; iii) both XIAP and cIAP1 were constitutively expressed in AEC, and protein levels were unaffected by cytokine treatment. In contrast cIAP2, initially weakly expressed, was strongly inducible by cytokine treatment; iv) No differences were observed between AEC from asthmatics and controls in terms of either basal IAP gene expression

levels or their response to cytokine treatment; v) siRNA-mediated depletion of cIAP2-transcripts allows AEC to progress into apoptosis after extended culture, conditions which also resulted in a decrease in both cIAP1 and Bcl2; vi) genetic polymorphism in the genes encoding, XIAP cIAP1 and cIAP2 do not associate with susceptibility for asthma. However, cIAP1 polymorphism may modulate disease severity within asthmatics.

This thesis contributes to the knowledge of IAPs and apoptosis in asthma, and provides evidence that they are important for sustaining AEC survival, and participate within a cooperative of endogenous regulators of apoptosis. There is no evidence of intrinsic dysregulation of IAPs in asthma, yet cIAP1 polymorphism may modulate asthma severity, and IAPs are central in maintaining a proapoptotic state in AECs exposed to asthma related cytokines. Epithelial activation and damage, coupled with non-progression to apoptosis may contribute to fragility of the AE observed in asthma. Therapies targeting the IAPs may therefore provide a means of ameliorating the disease by allowing AECs to progress into apoptosis.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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This research would not have been possible without the generosity of the Department of Medicine and Hospital Research Foundation. Their support allowed me to care for the things in life that matter most.

Finally, I wish to thank my wonderful wife Trish, and our gorgeous kids for their love and encouragement.

Publications, Presentations, and Achievements

Publications, presentations, and achievements made during this PhD are listed below.

Published Journal Articles

Roscioli E, Hamon R, Lester S, Murgia C, Grant J, Zalewski P. Zinc-rich inhibitor of apoptosis proteins (IAPs) as regulatory factors in the epithelium of normal and inflamed airways. Biometals 2013; 26(2):205-27. (Appendix 4).

Roscioli E, Hamon R, Ruffin R, Lester S, Zalewski P. Cellular inhibitor of apoptosis-2 is a critical regulator of apoptosis in airway epithelial cells treated with asthma-related inflammatory cytokines. Physiological Reports 2013: 1(5):e00123 (Chapter 3).

Roscioli E, Hamon R, Ruffin RE, Zalewski P, Grant J, Lester S. X-linked inhibitor of apoptosis single nucleotide polymorphisms and copy number variation are not risk factors for asthma. Respirology 2013; 18(4):697-703 (Chapter 4, Part A).

Lang C, Hansen M, Roscioli E, Jones J, Murgia C, Leigh Ackland M, *et al.* Dietary zinc mediates inflammation and protects against wasting and metabolic derangement caused by sustained cigarette smoke exposure in mice. Biometals 2011; 24(1):23-39 (Appendix 5).

Murgia C, Grosser D, Truong-Tran A, Roscioli E, Michalczyk A, Ackland M, *et al.* Apical localization of zinc transporter ZnT4 in human airway epithelial cells and its loss in a murine model of allergic airway inflammation. Nutrients 2011; 3(11):910-28. (Appendix 6).

Tan N, Tran H, Roscioli E, Wormald P, Vreugde S. Prevention of false positive binding during immunofluorescence of *Staphylococcus aureus* infected tissue biopsies. Journal of immunological methods 2012; 384(1-2):111-7 (Appendix 7).

Submitted Manuscripts

Roscioli E, Hamon R, Ruffin R, Zalewski P, Grant J, Lester S. *BIRC2* single nucleotide polymorphisms are protective of severe asthma in a Caucasian community-based cohort. International Journal of Immunogenetics 2013 (Chapter 4, Part B).

Manuscripts in Preparation

Tan N, Cooksley C, Roscioli E. Drilling A, Douglas R, Wormald P, Vreugde S. Small colony variants and phenotype switching of intracellular *Staphylococcus aureus* in chronic rhinosinusitis.

Tran H, Roscioli E, Tan N, Coocksley C, Zalewski P, Wormald P, Vreugde S. Increased expression and sub-cellular translocation of AIM2 protein in conjunction with caspase-1 activation in sinus epithelium of the asthmatic endotype of chronic rhinosinusitis.

Conference Presentations

“Expression and Function of Inhibitor of Apoptosis Proteins in Asthmatic and Non-Asthmatic Airway Epithelial Cells Treated with Inflammatory Cytokines.” Presented at The Queen Elizabeth Hospital Research Day; Adelaide, 2012. Accepted for the seminar series competition based on abstract submission.

“Transcript Levels of Inhibitor of Apoptosis Proteins in the Normal and Asthmatic Airway Epithelium.” Presented at the Thoracic Society of Australia and New Zealand conference in Brisbane; 2010.

“The Role of the Inhibitor of Apoptosis Proteins in Normal and Asthma Affected Airway Epithelium.” Presented at the Thoracic Society of Australia and New Zealand Young Investigator of the Year in Adelaide; 2010.

“The Role of the Inhibitor of Apoptosis Proteins in Normal and Asthma Affected Airway Epithelium.” Presented at The Queen Elizabeth Hospital Research Day seminar seriesin Adelaide; 2010.

Achievements

Merit-based entry and scholarship recipient ($24,000) for The University of Adelaide postgraduate certificate in Technology Innovation and Entrepreneurship. Awarded the postgraduate certificate in Technology Innovation and Entrepreneurship; 2012.

Runner-up in the Thoracic Society of Australia and New Zealand Young Investigator of the Year seminar series competition, South Australian branch, Adelaide; 2010.

Co-investigator for The Queen Elizabeth Hospital Research Foundation research grant. Awarded funding ($5,000) for genetics research presented in this thesis; 2010.

Awarded the Thoracic Society of Australia and New Zealand travel grant ($450), to participate in the Brisbane conference; 2010.

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Commonly Used Abbreviations

°C - Degrees celcius

AE - airway epithelium

AEC - Airway epithelial cell

ALI - Air-liquid interface

Bax - Bcl-2-associated X protein

Bcl2 - B-cell lymphoma 2

BEGM - Bronchial epithelial growth media

BLAST - Basic local alignment search tool

bp - base pair

cDNA - Complementary deoxyribonucleic acid

cIAP - Cellular inhibitor of apoptosisbp - Base pairs

DAPI - 4',6-diamidino-2-phenylindole

DISC - death-inducing signalling complex

Dox - Doxorubicin

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

g - Gravitational acceleration

gDNA - Genomic deoxyribonucleic acid

h - Hour

HPRT-1 - Hypoxanthine-guanine phosphoribosyltransferase

HRP - Horse radish peroxidase

IL - Interleukin

IFN-γ - Interferon- γ

IRF - Interferon regulatory factor

IL-1β - Interleukin-1β

IL-4 - Interleukin-4

IL-13 - Interleukin-4

kDa - Kilo Dalton

M - Molar

min - Minutes

ml - Millilitre

mm - Millimetre

mM - Millimolar

mRNA - Messenger ribonucleic acid

NF-κB - Nuclear factor kappa B

NHBE - Normal human bronchial epithelial cell

nm - Nanometre

PARP - Poly (ADP-ribose) polymerase

PBS - Phosphate buffered saline

PCR - polymerase chain reaction

PET - Polyethylene terephthalate

PI - Propidium Iodide

qRT-PCR - Quantitative reverse transcription real-time polymerase chain reaction

RT - Room temperature

s - Seconds

siRNA - Short interfering ribonucleic acid

SFB - Serum free blocker

Smac - Second mitochondrial activator of caspases

STAT - Signal transducer and activator of transcription

TBP - Tata box binding protein

TBST - Tris-buffered saline

TNFα - Tumour necrosis factor-α

TRADD - TNF receptor-associated death domain

µg - Microgram

µl - Microliters

XAF1 - XIAP associated factor-1

XIAP - X-linked inhibitor of apoptosis

Chapter 1: Introduction

1.1 Project Synopsis

Asthma is a complex syndrome composed of multiple sub-phenotypes, which can be shared by other respiratory diseases such as chronic rhinosinusitis (CRS), and chronic obstructive pulmonary disease (COPD). Although the inflammation is initially driven by T-helper type-2 (T_H2) cells, there are several overlapping inflammatory responses which become more prominent in severe asthmatics. In addition, many of the phenotypes presented by asthmatics are governed by complex gene-environment interaction. Consequently, a unifying phenomenon responsible for causing and potentiating asthma remains elusive.

Disease-related changes presented by the structural elements of the lungs are shared by most asthmatics. Epithelial cells which line the airways are extensively exposed to the external environment, and are the first line of defence for underlying pulmonary tissues. Hence, by necessity airway epithelial cells (AEC) are resistant to cytotoxic stimuli, and participate in both innate and adaptive immunological responses. Recently, convincing evidence has implicating the airway epithelium (AE) as a site which is involved in the pathogenesis of asthma. AECs of asthmatics are susceptible to injury, and exhibit apoptotic changes.

Although the AECs of asthmatics undergo apoptotic changes, dysfunction in mechanisms governing the apoptosis remain largely unknown. Here, the Inhibitor of apoptosis (IAP) proteins, X-linked inhibitor of apoptosis (XIAP), Cellular inhibitor of apoptosis-1 (cIAP1) and cIAP2 were examined for their contribution to the apoptosis reported in the airways of asthmatics. As asthma exhibits a significant genetic component, polymorphisms within the genes encoding XIAP and the cIAPs were also examined for susceptibility for the disease.

1.2 The Respiratory System

The respiratory system is principally composed of the airways and lungs, the musculoskeletal system of the thorax, and components of the nervous system which regulate breathing. Ventilation is a mechanical process of pumping respiratory gases between the environment and the terminal alveolus, via a series of conducting airways (1). The generations of the airways transition from a large, semi-rigid, cartilaginous structure at the trachea and bronchus, to a network of increasingly narrower smooth muscle and elastin-rich bronchioles, and terminate at the alveoli (Figure 1.1). Alveoli are composed of a specialised epithelium made up of alveolar epithelial cells (type I cells) for gas exchange, and surfactant secreting Clara cells (type II cells) which maintain alveolar compliance (2). In addition, a unique form of pulmonary macrophage (alveolar macrophages) regulates inflammation and clears potentially pathogenic particles from the alveoli (3). As alveoli are the site of gas exchange between the atmosphere and pulmonary capillaries, they represent the principle functional unit of the respiratory system (4). Distinct from the squamous alveolar epithelium, the epithelium of the conducting airway, are tall columnar epithelial cells, which perform a variety of activities critical for efficient respiration (5).

1.2.1 The Airway Epithelium

The conducting airways are lined with a pseudostratified layer of ciliated columnar epithelial cells, punctuated with mucin secreting goblet cells (Figure 1.2). Their primary function is to facilitate the passage of the atmosphere to the alveoli, and protect the underlying structures. As a result, the AE maintains an extensive interface between potentially harmful environmental agents and the tissues of the pulmonary system. Consequently, AECs participate in a wide range of activities involved in both the innate and adaptive immunity (6). Of note, the production of a complex mucus barrier, which clears via coordinated ciliary movements, minimises exposure to pathogens and provide conditions inhospitable to microbes. AECs also express pattern recognition receptors (PRR), such as the Toll-like receptors (TLR), which transmit pathogen-associated molecular patterns (PAMP) signals to the cell (7, 8). AECs respond by secreting potent immunomodulatory cytokines such as Thymic stromal lymphopoietin (TSLP),

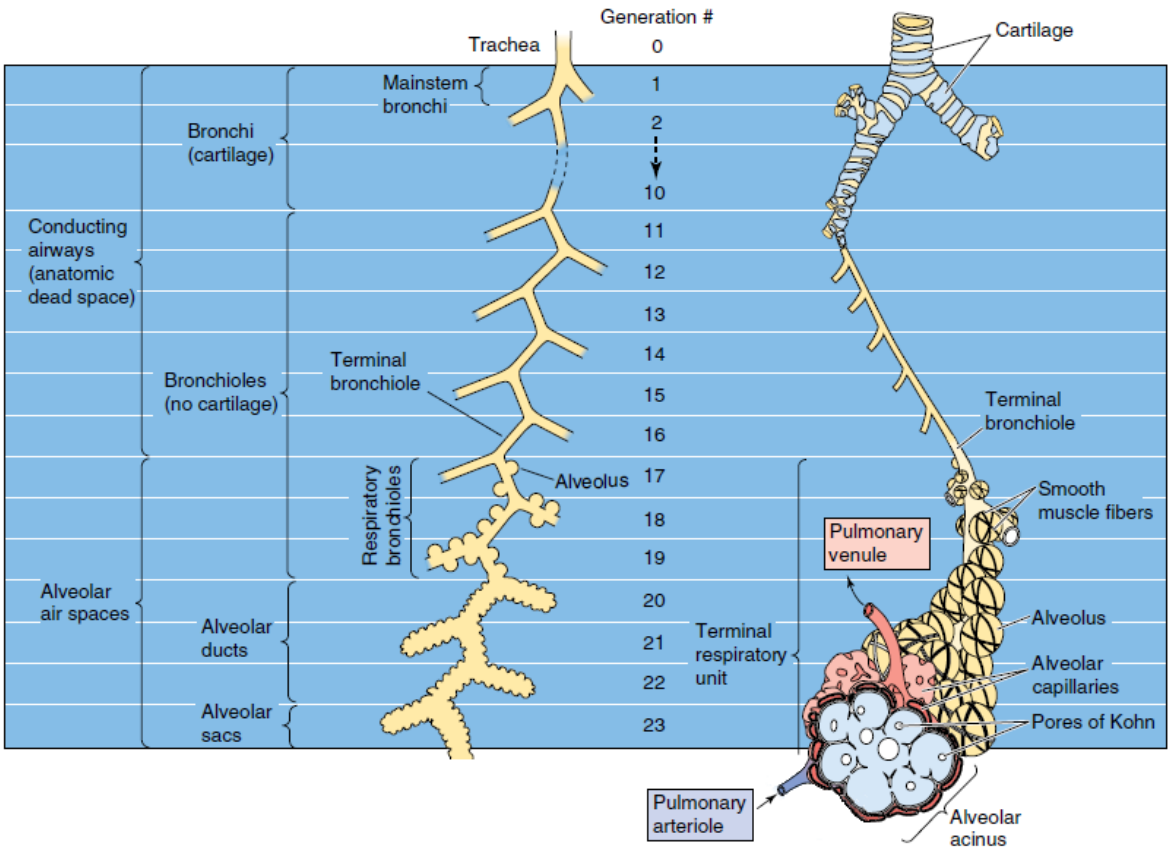


Figure 1.1: The structure of the airways

The human airways are composed of a series of tubular structures. With each successive generation, the airways reduce in size and rigidity, and terminate with alveoli. The cartilaginous trachea and bronchi progress to the bronchioles which contain no cartilage, fewer goblet cells, and an increasing proportion of elastin fibres and smooth muscle cells. The final seven generations for the airways are composed of the alveolar air spaces, which are highly vascularised, and provide a vast (approximately 70 m^2) surface area for gas exchange.

Figure modified from (2).

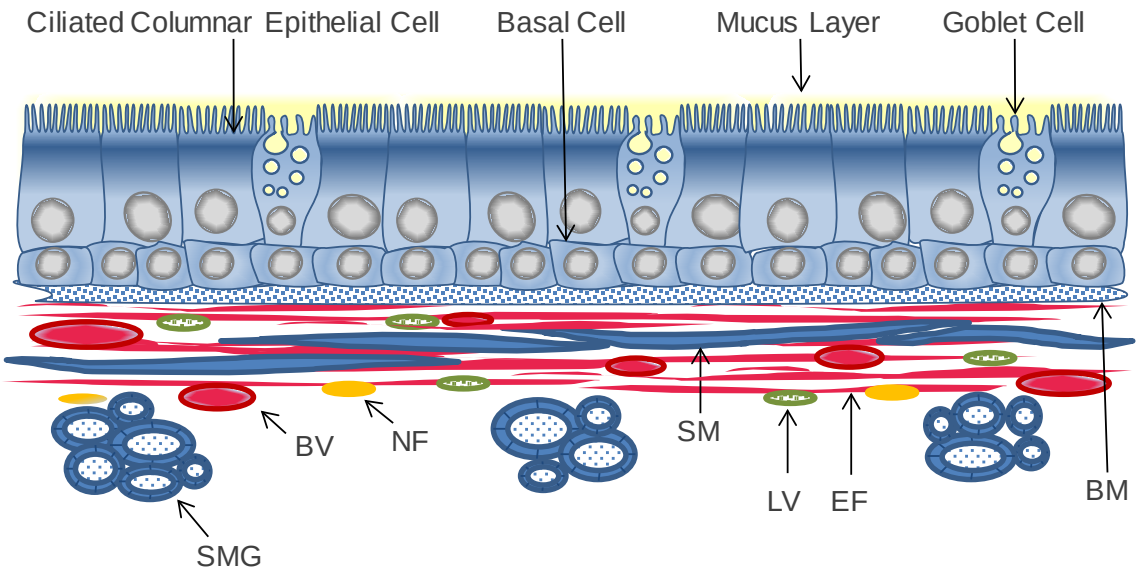


Figure 1.2: The airway epithelium and underlying structures

The airway epithelium is a pseudostratified layer of tightly bound ciliated columnar cells, mucus secreting goblet cells, and basal progenitor cells. A viscoelastic mucus blanket is propelled by co-ordinated ciliary beating towards the pharynx. The epithelium is supported by a fibrous basement membrane (BM), which overlies the structures of the mucosa and submucosa including blood vessels (BV), lymph vessels (LV), smooth muscle (SM), nerve fibres (NF), elastic fibres (EF), and submucosal glands (SMG). In the bronchioles, which lack lymph vessels and glands, the epithelial becomes cuboidal.

which is able to initiate the adaptive immune response by activating dendritic cells (DC) (9, 10).

In the normal state, the AE is highly resilient, exhibiting a remarkable capacity to regenerate through the plasticity of neighbouring AEC, most of which are able to serve as stem cells when the need arises (11). Consequently, the integrity of the AE is of central importance in the context of respiratory diseases such as asthma, where AECs are repeatedly subject to the inflammatory milieu. Accordingly, the hallmark signs of asthma are now hypothesised to originate from dysfunction in the homeostatic and regenerative potential of the AE (12).

1.3 Asthma

1.3.1 Asthma is an Inflammatory Disease of the Airways

Asthma is a chronic lung disease that inflames and narrows the airways. It affects millions of individuals worldwide, and therefore represents a major global medical and socioeconomic burden. The annual cost to manage asthma is approximately 18 billion dollars in the US alone (13). Sufferers exhibit a predisposition for bronchial hyperresponsiveness (14), and unwarranted immune activity at the airways (15). Although asthma is now known to be a heterogeneous disease comprising distinct sub-phenotypes (16), these subtypes all involve chronic inflammation and narrowing of the airways, punctuated by episodes of wheezing, breathlessness and chest tightness (17).

The inflammation is thought to be driven by T_H-dependent responses to intrinsic and extrinsic triggers (15), and involves several other regulatory and effector cells including epithelial cells, mast cells, eosinophils, and neutrophils (18, 19). Inflammation is accompanied by significant proteolytic and oxidative damage to the epithelium (20). Eventually, the airways become remodelled with smooth muscle hyperplasia and extensive collagen deposition that further limit airflow. It has been estimated that genetic susceptibility contributes approximately 50% to the risk factor of asthma, with the

remainder due to environmental and life style associated factors such as pro-oxidants, allergens and respiratory viruses (21, 22).

Affected individuals manage chronic symptoms and potentially lethal episodes of respiratory dysfunction with pharmaceuticals which are not curative, require daily administration, and can cause adverse effects (23, 24). The usual treatment for persistent asthma in humans involves a combination of inhaled corticosteroid and bronchodilators to manage inflammation and smooth muscle spasm, respectively. There is however a need to develop strategies to better control other aspects of the disease.

1.3.2 The Prevalence of Asthma

There are approximately 300 million individuals currently suffering from asthma worldwide (25), and this is expected to rise to 400 million by 2025 (26). Over the past 30-40 years the prevalence of asthma has doubled, affecting 1 in 10 adults and 1 in 3 children in several countries (13). This increase was for the most part potentiated by a considerable elevation in the rate of asthma between 1980 and 1990, in Westernised countries (27).

The largest global audit of asthma in children (aged 13-14 years, from 56 countries, between 1993 and 2003) comes from the International Study of Asthma and Allergies in Childhood (ISAAC, (28)), and for adults (aged 22-44 years, from 25 countries, between 1998 and 2002) the European Community Respiratory Health Survey (ECRHS; (29)). The ECRHS determined 5.3% of the world's population suffered from asthma over the census period (30). Both studies reported an overall increase in the rate and distribution of asthma, particularly in developed countries (20 to 60-fold vs. undeveloped countries), the highest being The United Kingdom, Australia, and New Zealand (13). In contrast to previous trends, results from ISAAC also showed the prevalence of symptomatic asthma had levelled off between phase I and III measures (31). Most significantly, the asthma epidemic reported for Westernised countries between 1980 and 1990, decreased by 0.42% between phase I and III. However, recent data from the USA report a 1.1% increase in asthma between 2001 and 2010, meaning a new elevation in global asthma is expected (13).

In Australia asthma affects approximately 15% of children and 12% of adults; in addition, about 20% of elderly asthmatics may be undiagnosed (32, 33). Both the ISAAC and ECRHS surveys ranked Australia in the top three for asthma prevalence compared to other countries (13, 30). In addition, the World Health Survey conducted between 2002 and 2003 (178,215 adults aged 18 to 45) found the prevalence asthma to be the highest in Australia at 21.0%, compared to a global average of 4.3% ((26); Figure 1.3). In line with these results, a recent study by Wilson *et al.* found asthma prevalence increased markedly between 1990 and 2003 in South Australia (7.3% for females and 1.6% for males), and was attributed to increases in weight, the incidence of diabetes, and reduced exercise (34). Environmental changes and host susceptibilities are yet to explain the disproportionately large incidence of asthma in Australia and other developed countries. Rather a 'package' of risk factors found in Westernised societies, such as urbanisation and those linked to the hygiene hypothesis, are thought to be central to this trend (31).

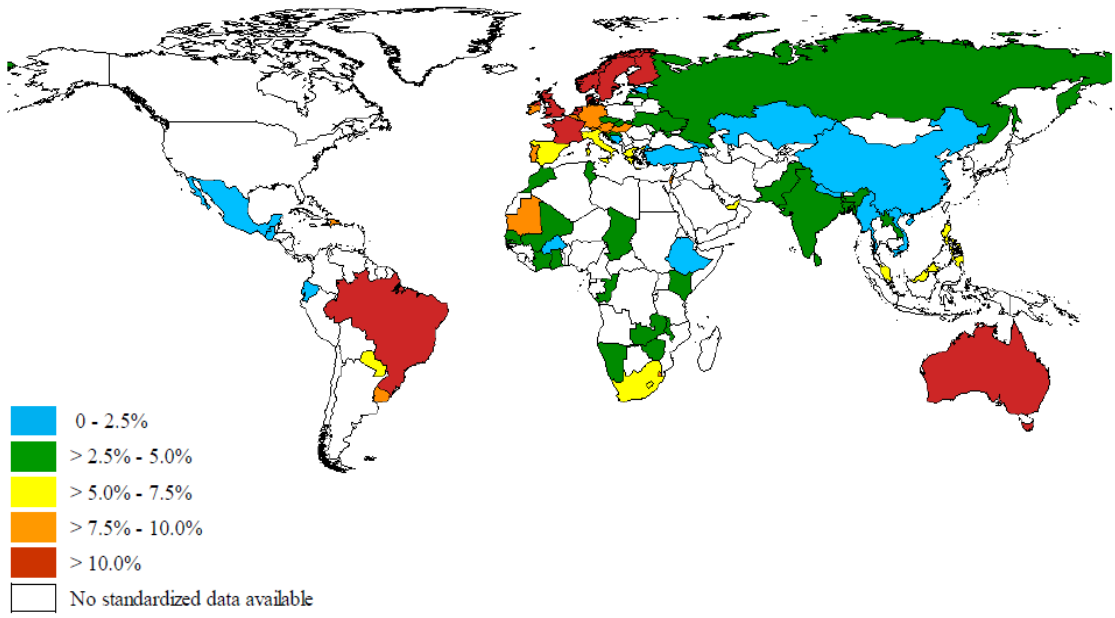


Figure 1.3: The global prevalence of clinical asthma

Figure from (26).

1.3.3 Asthma Aetiology

Asthma appears to manifest in genetically susceptible individuals as a result of exposure to risk factors including allergens, environmental irritants, and infection (35). However, a distinct cause for asthma remains unknown. A central reason for this is that asthma exhibits significant genetic and phenotypic heterogeneity (36), and its pathophysiology is influenced by complex gene-environment interactions, which are not completely understood (22, 37, 38). One theory is that childhood asthma arises as a result of exposure to respiratory viruses early in life (39). Indeed, a range of environmental and lifestyle risk factors have been associated with the development of asthma, most significantly, exposure to allergens such as cigarette smoke, pollen and microbes, obesity, exercise, excessive hygiene, and preterm birth (13, 40, 41). However, genetic factors are known to contribute over 50% to asthma risk (22). This indicates heritable traits are a central determinant of asthma, and extrinsic factors may play a more important role in triggering and perpetuating the disease (21).

1.3.4 Asthma Genetics

Genetic studies have become an important tool for unravelling the basis of disease. A genetic component to asthma has been well established in twin studies, and by its disease aggregation in familial groups (42), with heritability estimates ranging from 33-91% (43-47). Many genes have been associated with asthma (48), and susceptibility linked with phenotypes including elevated IgE, bronchial hyperresponsiveness, and eosinophilia (35). However, the identification of an underlying genetic susceptibility for asthma remains elusive. This is for the most part due to a range of complexities surrounding the nature of asthma genetics (49, 50). For example, gene-environment interactions which potentiate asthma, provide a significant challenge for identifying gene networks responsible for its pathogenesis (37). Asthma also exhibits significant genetic heterogeneity, whereby one of a number of genetic polymorphisms maybe associated with a single disease-related phenotype (51, 52). In addition, some phenotypes commonly related with asthma, such as elevated serum IgE and eosinophilia, are not shared by a significant proportion of asthmatics (53, 54). Consequently asthma is now recognised as a complex genetic disease (55), and the identification of underlying genetic susceptibility is ongoing.

Over the past decade the discovery of asthma candidate genes increased from approximately 100 in 2007 (56), to over 715 in 2011 (48). This is for the most part due to improved methods for analysing polymorphisms in genomic DNA (gDNA). There are several methods for elucidating susceptibility genes in diseases such as asthma, each with distinct advantages (reviewed in (55) and (36)). Broadly they include, positional cloning, candidate gene studies, genome wide association studies, and more recently whole genome sequencing (49). However, for the most part these techniques exploit the presence of single nucleotide polymorphisms (SNP), to track segregation patterns and to identify susceptibility genes (57).

Chromosomal regions found to encode a disproportionately high number of asthma susceptibility genes include, 2q33, 4q, 5q12, 5p15, 5q23-31, 6p21-24, 11q13-21, 12q12-24, 13q12-14, 16q21-23, 17q12-21and 19q (36, 55, 56). The most consistently replicated regions include 5q23-31, 5p15 and 12q14-24.2 which encode asthma susceptibility genes *IL-3*, *IL-4*, *IL-5*, *IL-9*, *IL-12β*, *IL-13*, *IFNγ*, *iNOS*, and *FCεRIβ* (58). Although a number of asthma candidate genes have been identified, many still require replication in independent studies, or await functional assessment (56). Genes related to the pathogenesis of asthma which have been validated in multiple studies include *IL-4*, *IL-13*, *ADRB2*, *TNFα*, *HLA-DRB1*, *HLA-DQB1*, *FCεRIβ*, *IL-4RA*, *CD14*, and *ADAM33* (59). The challenge for asthma genetics is to identify polymorphisms in a network of genes which are predictive for disease, and can be applied to individuals with different genetic backgrounds.

1.3.5 The Pathophysiology of Asthma

1.3.5.1 Asthma is driven by T helper cell inflammatory responses

Until recently, the pathogenesis of asthma was primarily attributed to T_H2 immunological response in the airways characterised by inflammation, eosinophilia, and increased serum IgE. Varying complexities of the T_H2 response have been proposed (e.g. Figure 1.4.A). Central to this is model, the exposure of allergens to naïve helper T-cells (T_H0) through the major histocompatibility complex class II molecules of antigen presenting cells (APC), such as dendritic cells (14), leads to differentiation of the T_H2 pattern of immuno-surveillance (36). T_H2 cytokines (e.g. IL-4, -5, and -13) promote the recruitment and

activation of eosinophils, B-cells, and mast cells, which secrete further factors to produce the hallmark signs of asthma, such as airway remodelling (60). Allergen sensitisation is mediated by a pool of allergen specific memory $CD4^+$ T-cells (and B-cells), which accumulate in the lymphoid tissues, and require a significantly reduced level of DC interaction to produce a potent response (21). In addition, destruction of the airway epithelium allows a larger load of allergen to activate cells of the adaptive immune response (21). Continued, unresolved inflammation produces marked changes to the airways such as neovascularisation, myocyte hypertrophy, and submucosal gland hypersecretion (Figure 1.5) (51, 60).

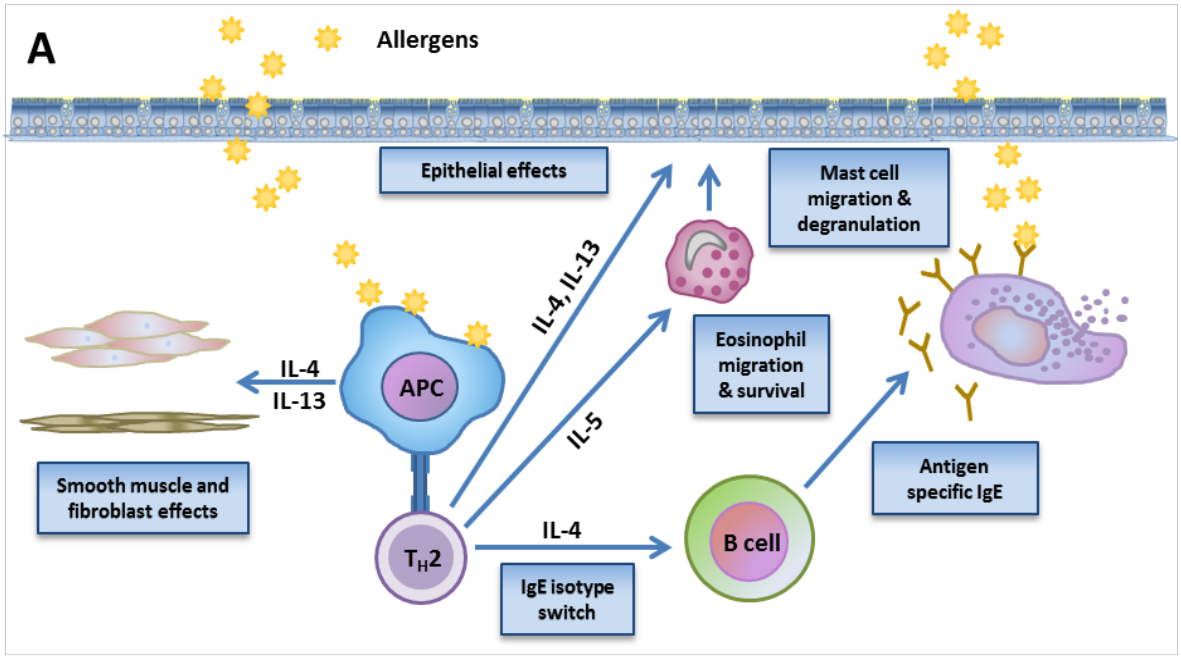
However, increased attention on the heterogeneity of asthma, has led to the recognition of asthma as a disease of overlapping phenotypes. In many cases (approximately 50%), asthma exhibits a T_H1 (or T_H17) inflammatory responses with minimal eosinophilia or serum IgE, and pathogenesis is more strongly influenced by the innate immune response (Figure 1.4.B) (reviewed in (16) and (6)). An individual is diagnosed with clinical asthma when they exhibit airway hyperresponsiveness and inflammation, with reversible airway obstruction (61, 62). As the inflammation is rarely assessed, this clinical definition does not distinguish the T_H1 and T_H2 phenotypes, both of which produce the hallmark signs of asthma, such as airway remodelling (16). The T_H1 mode of inflammation is prevalent in the non-allergic forms of asthma, particularly in corticosteroid refractory adults, and is characterised by neutrophilia and secretion of the pleotropic cytokine Interferon-γ (IFNγ) (16).

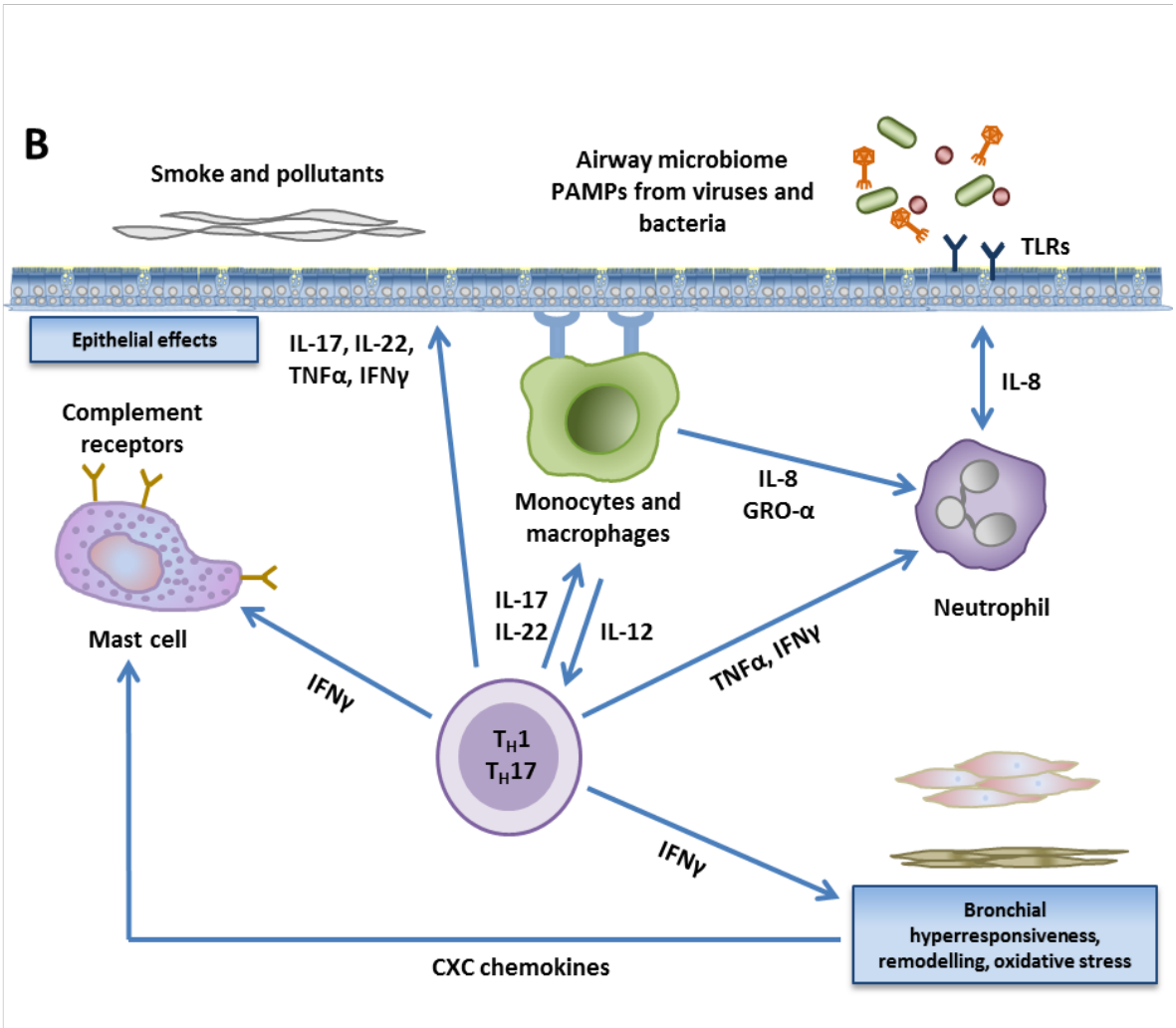
Attributing the pathogenesis of asthma to any one T_H response over-simplifies a complex disease. The cytokine profile expressed by T_H cells can be modulated by the inflammatory milieu, whereby T_H-17 (produce IL-17 and IL-22) cells can express both T_H1 and T_H2 cytokines (63, 64), and T_H1 cells can be modified to secrete IFNγ, and T_H2 cytokines such as IL-4, IL-5, IL-13 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (65, 66). Adding to the complexity, a new subset of T-cells which secrete IL-9, T_H9 cells, have been shown to drive mucus production, subepithelial fibrosis, and mast cell development (6). The activity of multiple T_H responses may explain the destructive changes observed in the airways of severe asthmatics (67).

Figure 1.4: Helper T cell-regulated immunological responses in asthmatic airways

The T_H pathways produce inflammation in the airways of asthmatic individuals. **A**: Antigen presenting cells (APC), exposed to allergens, interact with naïve helper T cells, leading to differentiation of the T_H2 linage. The major cytokines of the T_H2 response, IL-4, IL-5 and IL-13, recruit further inflammatory cells, which cooperate to cause airway remodelling by effecting smooth muscle, fibroblasts and epithelial cells in the airways. The inflammation is characterised by the presence, and sustained activity of eosinophils. **B**: In this model, T_H1 and T_H17 responses are initiated by environmental irritants, and pathogen-associated molecular patterns (PAMP), which interact with Toll-like receptors (TLR). This form of inflammation is characterised by the activity of IFNγ, neutrophilia, and smooth muscle and fibroblast effects. This form of inflammation is common to severe asthmatics, and strongly influences bronchial hyperresponsiveness and airway remodelling. IFNγ, Interferon-γ; TNFα, Tumour necrosis factor-α; GRO-ɤ, Growth regulated oncogene-α.

Figures adapted from (16) and (6).


A
Allergens
Epithelial effects
Mast cell migration & degranulation
IL-4, IL-13
Eosinophil migration & survival
IL-4
IL-13
APC
IL-5
Smooth muscle and fibroblast effects
Antigen specific IgE
IL-4
B cell
TH2
IgE isotype switch


B
Smoke and pollutants
Airway microbiome PAMPs from viruses and bacteria
TLRs
Epithelial effects
IL-17, IL-22, TNFα, IFNγ
IL-8
Complement receptors
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IL-8 GRO-α
Neutrophil
Mast cell
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IFNγ
TNFα, IFNγ
TH1 TH17
IFNγ
Bronchial hyperresponsiveness, remodelling, oxidative stress
CXC chemokines

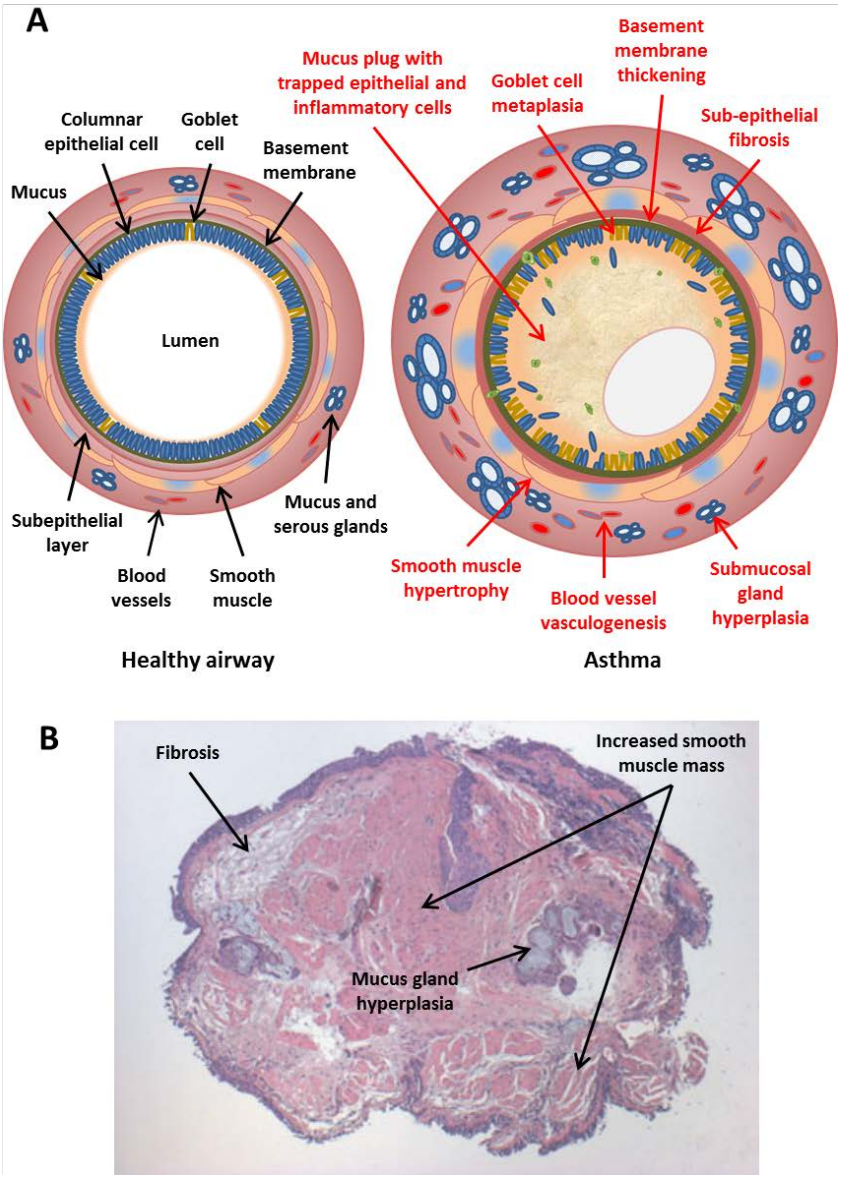

Figure 1.5: Airway remodelling in asthma

A: A cross section, schematic representation comparing a non-asthmatic and asthma-affected airway. Airways of asthmatic individuals exhibit excessive mucus in lumen, due to goblet cell metaplasia and hyperplasia of submucosal glands. Prolonged infiltration and activity of inflammatory cells remodel the airways, and is characterised by smooth muscle hypertrophy, epithelial destruction, and fibrosis of the basement membrane and subepithelial layer. **B**: Histological assessment of a bronchiole from a severe asthmatic displays the hallmark signs of airway remodelling, including fibrosis, smooth muscle hypertrophy, and hyperplasia of mucus glands. Figure 1.5.B is from (48).

Hence, a range of inflammatory cells participate in the development of asthma, and give rise to similar pathological features. This raises questions of whether a cell, or group of cells, is fundamental to disease pathogenesis. Over the past decade, the cells of the airway epithelium have come under intense scrutiny to determine whether they play a unifying role in the development of asthma.

1.3.5.2 The airway epithelium is involved in the pathogenesis of asthma

It was not until the mid-1990's that the AE was described as a site which can potentiate asthma (68). A number of proinflammatory factors are now known to be produced by activated AECs in the context of asthma such as IL-5 (64), IL-33 (69), and TSLP (70). Indeed, AECs are central effectors of asthma pathogenesis (reviewed in (71)), and epithelial damage and dysfunction is associated with aspects of the disease shared by the majority of sufferers (72). Currently, AE potentiation of asthma is thought to stem from a fragile phenotype characterised by (for example) deficits in tight junction (TJ) formation (65), and an immature, injury susceptible epithelium (66). Continued repair and injury sustains the activation of the epithelium, prolonging the release of cytokines and growth factors which contribute to the disease (73).

As previously stated, the AE is capable of efficient repair. When the AE is damaged, epithelial cells interact with multi-potent mesenchymal cells to promote rapid regeneration, establishing a region termed the epithelial-mesenchymal tropic unit (EMTU; (74)). Among other activities, the EMTU facilitates the function of the surrounding cells such as myocytes, and fibroblasts, to drive rapid re-epithelialisation (21). Conversely, injury to the fragile epithelium of asthmatics heals slowly, and AECs over express markers of activation including CD40, Epidermal growth factor receptor (EGFR) and TLRs (67). Functional disturbance in the EMTU is hypothesised to perpetuate a cycle of damage and aberrant repair, causing prolonged AEC activation, and inappropriate secretion of immunomodulatory agents (10, 75). Histological examination of the epithelium from asthmatics shows interruptions in the epithelial barrier, as a result of unresolved damage and premature apoptosis (Figure 1.6). Adding to this, accelerated turnover of AECs increases the number of undifferentiated, 'immature' cells, exposed to the environment, which provide a new source of injury susceptible epithelial cells (66). Consequently,

models which place a chronically injured epithelium at the forefront of asthma pathophysiology have been convincingly linked with bronchial hyperreactivity, infiltration of inflammatory cells, and airway remodelling (9, 10, 76).

The production of TSLP by AECs represents an important linkage between the adaptive and innate immunological responses in asthma. TSLP is secreted into the underlying pulmonary tissues by activated AECs at the EMTU, and occurs after engagement of TLRs with antigens such as products from bacteria and viruses (77) (Figure 1.7). TSLP upregulates the expression of co-stimulatory molecules (CD80 and CD40) on DCs (78) which activate T_H2 cells. This induces the production of cytotoxic $CD8^+$ T-cells, capable of secreting IL-4 and IL-13 (67, 77). Epithelial cell-derived TSLP also promotes maturation of pre-B-cells, and increases the production of proinflammatory cytokines in mast cells (71). In addition, TLR engagement causes the production of further proinflammatory cytokines, adhesion molecules, and β-defensins (79). Hence, AECs have the potential to initiate and maintain a cytokine pattern capable of supporting chronic inflammation, independent of prior T-cell involvement. This may, in part, provide some explanation for the limited effectiveness of current therapeutics which primarily target inflammatory cell in asthmatic airways (71).

Hence, the airway epithelium can be implicated with the development of asthma, and its prolongation by initiating the innate and adaptive immunological responses. Impairment of the epithelial barrier function potentiates the disease further, by exposing the mucosal layer to allergens, and disrupting mucociliary clearance (80, 81). Hence, although asthma is a heterogeneous disease, a common feature may be an activated and injury susceptible epithelium (21, 71).

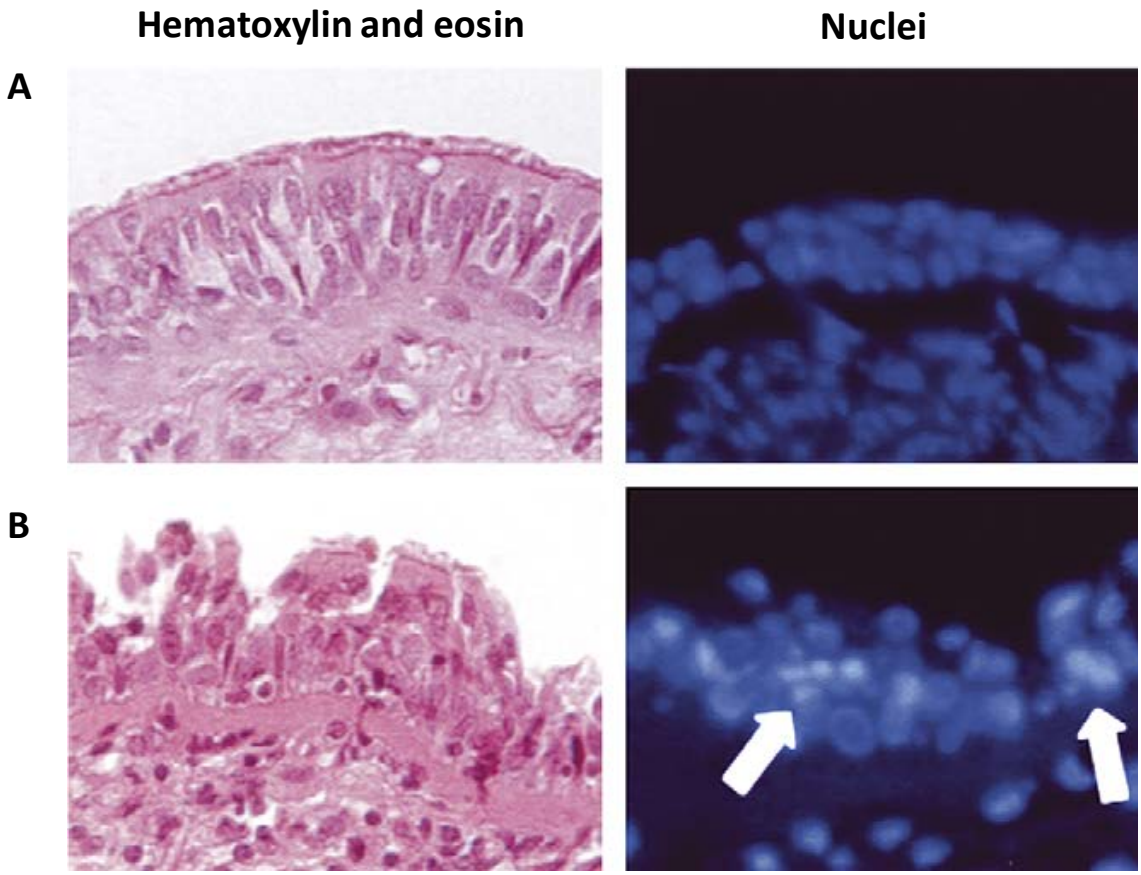

Figure 1.6: The bronchial epithelium in asthmatic and healthy individuals

A: The epithelium in healthy airways are highly organised, and provide a continuous epithelial cell layer. Condensed nuclei, indicative of cell death, are absent suggesting efficient clearance (efferocytosis) of apoptotic cells. **B**: Conversely, the airways epithelium in asthmatics can be damaged, producing regions where the atmosphere can contact underlying structures. Bright regions of condensed nuclei are evident (arrows) after DNA staining (Hoechst). This indicates apoptotic changes are a relatively frequent event, and apoptotic cells are not effectively removed.

Figure from (82).

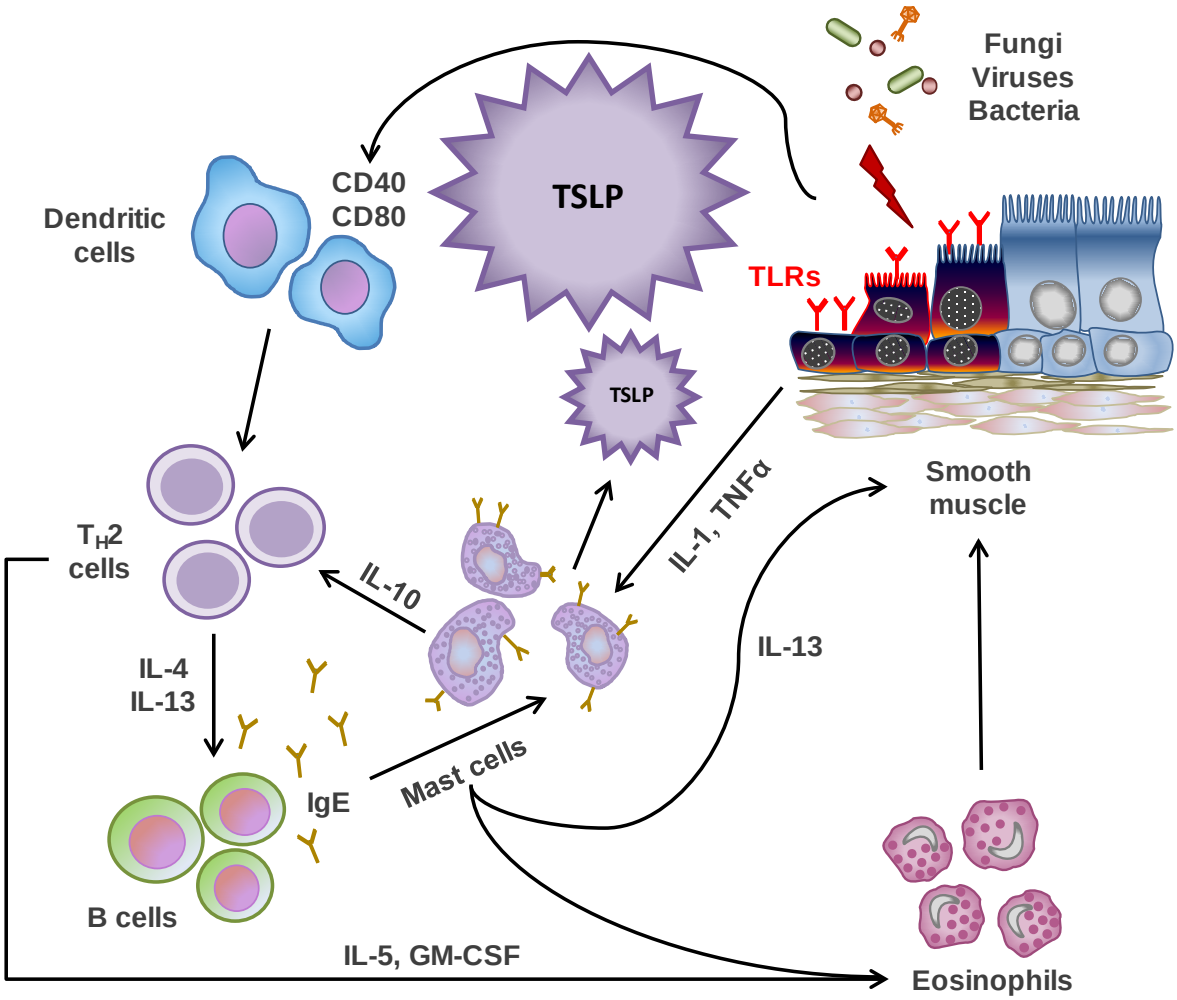

Figure 1.7: AEC-derived TSLP can initiate airway inflammation

Damaged airway epithelial cells express Toll-like receptor-2, -3, -8, -9, which interact with microbial products, leading to the secretion of Thymic stromal lymphopoietin (TSLP). TSLP induces the upregulation of co-stimulatory receptors on dendritic cells, which promote the differentiation of naïve T-cells into cells of the T_H2 linage, in the absence of antigen presentation. The ensuing inflammation is associated with the production of IgE, mast cell activation and recruitment of eosinophils, seen in the airways of many asthmatics.

Figure adapted from (71).

1.3.5.3 IFNγ and TNFα in the pathogenesis of asthma

IFNγ and TNFα are proinflammatory cytokines that are elevated in the airways of asthmatics, particularly as the disease becomes more severe and infiltrating T_H1 cells increase (83, 84). These cytokines are also well known to potentiate each other's effects through cross-talk of intracellular signalling pathways and by upregulating their respective receptors (85-87). Given that these cytokines also exhibit proapoptotic effects (discussed below), they were a focus of the investigations in this thesis.

1.3.5.4 IFNγ signalling

Effects brought about by IFNγ (type II interferon) involve modulating transcription of genes which regulate several aspects of cellular function, particularly those associated with inflammation and immunity. IFNγ is secreted by a range of cells including T_H1 cells, natural killer cells, natural killer T-cells, cytotoxic lymphocytes, B-cells and APCs (88-95). Normally, IFNγ secretion provides resistance to parasites, bacteria, and viruses (51, 60-62, 96), by influencing the immunomodulatory activities of target genes in, T_H2 cells, neutrophils, macrophages, mast cells, cytotoxic T-cells, professional and non-professional APCs, and epithelial cells (reviewed in (69)). Activation of the IFNγ-receptor (IFNγR) complex transmits signals through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, and through Interferon regulatory factor-1 (IRF-1). These signalling pathways direct the expression of IFNγ inducible genes involved in immune surveillance, antigen presentation, inflammation, and apoptosis ((69); Figure 1.8). However, it has recently been appreciated that much of the effects attributed to the activation of the IFNγR arise from cross-regulation of other signalling pathways, meaning the activity of STAT and IRF-1 cannot explain the breadth of IFNγ's influence (reviewed in (70)). This can complicate identifying clear biological consequences of IFNγ signal transduction in the context of disease, where many factors can contribute to produce multiple, intersecting signalling events. Consequently, IFNγ can either mediate or suppress disease processes such as autoimmunity, depending on variations in the relative contribution of exogenous and endogenous signalling events, cell type, and genetic factors (70).

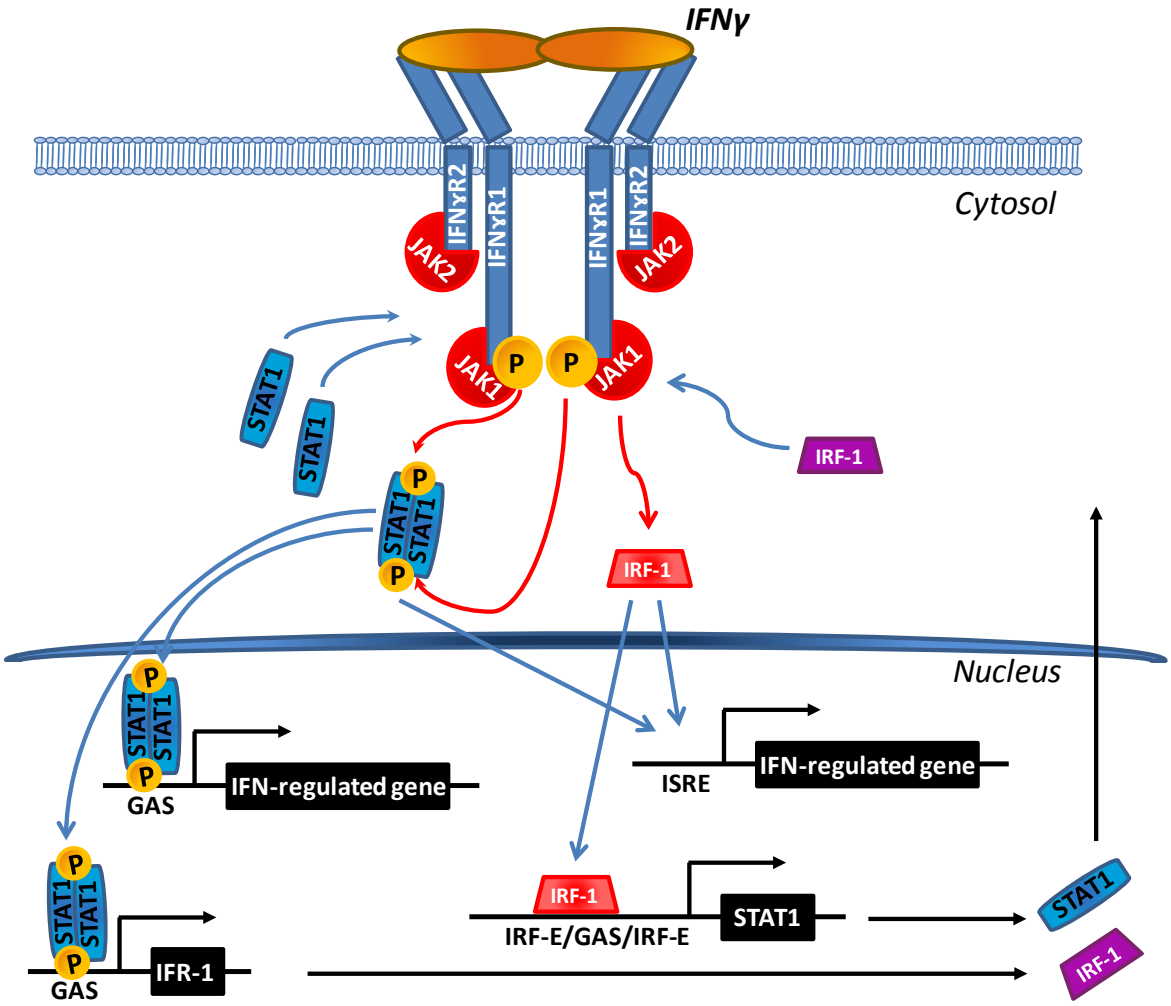

Figure 1.8: IFN-γ signal transduction

IFNγ homodimers bind the preassembled IFNγR-1 and -2 chains, leading to a conformational change in the cytosolic subunits of the receptor complex, and association of downstream signalling molecules. Transphosphorylation of Janus kinase-1 (JAK1) by JAK2 enables JAK1-P to phosphorylate dimerised STAT1. Phosphorylated STAT1 is able to dissociate from the receptor complex and translocate into the nucleus where it binds the IFNγ-activation site (GAS) promoter, triggering the transcription of interferon-regulated genes, including transcription factors such as IRF-1. IRF-1 is also activated by the IFNγ receptor complex, and like STAT1, is able to bind IFN-stimulated response elements (ISRE). Additionally, IRF-1 is able to bind the IRF-E/GAS/IRF-E element, which is a positive regulator for the production of further STAT1 transcripts. There are over three hundred IFNγ-regulated genes, and the relative contribution of their influences directs the cellular response.

Figure adapted from (69, 70).

1.3.5.5 The role of IFNγ in asthma

Accumulating evidence demonstrates that T_H1 cytokines play a critical role in the development of asthma, and therefore implicate IFNγ as an influential endogenous mediator of asthma pathogenesis. IFNγ is increased in the lungs of asthmatics, where it promotes mast cell responsiveness, neutrophilia, macrophage activation, and degenerative changes to the airway epithelium (6, 16, 19, 84, 87, 97). Several studies have identified IFNγ as an important cytokine contributing to asthma in the scope of their investigations. Work by Kumar *et al.* showed the activity of IFNγ contributed to activation of pulmonary macrophage, airway hyperresponsiveness, and steroid resistance, independent of the T_H2 cytokines (87). In another example, mice challenged with lipopolysaccharide and OVA developed bronchial hyperresponsiveness (BHR) and neutrophilia after administration of IFNγ-secreting T_H1 cells (98). In addition, genetic studies of IFNγ and IRF-1 polymorphisms have reported significant association with susceptibility to both asthma, and disease severity (99, 100).

The interaction of IFNγ with the T_H2 cytokine response has been shown to either oppose, or potentiate the effect of T_H2 cells, a situation which likely reflects differences between murine models of asthma (discussed in (84)). IFNγ may be increased in asthmatics who exhibit a T_H2 cytokine profile (101), which supports a multiple T_H cytokine model in human disease. Furthermore, IFNγ has been shown to contribute to pathogenesis and disease severity in conjunction with IL-4, IL-5 (102), and IL-13 (103). An elegant study by Yu *et al.* compared a murine model of chronic asthma, with mast cell deficient mice, and showed IFNγ potentiated both eosinophilic and neutrophilic responses in a mast cell-dependant manner (84). Hence, IFNγ has a role in several aspects of asthma pathogenesis, and its effects are moderated by the local cellular repertoire, and cross-talk with other signalling pathways.

Surprisingly, there are relatively few investigations which directly examine the effect of IFNγ engagement with AECs in the context of asthma. Most of INFγ's influence with

regard to AEC has been attributed to cytotoxic effects of IFNγR engagement, which are expanded on in subsequent sections.

1.3.5.6 TNFα signalling

TNFα is a pleotropic cytokine, noted for its potent proinflammatory influences, and participation in complex signalling pathways which produce a number of biological consequences, the breadth of which are still not fully understood (88). TNFα is produced as a transmembrane protein, but is also secreted (when cleaved from the membrane by TNFα-converting enzyme) by T_H1 cells, macrophages, mast cells, and neutrophils (88, 89, 104). The TNFα-receptor (TNF-R) is expressed on most nucleated cells, and it's cytosolic 'death domains' allow recruitment of (for example) Fas-associated death domain (FADD), and TNF receptor–associated death domain (TRADD). While bound to the TNF-R, TRADD displays docking sites for adapter proteins such as Receptor interacting protein (RIP), and TNF receptor-associated factor 2 (TRAF2), as depicted in Figure 1.9. This signalling complex ultimately leads to activation of Mitogen-activated kinase (MEK), and Mitogen-activated protein kinases (MAP kinase; e.g. p38-MAPK and c-Jun N-terminal kinase), and the formation of the IκB kinase (IKK) complex which facilitates the translocation of NF-κB to the nucleus (90, 94). MAPK and NF-κB are influential modulators of inflammation and cell survival, and are responsible for much of TNFα's proinflammatory influences (93, 94). Apoptosis as a result of TNF-R engagement is in part mediated by FADD, which after associating with the TNF-R complex, is able to recruit and promote autolytic activation of procaspase-8, thereby forming the death inducing signal complex (DISC; (105)). The DISC is negatively regulated by (for example) Cellular inhibitor of apoptosis-1 (cIAP1) and cIAP2, which are recruited to TRAF2, and the activity of FLICE-like inhibitory protein (FLIP; (90, 105)).

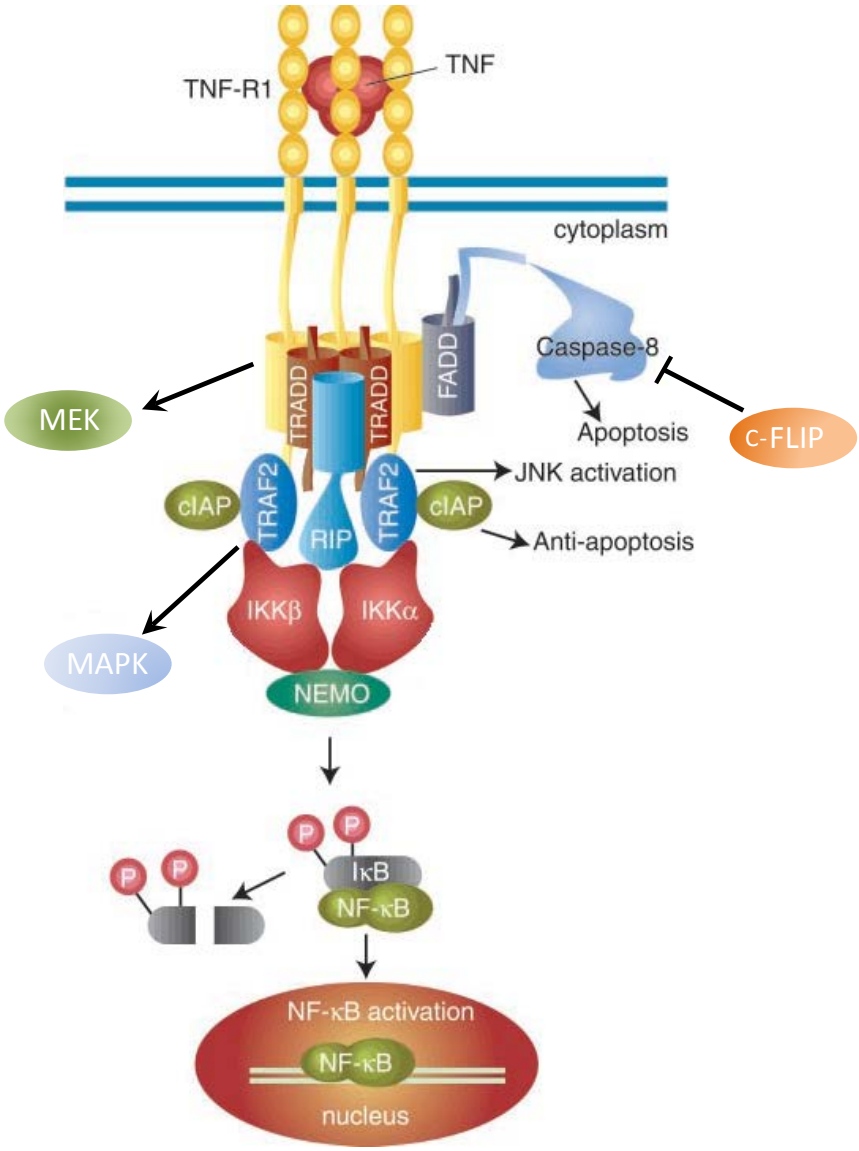

Figure 1.9: TNFα signal transduction

Engagement of soluble TNFα to the TNF-R promotes the recruitment of the adapter proteins TRADD, TRAF2, FADD and RIP to the receptor intracellular domains. The adapters provide docking sites and phosphorylation activity which activate (for example) Mitogen-activated kinase (MEK), NF-κB, Mitogen-activated protein kinases (MAPK), and c-Jun N-terminal kinase (JNK). Formation of the IκB kinase complex composed of IKKα, IKKβ and NEMO is responsible for NF-κB activation, which directs the transcription of genes involved in inflammation. The death inducing signal complex is formed by the association of the TNF-R, Fas-associated protein with death domain (FADD), and procaspases-8, leading to the activation of the caspase cascade and apoptosis. The cIAPs and Cellular FLICE-like inhibitory protein (c-FLIP) operate at the receptor complex to inhibit caspase-8 mediated apoptosis. Figure adapted from (90).

1.3.5.7 The role of TNFα in asthma

TNFα is a potent proinflammatory cytokine that influences many aspects of the inflammatory response (Figure 1.10). TNFα is a master regulator of proinflammatory cytokine production, activation and recruitment of inflammatory cells, and is linked with the pathogenesis of a number of chronic inflammatory disorders such as asthma, rheumatoid arthritis, psoriasis, and Crohn's Disease (106-108). In asthma, TNFα has been shown to be elevated systemically in the airways, particularly in severe, refractory asthma, characterised by the presence by monocytes, macrophages, and T_H1 and T_H17 cells (89, 109, 110). TNFα it is also involved in the recruitment and activity of eosinophils (111). Indeed, in mouse models of allergic asthma, TNFα mediates an increase in the abundance of neutrophils, eosinophils, and activated T-cells in the airways (89). There are a number of other asthma-related cytokines which are produced in response to TNFα signalling including (but not restricted to) IL-8, IL-6, IL-1β, GM-CSF, Transforming growth factor-α (TGFα) and eotaxin (111, 112).Common asthma-related phenotypes associated with TNFα activity include BHR, airway remodelling, corticosteroid-resistance, and severe exacerbations of the disease (89, 104, 113).

Hence, TNFα is a major cytokine that influences several characteristics of the asthma paradigm, particularly in refractory disease. In support of this, genetic polymorphisms in the gene encoding TNFα correlate with an increased susceptibility for asthma and elevated serum-TNFα (114-116). Although TNFα plays an influential role in airway inflammation, treatment modes targeting TNFα for the management of asthma have met with limited success, and likely reflects varying influences of the sub-phenotypes which potentiate the disease (113).

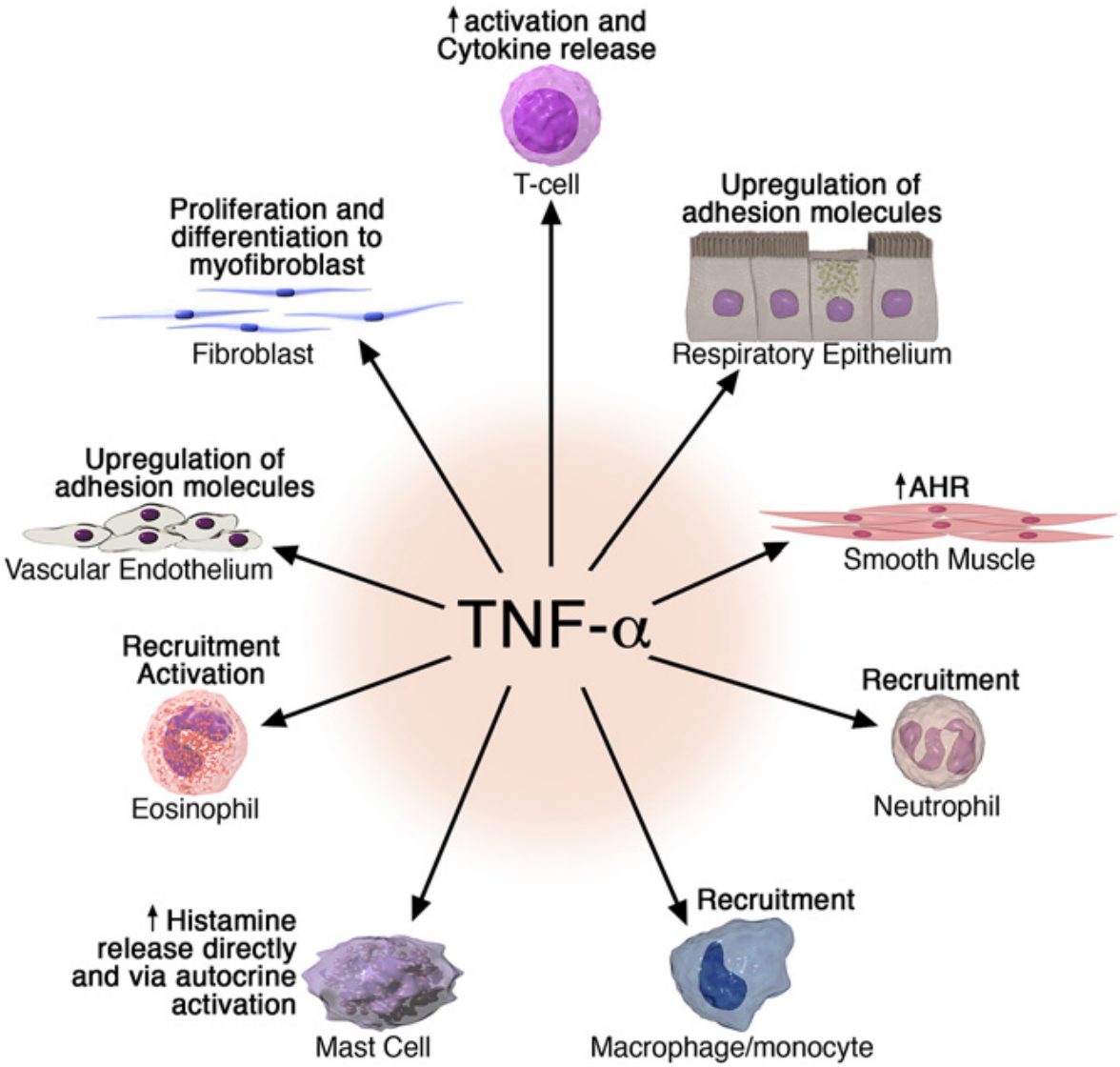

Figure 1.10: Disease-related roles of TNFα in asthma

Figure from (113).

1.3.6 Current Asthma Treatments

Management plans for asthma rely on accurate diagnosis, follow up tests to confirm diagnoses, and the administration of bronchodilators and steroid-based preventative therapeutics (described in (117)). Changing daily activities and other steps to avoid allergens, and immunotherapy to desensitise individuals to specific allergens, can ameliorate asthmatic responses, but have limited benefits (118). Usually the first treatment mode for persistent asthma is the administration of inhaled corticosteroids (ICS; e.g. fluticasone proprionate), which suppress the expression of inflammatory genes including cytokines, inflammatory enzymes, adhesion molecules, and inflammation-associated receptors (118). Treatment for poorly controlled asthma can be supplemented with a long acting β-agonists (e.g. Salmeterol) or leukotreine modifiers (51). For the most part, steroid-based, and combination therapies with long-acting β-agonists, are the mainstay of asthma treatment (89). These treatment modes are not curative, and many individuals become resistant to their effects. Indeed, corticosteroid-refractory asthmatics represent 10% of all sufferers and account for 30% of total costs in asthma-related treatment in the USA (119). The effects of corticosteroids are broad and non-specific, and generally target only the T_H2 form of the disease (16). Hence, the heterogeneity of asthma present challenges for the discovery of treatment modes that are applicable for all asthmatics (16).

To counter these issues a number of molecular agents targeting proteins critical to asthma pathogenesis have been developed (reviewed in (120, 121)). They include, antibodies directed to CD4 (Keliximab and Clenoliximab), TNFα (Infliximab), α-4-intergrin (Natalizumab) and IgE (Omalizumab), and inhibitory peptides which block IL-4 (Nuvance) (122). There are also synthetic peptides being developed such as the anti-inflammatory IL-10, and IL-2 which maintains T-cell and eosinophil anergy (122). Omalizumab has been successfully applied to one third of patients with persistent asthma despite aggressive pharmacotherapy (71). However, the great majority of other new therapeutics have limited clinical effectiveness and often target signalling pathways with other important functions (122, 123). There is a need for novel, curative therapeutics targeting other sites which potentiate the pathogenesis of asthma. The recent discovery of the importance of the AEC in asthma pathogenesis, may help direct the development of

novel treatment modes which could complement current therapeutics (reviewed in (12)). This has begun with the development of anti-TSLP therapeutics (124), which if successful, may present the vanguard of treatment modes targeting an epithelial origin of the disease. Another important aspect of AEC biology in asthmatics is their propensity to undergo apoptotic changes, which may be fundamental to the disease-related phenomenon presented by the epithelium.

1.4 Apoptosis

1.4.1 Apoptosis and the Caspases

Apoptosis is a highly regulated and non-inflammatory form of cell death, which is vital for normal homeostatic regulation, particularly during development and for clearance of potentially harmful cells (125, 126). Apoptosis occurs through a process of programmed cell death, which like autophagy, is a genetically determined activity (127). The apoptotic phenotype is characterised by the activity of a closely related family of aspartate-specific proteases called caspases (Figure 1.11), which cleave hundreds of substrates throughout the cell (128). Although there are other forms of caspase-independent apoptosis (reviewed in (129)), caspase-activation is responsible for most of the phenotypes associated with apoptosis (Figure 1.12) (130).

The caspases involved in apoptosis (the others are involved in inflammation) are present in viable cells as proenzymes and participate in two primary signalling cascades. The intrinsic apoptosis pathway initiates when cell death signals cause the release of cytochrome *c* from the mitochondria, which activates the procaspase form of caspase-9 via the formation of a multiprotein complex called the apoptosome (131). The extrinsic pathway is initiated by the activation of the death receptors of TNF family, resulting in the recruitment of procaspase-8 to the DISC. This recruitment occurs via binding of caspases to an adaptor protein, FADD and results in the activation of caspase-8 (131). Once activated, the upstream (initiator) caspases directly, or indirectly trigger the activation of effector (or executioner) caspases-3 and -7. These then cleave critical cellular proteins that

begin the process of dismantling the cell into apoptotic bodies, which are cleared by macrophages or other cell types (132). It is now understood that overcoming the negative regulation of caspases may be an important event in the cell death process (133).

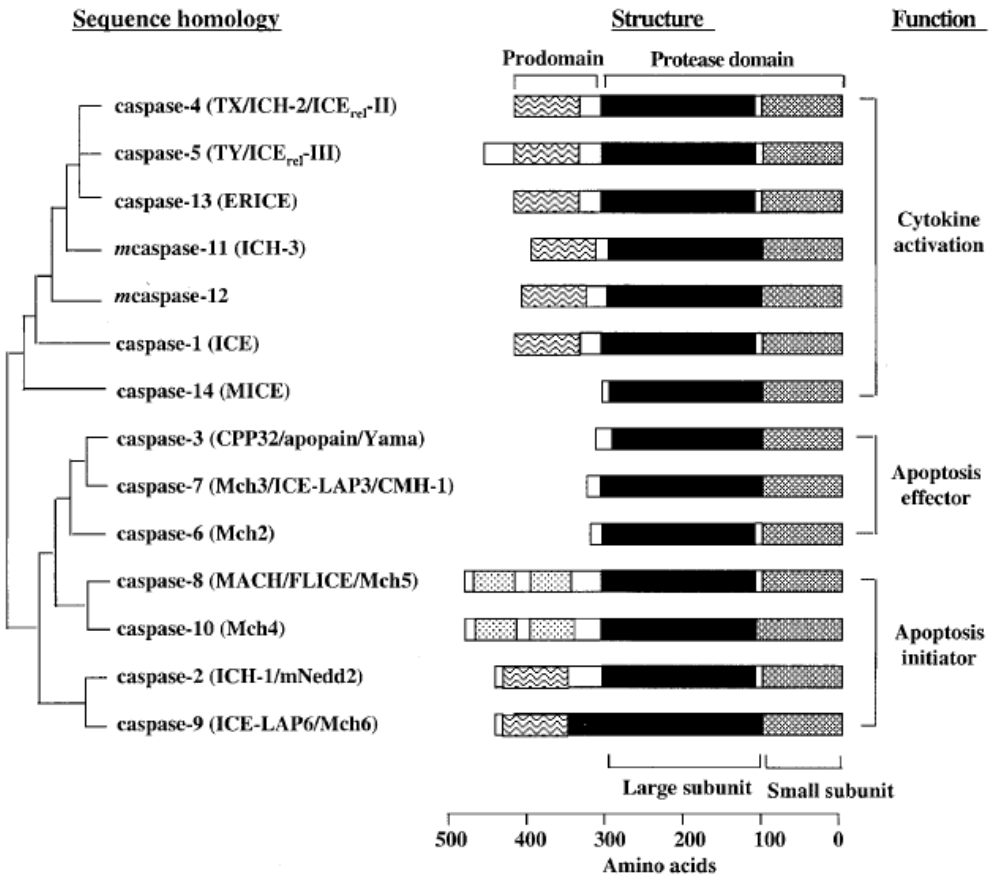

Figure 1.11: The mammalian family of caspases

Prior to cleavage (i.e. activation), procaspases exist as zymogens, composed of a prodomain, large subunit, and small subunit. The large and small subunits dimerise after procaspases are cleaved, and dimers-dimer pairs form the active caspases tetrameter. Many caspases are also involved in proteolytic maturation of inflammatory cytokines. In addition to the caspases found in humans, murine caspase-11 and -12 are shown, which are not found in *Homo sapiens*.

Figure adapted from (134).

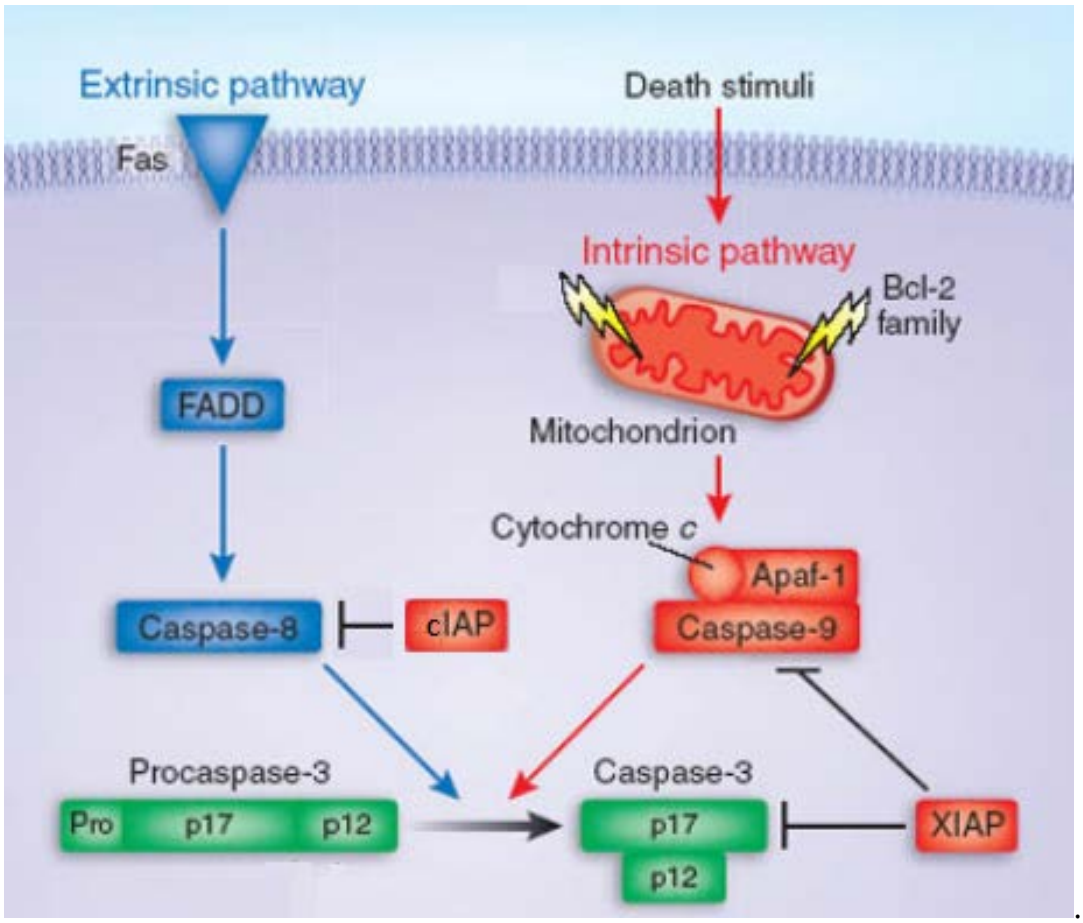

Figure 1.12: The pathways to apoptosis and procaspase-3 processing

The extrinsic pathway activates after death receptor engagement with ligands including Fas-ligand, TNFα, and TNF-related apoptosis-inducing ligand. The death inducing signal complex (DISC) forms around the intracellular receptor domain by the association of Fas-associated protein with death domain (FADD), with caspase-8. Caspase-8 can be inhibited by the cIAPs while in the DISC. The intrinsic (mitochondrial) pathway is initiated by cellular stress, such as DNA damage and irradiation. Depolarisation of the mitochondria leads to the release of cytochrome *c*, which enables the association of Apaf-1 with caspase-9. The resulting complex is called the apoptosome. Mitochondrial depolarisation is inhibited by Bcl2, and potentiated by Bax (not shown). Both the DISC and the apoptosome potentiate the processing of executioner caspases such as procaspase-3, through the activity of caspase-8 and caspase-9, respectively. Caspase-3 is formed when initiator caspases cleave procaspase-3, releasing the p17 and p12 subunits (the prodomain is released by autocatalysis). The p17 and p12 subunits form heterodimers, and two heterodimers form the active caspase-3 complex (the caspase-3 heterotetramer is not shown). Although the cIAPs have been shown to inhibit executioner caspases, this role is primarily attributed to XIAP. XIAP potently suppress caspase-9, as well as executioner caspases-3 and -7. Figure adapted from (135).

1.4.2 The Relationship Between Apoptosis, Autophagy, and Necrosis

In addition to apoptosis, the other two major forms of cell death are autophagy and necrosis. Autophagy is programmed cell death (i.e. involves gene expression), which is characterised by the formation of the autophagosome. The autophagosome is responsible for digesting organelles and protein complexes to generate adenosine triphosphate (ATP; i.e. cellular energy), and biosynthetic intermediates during periods of starvation (127). Prolonged starvation eventually leads to death due to critical depletion of cellular machinery, and is characterised by loss of organelles and the presence of autophagosome regulators phosphatidylinositol-3-kinase (PI3K) and beclin-1 (BECN1) (136). Hence, unlike apoptosis, autophagy is potentiated by distinct mechanisms, and autophagy can inhibit apoptosis through the degradation of death inducing protein complexes (137). However, autophagy and apoptosis exhibit considerable cross-talk (reviewed in (138)). For example, these forms of cell death share similar regulatory molecules including Bcl2, Atg5, caspase-8 and FADD-like apoptosis regulator (139-141). A significant question is whether these phenomena occur independently or if apoptosis switches to autophagy when caspases are inhibited or ATP is depleted (138, 142). Figure 1.13 illustrates some of the morphological features distinguishing apoptosis and autophagy.

Necrosis differs from apoptosis and autophagy in that it is an inflammatory form of cell death which results from trauma or injury. Events which lead to necrosis include ischemia (143), and prolonged exposure to reactive oxygen and nitrogen species (144). Necrosis is potentiated by a loss of plasma membrane integrity which allows influx of extracellular fluid and ions. This swells the cell and its organelles, and leads to (for example) cytosolic leakage and an overload in mitochondrial calcium levels (145). Although necrosis requires less ATP than apoptosis, it is a controlled cellular activity, as modulation of gene expression is observed during necrotic processes (146). Interestingly, factors such as Fas-receptor, TNF-related apoptosis-inducing ligand (TRAIL), and RIP1 can lead to apoptosis and necrosis (147). Many of the differences between apoptosis and necrosis are observed through alterations in the plasma membrane (Figure 1.13). While the integrity of the plasma membrane deteriorates early during necrosis, this is a late event in apoptosis.

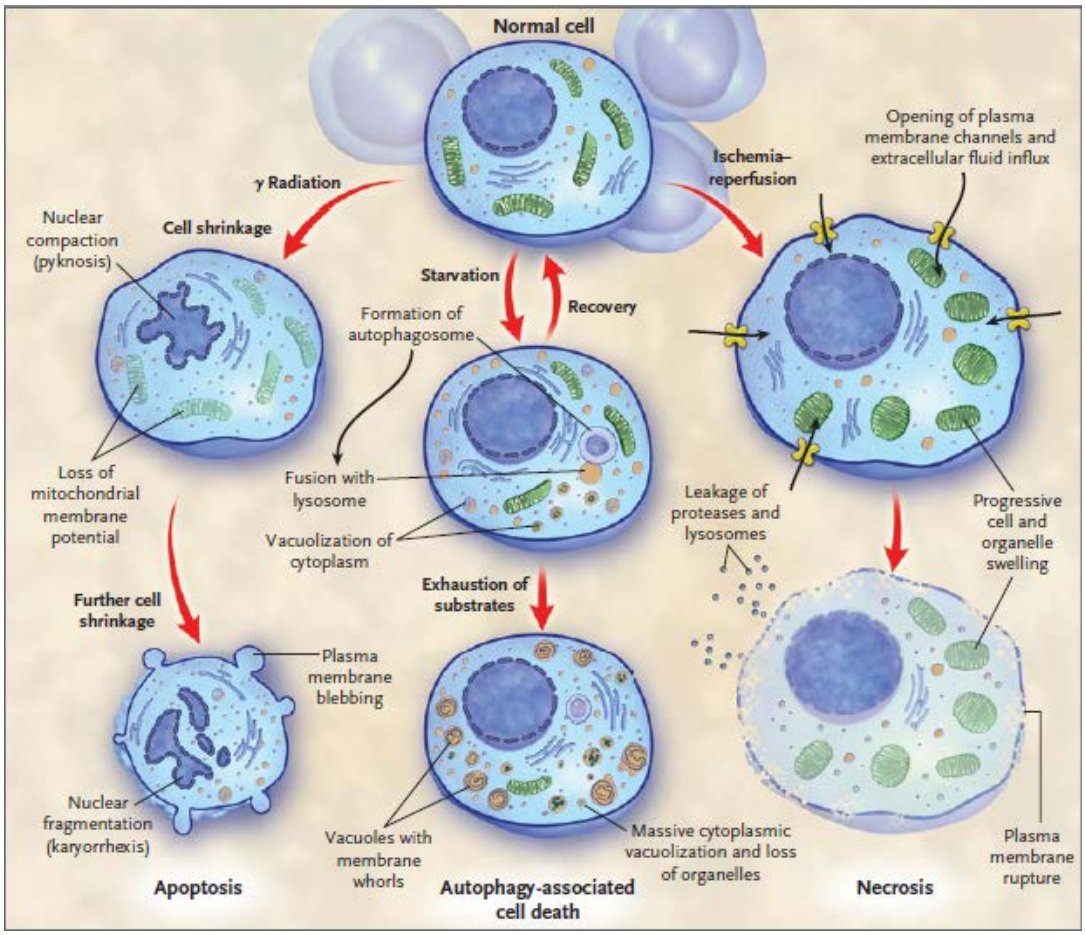

Figure 1.13: Distinctions between apoptosis, autophagy, and necrosis

Cells undergoing apoptosis exhibit disassembly of cytoskeletal structures making cells become smaller, they lose mitochondrial membrane potential, and the nucleus becomes compacted. These events are directed by the proteolytic activity of caspases, which cleave hundreds of protein substrates. Membrane budding and DNA fragmentation occur late during apoptosis. Eventually the cell becomes a series of membrane bound apoptotic bodies (not shown) which expedites their clearance by macrophages and neighbouring cells (efferocytosis). Autophagy occurs when nutrition is depleted, and can be reversed if the cell is exposed to new sources of energy and substrates for biosynthesis. Extended period of starvation leads to the formation of lysosomes and autophagosomes which digest organelles and proteins. When substrates are exhausted, the cytoplasm exhibits severe loss of organelles and the formation of large vacuoles. Similar to apoptosis, degradation of the plasma membrane occurs late during autophagy, and allows residual cellular components to be cleared. Cells undergoing necrosis allow the influx of extracellular fluid, and efflux of cytoplasmic contents, which enlarges the cell and the organelles, and causes the formation of irregularities in the plasma membrane called blebs. When the volume of the cell becomes excessive, the plasma membrane ruptures the cytoplasm is released into the extracellular environment. Figure from (127).

Changes to the plasma membrane are controlled during apoptosis, and allow the formation of apoptotic bodies which facilitate efferocytosis (127).

There is mounting evidence that apoptosis, necrosis and autophagy (and other forms of cell death) are not mutually exclusive, and they share a surprising amount of similarity. Ultimately, the mode of cell death may depends on a number of factors such as the availability of ATP, the nature and magnitude of the cytotoxic stimuli, the activity of caspase inhibitors, and the biochemical and molecular attributes of the cell (127, 148, 149).

1.4.3 Aberrant Apoptosis in Disease

Apoptosis occurs rapidly and is difficult to detect in healthy tissues (150). Apoptotic cells release factors which allow for recognition, response, and removal of these cells by phagocytosis (reviewed in (132)). Cells which progress efficiently into apoptosis exhibit anti-inflammatory effects characterised by a decrease in the pro-inflammatory cytokines TNFα, IL-1β, and IL-8, and an increase in anti-inflammatory mediators including IL-10 (132). Apoptotic cells suppress the disease-related activities of neighbouring macrophages (151) and mast cells (152). In addition, apoptosis promotes wound healing, which has been directly linked with the activity of caspase-3 and -7 (153). Hence, the mechanisms of programmed death can potentiate recovery and regeneration of cells in the surrounding environment.

Accelerated apoptosis and delayed clearance of dying cells have implications for disease progression and severity. Persistence of apoptotic cells contributes to systemic autoimmune diseases via autoantigen exposure, suppression of regulatory T-cells production, and the elevation proinflammatory responses (132). Delayed clearance of apoptotic cells also results in the release of cytotoxic cellular factors, such as ATP and DNA, which may activate the immune pathways through TLRs (154, 155). Resistance to apoptosis is a hallmark of cancer (156). Delayed apoptosis in cancer allows metastatic cells to survive the host's immune defence and cytotoxic therapeutics, enabling metastatic cells to establish secondary tumours (157). Accelerated or widespread apoptosis also has pathological consequences. This is evident in diseases such as Alzheimer's Dementia and diabetes, in which apoptosis of neuronal and pancreatic islet β-cells (respectively), have

severe consequences (158, 159). In addition to abolishing the function of a tissue, accelerated apoptosis may expose immature cells to disease-related factors, and elicit an inflammatory response (132, 147).

A number of systemic autoimmune conditions have now been associated with aberrations in apoptosis, including, systemic lupus erythematosus, lymphoproliferative disorders, and rheumatoid arthritis (126, 127, 132). However, less is known about of apoptosis in respiratory diseases such as asthma.

1.4.4 Apoptosis of Airway Epithelial Cells in Asthma

The AE in healthy airways maintains an impermeable barrier and secrete a number of regulatory factors which promote lung function (11, 160). Epithelial damage, in both symptomatic and non-symptomatic asthmatic lungs, has been recognised for over a century (68). This has led to the concept that the asthmatic phenotype involves a combination of immune sensitisation and injury susceptible epithelium (21, 71) (Figure 1.14), otherwise known as AE fragility

AE fragility may be associated with an elevated rate of epithelial cell death by apoptosis (161), which increases with the clinical severity of the disease (162). Excessive AE apoptosis can initiate, and sustain, signalling pathways leading to disease pathogenesis (163). Normal AE cells are relatively refractory to a number of apoptotic stimuli (164, 165), and survival mechanisms maintain the integrity of the epithelial barrier exposed to high levels of reactive oxygen species (ROS) and to death receptor ligands secreted by immune cells during inflammation. It is increasingly recognised that inappropriate apoptosis may contribute to the shedding of AE thereby increasing the exposure of underlying tissue to the toxic effects of inhaled oxidants, allergens and pathogens. ROS, corticosteroids, and proinflammatory cytokines are proapoptotic triggers to AECs (166). Trautmann *et al.* (167), using co-cultures of T-cells, eosinophils, and AEC, demonstrated a marked increase in cytokine-induced AEC apoptosis, findings which are supported by excessive apoptosis in bronchial biopsies from asthmatic patients. Asthma affected AEC are also vulnerable to oxidants and other apoptosis-inducing agents (168), and exhibit abnormalities in the production of the anti-apoptotic molecule Bcl2 (162). Other factors

shown to promote apoptosis of the AE include dysregulated zinc homeostasis (169), decreased production of E-cadherin (97), and heightened sensitivity to disease related agents such as Fas ligand (170). In addition, accumulation and inefficient efferocytosis of damaged AEC may be harmful for adjacent cells and prolong inflammation (132, 171).

Complementary to these phenomena, a reduced rate of apoptosis in eosinophils, T-lymphocytes, neutrophils and macrophages is characteristic of asthma, and prolongs airway inflammation (172-174). Whether elevated AEC apoptosis potentiates the fragile AE phenotype, or is a distinct phenomenon, remains unclear (170). Further to this, little is known about the function of endogenous suppressors of the caspase cascade in the inflamed airways, and whether they exhibit deficits which may explain the aberrant apoptosis.

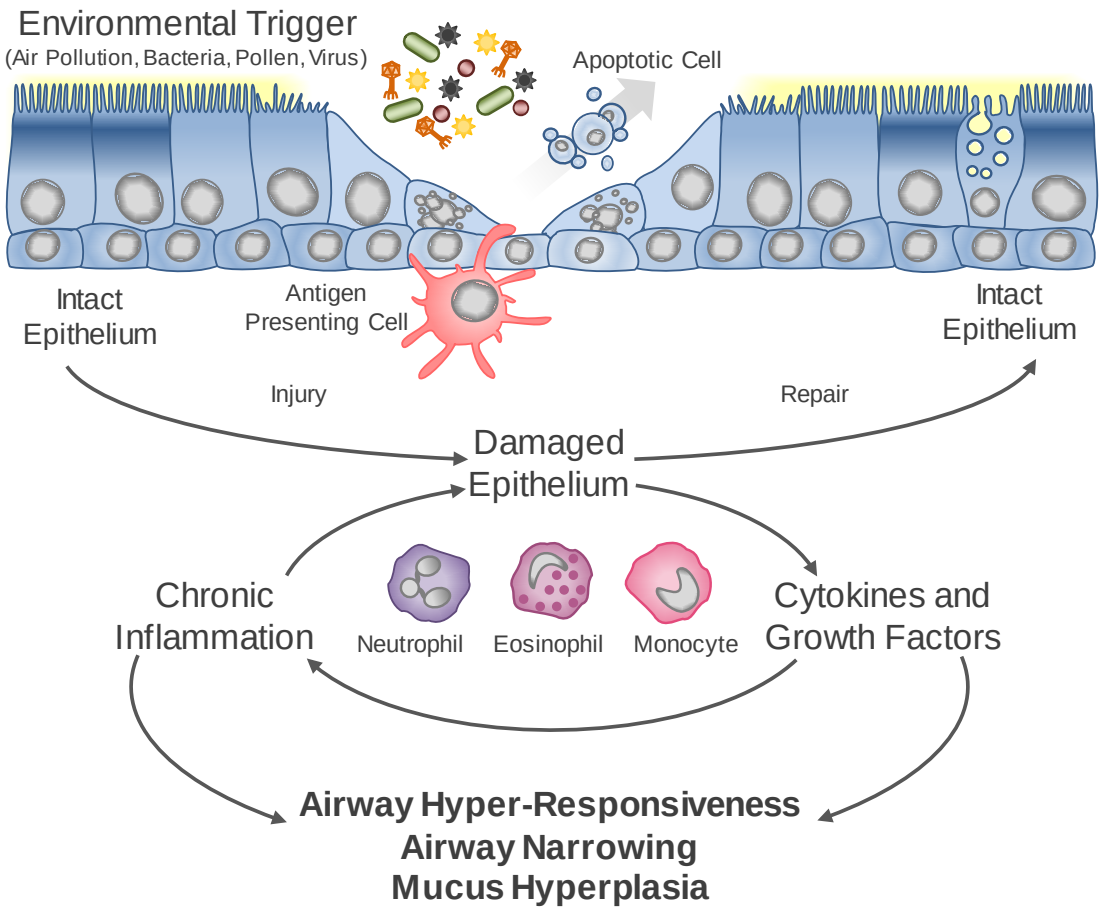

Figure 1.14: A schematic representation of airway epithelial damage in asthma

Epithelial cell contact with a range of environmental triggers enhances epithelial cell damage, and an inflammatory response involving recruitment of neutrophils, eosinophils and monocytes. Recruited inflammatory cells release cytokines and growth factors that stimulate airway remodelling and prolong the inflammation. Recurrent damage to the epithelium leads to mucus hyperplasia, airway narrowing and airway hyperresponsiveness which is associated with asthma.

Figure adapted from (71).

1.4.4.1 TNFα and potentiation of AEC apoptosis in asthma

As discussed previously, TNFα is able to potentiate apoptotic signalling through the binding of the TNF-R, which leads to the formation of the DISC, and the activation of caspase-8. Prosurvival effects are also initiated by TNFα through the activation of NF-κB and upregulation of IAP proteins (163), indicating the balance of prosurvival and apoptotic signals may determine life and death outcomes (reviewed in (175, 176)). TNFα elicits apoptosis in a number of cell types including pituitary cells (177), insulin producing islet cells (178, 179), intestinal epithelial cells (180), and immune cells (181, 182). In most cases, TNFα's capacity to induce apoptosis has been found to be caspase-dependent (e.g. (183)).

TNFα has diverse effects on AEC survival and function (summarised in Table 1.1). In general, TNFα increases the expression of proinflammatory cytokines and adhesion molecules in AEC. However, given the importance of TNFα in promoting inflammation and influencing cell survival, there has been surprisingly little research examining its apoptotic effects in AEC (170). The most convincing evidence of TNFα's capacity to induce apoptosis in AEC comes from two studies by Trautmann *et al.*, who reported that TNFα induced DNA fragmentation, and epithelial shedding due to downregulation of cell adhesion molecules (97, 167). Importantly, they showed the apoptotic potential of cell lysates from activated T-cells and eosinophils was inhibited by blocking antibodies to TNFα and IFNγ (169). TNFα was also shown to increase the production of adhesion molecules however, caspase activity was not examined. Further, IFNγ was an important co-stimulus in this process, indicating that interaction between IFNγ and TNFα pathways may be important for AEC apoptosis. Indeed, IFNγ and TNFα have been shown to potentiate each other's apoptotic effects on a number of occasions (e.g. (184, 185)).

Table 1.1: Disease-related and apoptotic effects of AECs exposed to TNFα

Disease-related effects	**Reference**
Dose dependent increase in ICAM-1 expression in hAECs	(186)
Induction of ICAM-1 surface expression in hAEC	(187)
Upregulation of IL-4 and downregulation of TGF-β in 16HBE14o- secondary AECs	(188)
Induction of RANTES in hAECs through MAP kinase	(189)
Induced Clara cell secretory protein expression in hAECs	(190)
Induction of IL-5 in hAECs and A549 secondary cells	(64)
Induction of ICAM-1 expression in hAEC	(191)
Induction of IL-8 expression in hAECs	(192)
Hypersecretion of mucin and induction of MUC-2 in hAECs	(193)
Proapoptotic effects	
Loss of E-cadherin, but an increased expression of ICAM-1 in hAECs	(97)
Mitochondrial damage and apoptosis in hAECs	(184)
Caspase-3 activation and IL-8 expression in hAECs via phosphorylation of MAPK	(194)
Production of cytotoxicity effects in human alveolar epithelial cells	(195)
Induction of apoptosis of murine AECs	(196)
Induction of apoptosis through anoikis and DNA fragmentation in hAECs	(167)

Abbreviations: hAEC, human airway epithelial cell; RANTES, Regulated upon activation normal T cell expressed and presumably secreted; ICAM-1, Intercellular adhesion molecule-1; MAPK, Mitogen-activated protein kinases; MUC-1, mucin-1; TGF-β, Transforming growth factor-β.

1.4.4.2 IFNγ and potentiation of AEC apoptosis in asthma

Similar to TNFα, IFNγ is able to elicit both cytotoxic and prosurvival effects. The outcome of IFNγ signals depends largely on the cell type, cross talk with other signalling pathways, and the relative influence of the hundreds of IFNγ regulated genes (reviewed in (197)). IFNγ can influence apoptosis by upregulation and activation of procaspase-8, and induction of a number of apoptotic genes including *TRAIL*, *Fas ligand*, *Bax*, *p21*, *p53*, *XAF1* and *TNFα* (197-199). IFNγ is able to induce the formation of the DISC via cross talk with FasL (200) and TNFα (201) signalling pathways, and by inducing the expression of (for example) FasL, TRAIL, and TNFα, which engage death receptors through an autocrine loop (202). Recruitment and activation of procaspase-8 at the DISC can lead to initiate the caspase-cascade. Further to this, IFNγ strongly induces the expression of procaspase-8 through an interferon-stimulated response element (ISRE) located in the *procaspase-8* gene promoter region (202). IFNγ has been observed to elicit an apoptotic response in a number of cell types. For epithelial cells, IFNγ signalling can initiate programmed cell death in the epithelium of the intestines (203), optic lenses (204), salivary glands (205), and blood vessels (206). Although IFNγ is a major inflammatory cytokine in asthma, many of its deleterious effects for the airway epithelium have been associated with apoptosis (summarised in Table 1.2).

Table 1.2: Disease-related and apoptotic effects of AECs exposed to IFNγ

Disease-related effects	**Reference**
Mice treated with IFNγ exhibited subepithelial fibrosis and airway remodelling	(207)
Epithelial damage and secretory cell hypertrophy contributed to the initiation of airway hyperresponsiveness in in mice	(208)
Proapoptotic effects	
IFNγ-STAT1 (but not Fas ligand or IL-13) induced caspase-dependent apoptosis of hAEC derived from asthmatics and controls	(209)
Mitochondrial damage and apoptosis in hAECs	(184)
Induction of apoptosis through anoikis and DNA fragmentation in hAECs	(167)
DAP3 mRNA expression increased ten-fold with in NHBEs	(210)
Loss of E-cadherin, but an increased expression of ICAM-1 in hAECs	(97)
Increased TUNEL and annexin-V staining in mouse AECs	(211)
IFNγ-STAT1 signalling increased Bax localisation to the ER in mouse AECs	(212)
Induction of Bik mediating apoptosis in AECs of mice	(213)
IFNγ (not Fas or TNFα) induced caspase-dependant apoptosis in A549 AECs	(214)

Abbreviations: hAEC, human airway epithelial cell; DAP3, Death associated protein-3; ER, endoplasmic reticulum; NHBE, normal human bronchial epithelial cell; ICAM-1, STAT1, Intercellular adhesion molecule-1; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling.

1.5 The Inhibitor of Apoptosis (IAP) Family

1.5.1 Introduction

The IAPs were first identified by Louis Millers' group in 1993 in baculoviruses (215, 216) and are characterised by the inclusion of 1-3 baculoviral inhibitor of apoptosis repeat (BIR) modules (217). The IAP family, of which there are eight in humans, include X-linked IAP (XIAP), the Cellular inhibitor of apoptosis (cIAP1and cIAP2), IAP-like protein-2 (ILP-2), Melanoma-IAP (ML-IAP), "Domain found in NAIP, CIITA, HET-E and TP-1" (NACHT), Neuronal apoptosis inhibitory protein (NAIP), and Testis Specific-IAP (Ts-IAP). One of the defining qualities of the IAPs is that they are able to directly interact with caspases to inhibit their activity (218, 219). Of the IAPs, XIAP, cIAP1 and cIAP2 have been examined most rigorously, due to their ubiquitous expression and relevance to the pathogenesis and treatment of cancer (220). Similarly, this thesis focuses on the potent caspase inhibitor, XIAP, and its closest paralogues, cIAP1 and cIAP2.

1.5.2 IAP Structure and Function

The IAP family are so named because of their capacity as natural inhibitors of apoptosis (218). As IAPs were first identified in baculoviruses (216), the functional domain common to all IAPs, a zinc (Zn) finger structure of approximately 70 amino acids, was named baculovirus IAP repeat (215). Proteins containing BIR domains have since been identified in a variety of eukaryotes including yeasts, nematodes, insects and mammals (218). The IAP BIR domain, characterised by the amino acid sequence $CX_2CX_{16}HX_{6-8}C$ (221), allows IAPs to participate in protein-protein interactions, signalling, and caspase inhibition. XIAP, the cIAPs, and NAIP contain three consecutive BIR domains, separated by "linker regions" (222), while the remaining IAPs possess a single BIR domain (Figure 1.15). The BIR2 interaction groove and the BIR1-BIR2 linker region of XIAP are involved in the inhibition of executioner caspases-3 and -7 (223), with stabilising interactions provided by the BIR domains (224-227). XIAP, cIAP1 and cIAP2 BIR2 and BIR3 domains also provide docking sites for IAP antagonists such as Second mitochondrial-derived activator of caspases (Smac), XIAP-associated factor-1 (XAF1), and High-temperature requirement A2 (HtrA2) (228-230). Consequently, IAP antagonists such as Smac and XAF1 can bind

XIAP and the cIAPs to prevent their activity. Although the structure of XIAP BIR2 and BIR3 are comparable, it is BIR3 that is responsible for high affinity binding and inhibition of caspase-9 (231, 232). Similarly, differences in the BIR1 domain of the cIAPs (compared to other IAPs) enable them to bind TRAF2 (e.g. Figure 1.9), and modulate survival signalling initiated by the TNF-R complex (233-236). Hence, BIR domains are central to IAP function, with zinc (Zn) providing the structural configuration necessary for their activity (237).

Five IAPs also possess a conserved C_3CH_4 Zn finger in a "Really interesting new gene" (RING) domain (Figure 1.15), which coordinate two Zn ions (238, 239). The RING domain recruits E2 ubiquitin conjugating enzymes allowing IAPs to act as E3 ubiquitin ligases. As E3 ligases, IAPs are able to participate in ubiqitin-mediated signalling or transfer ubiqutin to substrates including themselves (autoubiquitination), marking them for proteasomal degradation (239-241). For example XIAP has been shown to ubiquitinate IAP antagonists, caspase-3, and other proteins involved in cell survival (239, 241, 242). The cIAPs ubiquitinate specific substrates when co-localised to the TNF-R signalling complex such as the TRAFs (243-245), death inducing multiprotein platforms including the ripoptosome (246), and necrosome (247). A recently identified module present in the linker region between the BIR and RING domain in four IAPs, termed the ubiquitin associated domain (UBA), allows IAPs to be conjugated to ubiquitin (248, 249). Ubiquitination of the UBA was initially found to promote cell survival and activation of NF-κB (248), and may also expedite the proteasomal degradation of the cIAPs (250), as well as cIAP-bound antagonists such as Smac (251).

The CARD (caspase activation and recruitment domain) motif located between the UBA and RING domain, is contained only in the cIAPs (Figure 1.15). The CARD domain was initially predicted to bind caspases (219), but its function was otherwise unclear (252). Recently however, Lopez *et al.* found the CARD domain may inhibit cIAP1 E3 ligase activity, increasing cIAP1 half-life by suppressing its autoubiquitination (251). They suggest E3-mediated autoubiquitination of cIAP1 is restored upon binding to an IAP antagonist, allowing the complex to be degraded by the proteasome. NAIP contains a unique module called the NACHT domain, which was initially hypothesised to confer anti-

apoptosis properties (253). Reports from one group suggest the ATP binding properties of the NACHT domain may be required for NAIP to inhibit caspase-9 (254, 255). However, further biochemical analyses of the NACHT domain are needed to fully understand the significance of this module.

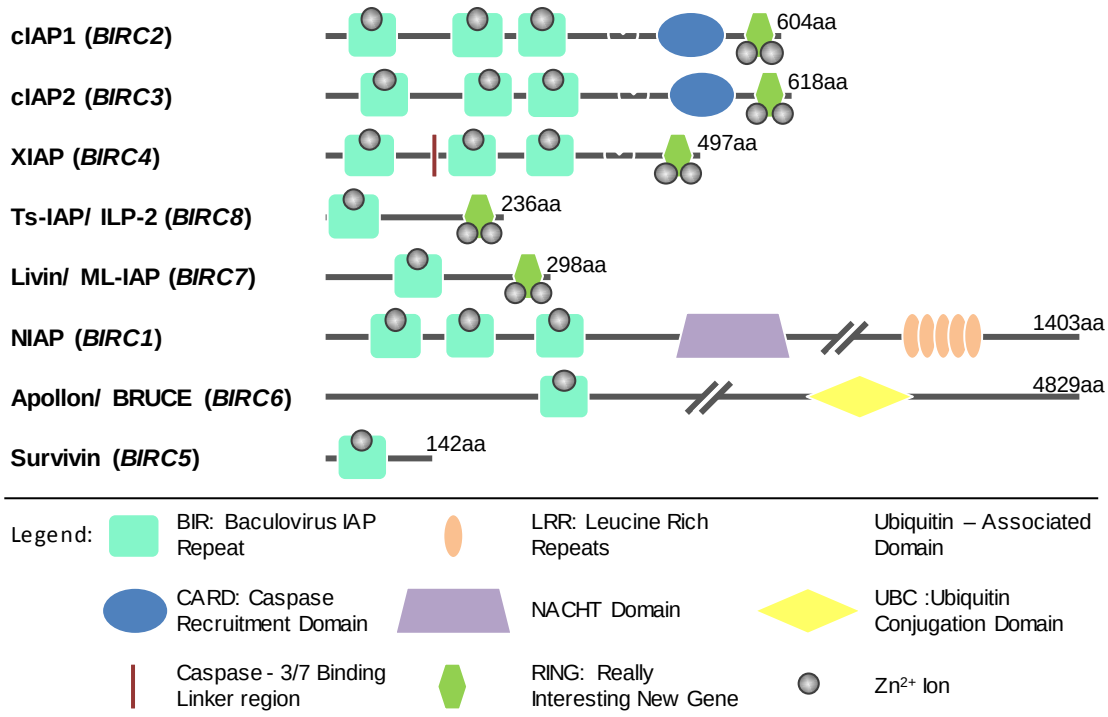

Figure 1.15: The human Inhibitor of apoptosis protein family

Inhibitor of apoptosis (IAP) family members contain at least one baculoviral IAP repeat domain, each containing a zinc (Zn) ion. The RING domain incorporates two Zn ions and has E3 ligase activity that signals target proteins for proteasomal degradation, while the ubiquitin associated domain (UBA) allows IAPs to be ubiquitinated. The Caspase Activation and Recruitment Domain (CARD) mediate protein–protein interactions. Apollon contains an ubiquitinconjugation domain, common to ubiquitin-conjugating enzymes. BIRC, baculoviral IAP-repeat-containing; cIAP, cellular inhibitor of apoptosis protein; ILP-2, IAP-like protein-2; ML-IAP, melanoma-IAP; NACHT, “domain found in NAIP, CIITA, HET-E and TP-1;” NAIP, Neuronal Apoptosis Inhibitory Protein; Ts-IAP, Testis Specific-IAP; XIAP X-linked IAP.

1.5.3 IAPs as Endogenous Inhibitors of Caspases

IAPs are unique as protein inhibitors of apoptosis in that they are able to physically interact with caspases (256, 257). In addition IAPs are able to suppress apoptosis induced by a variety of stimuli acting via intrinsic or extrinsic apoptosis pathways (258). XIAP is a potent inhibitor of apoptosis by blocking the proteolytic activity of caspase-3, -7 and -9 (Figure 1.16) (223, 259, 260). In a cell-free model of apoptosis, and in related studies, exogenous XIAP was shown to bind to, and inhibit, active caspases and procaspase activation (256, 261-263). XIAP is able to suppress caspases by one or more of a variety of mechanisms, although different members of the family may have distinct mechanisms (264). These include, i) binding to and inactivation of the active catalytic site of effector caspases-3 and -7, ii) binding to the processed amino terminus of monomeric caspase-9 and thereby preventing the dimerization required for effector caspase activation, iii) sequestering mitochondrial IAP-antagonists and thereby preventing their pro-apoptotic function or acting as antagonists for other IAPs, and iv) targeting procaspases or caspases for ubiquitination and degradation by the proteasome. XIAP may be the only IAP able to perform all these functions, and is able to bind caspases with high affinity, therefore it is widely considered the most potent regulator of apoptosis amongst the IAP family (223, 265). Indeed, when expressed in sufficient levels XIAP is the only caspase inhibitor able to prevent the convergence of extrinsic-to-intrinsic apoptosis pathways facilitated by the truncated Bid (tBid). Truncated Bid is able to stimulate Bax and Bak-mediated cytochrome *c* release from the mitochondria (258). While XIAP is clearly involved in regulation of caspases, less information is available about the role of other members of the family as caspase inhibitors.

cIAPs have been implicated in direct caspase interactions and inhibition of apoptosis, although whether they restrict caspase enzymatic activity, or primarily use other mechanisms to alter the apoptotic threshold, requires further investigation. Like XIAP, cIAPs have been shown to both inhibit executioner caspases directly (for example (219, 266)), and potentiate caspase degradation using the E3 ubiquitin ligase activity of the RING domain (267, 268). The cIAPs are also known for their capacity to inhibit caspase-8

activity by interacting with membrane bound death inducing complexes (233, 269, 270). Interestingly, Burke *et al.* found cIAP1 is able to associate with the apoptosome-caspase-9 complex to prevent activation of procaspase-3 via steric hindrance, demonstrating a novel mechanism of caspase inhibition (252). However, an elegant study by Eckelman *et al.* showed that, although cIAP BIR1-3 fragments are able to bind caspases in a cell free model of caspase-9 activation, they lack critical residues present in XIAP to prevent caspase enzymatic activity (271). Given that cIAPs associate with caspases, and many aspects of IAP and caspase biochemistry are not well understood, this suggests their capacity to affect caspase activity is potentiated through alternative mechanisms. It is generally accepted that the cIAPs regulate caspases directly through their ubiquitination activity, and indirectly by sequestering IAP-inhibitors. Additionally, the cIAPs inhibit caspases indirectly by (as previously mentioned) modulationof the TNF-R signal complex to promote the expression of NF-κB-regulated prosurvival genes (245, 272).

As XIAP is a more effective caspase inhibitor than the cIAPs (218, 219, 223), and in contrast to the cIAPs, does not associate with TRAFs, which implies non-redundant roles for these proteins (273). A likely scenario is that multiple IAPs are required to maintain the apoptotic threshold (274), and employ overlapping mechanisms to inhibit caspase activity. This observations are consistent with other reports which describe apoptosis related to decreased IAP surveillance requires the depletion of more than one IAP (274, 275). However, depletion of cIAPs, without a reduction in XIAP, sensitises cells to apoptosis (e.g. (276, 277)). cIAPs also specialise in the regulation of multiprotein death inducing signalling platforms, including the necrosome (which induces a form of programmed necrosis called necroptosis (247)), the inflammasome (278), and the ripoptosome (279). In addition, it has recently been shown that double knockout of cIAP1 and cIAP2 is embryonically lethal in mice (274).

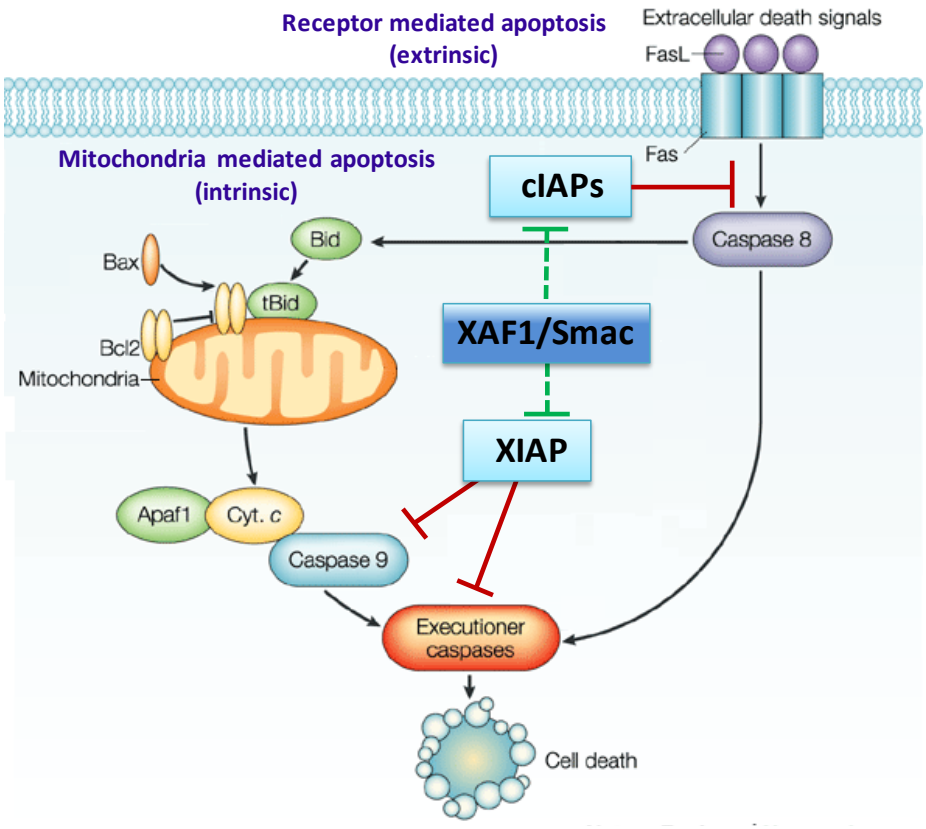

Figure 1.16: Caspase activity is regulated by the IAPs

Apoptosis can follow the extrinsic pathway, which involves engagement of soluble ligands, such as Fas ligand, to death receptors. The recruitment and activation of caspase-8 at the receptor complex lead to the formation of the death inducing signal complex. Caspase-8 activates executioner caspases such as caspase-3 and -7. The proteolytic activity of executioner caspases potentiates the final stages of apoptosis by cleaving substrates such as Poly (ADP-ribose) polymerase and the cytoskeleton. Caspase-8 can also truncate Bid (tBid), which translocates to the mitochondria and induces the release of cytochrome *c*. The intrinsic pathway is initiated by cellular stress factors such as reactive oxygen species and irradiation, which causes depolarisation of the mitochondrial membrane through the activity of (for example) Bax. This process is negatively regulated by Bcl2. Cytochrome *c* released from the mitochondria potentiates the formation of the apoptosome, which complexes with Apoptotic protease activating factor-1 (Apaf1) and caspase-9, which is the initiator caspase of the mitochondrial pathway. As for caspase-8, caspase-9 processes executioner caspases, which then dismantle the cell. XIAP is able to inhibit the activity of caspase-9, -7 and -3, whereas the cIAPs primarily increase the activation threshold of caspase-8 at receptor complexes. XIAP and the IAPs can be antagonised by the activity of Second mitochondria-derived activator of caspases (Smac), and XIAP-associated factor-1 (XAF1). Figure adapted from (280).

Similarly, each of the remaining IAP family members have been reported to inhibit caspases directly, but do not share all of the critical residues contained in XIAP (223, 271). Hence, although further efforts are required to elucidate the mechanisms by which each IAP physically interact with caspases (266), it is clear they are critical regulators of caspases and the apoptotic threshold. However, the IAPs also use other means of maintaining cell survival.

1.5.4 Other Survival-Related Functions of IAPs

Although members of the IAP family are best known for their capacity to inhibit caspases, they also participate in other pro-survival activities (169). IAPs are involved in survival activities independent of caspase inhibition, by co-operating in a surprising range of pro-survival signalling events (281, 282). Cell cycle progression is, in part, regulated by XIAP which has been shown to support endothelial cell survival via regulation of cyclins A and D1, and by promoting cyclin-dependant inhibitor activity (283). In the same investigation, Levkau *et al.* showed that XIAP is able to activate NF-κB in human endothelial cells which facilitates transcription of survival genes. Indeed, XIAP is known to interact with a range of molecules which prolong cell life, of note, Transforming growth factor-β (TGFβ), transforming growth factor-β-activated kinase (TAK1), the Akt/PI3K pathway, and JNK (273, 284). Sanna *et al.* demonstrated that (in addition to XIAP), NAIP and Livin were able to bind TAK1 thereby activating JNK1 to prevent caspase-1 and TNFα induced apoptosis, without directly inhibiting caspases (285). Several IAPs are also critical during embryogenesis and foetal growth, where they coordinate apoptosis, proliferation and mitosis. For example, knockout of the IAPs Survivin or Apollon in mice is lethal, likely attributable to their regulation of cell cycle progression and cell division (286-288). In addition, the IAPs are now understood to regulate immunity and several aspects of the inflammatory response, which is in part due to their participation in the canonical and non-canonical NF-κB signalling pathways (289).

As previously mentioned, in addition to the apoptosome, the IAPs are also known to regulate several other multiprotein death inducing platforms including the inflammasome, ripotosome and the necrosome. XIAP and the cIAPs are negative regulators of the ripoptosome, a death inducing platform which is activated as a result of IAP antagonism,

genotoxic stress, and inflammatory triggers, leading to necroptosis (246, 279, 290). The exact mechanism of IAP inhibition of the ripoptosome is not completely understood, but involves ubiquitination of its components (such as RIP1) for proteosomal degradation. In addition, a related necroptosis inducing platform, labelled the necrosome, is negatively regulated though inhibition of RIP1 and RIP3 activity by cIAP1 and cIAP2 (247, 291). In addition, XIAP participates in maintaining homeostatic levels of a variety of metals commonly encountered by the cell, including zinc, copper, and calcium (169).

1.5.5 IAPs and Zinc

IAP activity relies on the co-ordination of Zn within their protein structure. As discussed earlier, the three BIR domains of XIAP possess Zn-finger like motifs which chelate a single Zn, via three Cys and one His residue (292). The C-terminal RING domain, critical for ubiquitination, contains two Zn ions, one coordinated by three Cys and one His residue, and the other by four Cys residues (232). Therefore, XIAP and the cIAPs contain five Zn ions, while other members of the family are also rich in Zn, although differing according to the number of BIR domains and whether they possess a RING domain. Zn is an inhibitor of apoptosis in AECs exposed to asthma-related inflammation (166). The relationship between Zn and IAPs, in the context of AEC apoptosis, was reviewed as part of this thesis (Roscioli *et al.*, 2013; Appendix 4), because of the interest and expertise of our Group in relation to zinc. However zinc was not included in the experimental plan.

1.5.6 IAP Genetics

XIAP is encoded by the *Baculoviral IAP repeat-containing-4* (*BIRC4*; chromosome Xq25) gene, cIAP1 the *BIRC2* gene, and cIAP2 the *BIRC3* gene (both located on 11q22-q23). Overall, there are few IAP-related genetic disease association studies, and the majority of these so far have studied BIRC4 polymorphism.

The X-chromosome displays unique genetics such as random inactivation of paternal or maternal X-chromosomes in women, and is linked to a disproportionately high number of diseases (293). Studies relating *BIRC4* mutations or polymorphisms to disease are summarised in Table 1.3. *BIRC4* mutations leading to decrease in XIAP protein levels are associated with susceptibility to X-linked lymphoproliferative syndrome (294), and may

exert a disease modifying effect in Wilson disease (295). Furthermore, a non-synonymous SNP causing a Q423P amino acid substitution in XIAP is reported to be associated with periodic fever (296). Collectively these findings suggest that XIAP plays a significant role in regulating lymphocyte function and survival. However, there is no clear evidence that *BIRC4* polymorphism is linked to disease, with the exception of rare *BIRC4* mutations and X-linked lymphoproliferative syndrome. Although not examined in a disease association context, two additional studies have suggested there may also be relevant quantitative genetically determined variation in XIAP expression. A genome wide expression analysis reported that several *BIRC4* SNPs were associated with variable XIAP expression (297). In the other study, structural genomic variation (copy number variation, CNV) has been reported in a 9.125 kb duplicated region encompassing *BIRC4* and the flanking *STAG2* gene (298).

Although the X-chromosome carries asthma susceptibility genes such as *CYCLTR1*, *TLR8*, and *TLR9* (299, 300), currently there are no reports which examine the influence of *BIRC4* genetic variations in asthma or other respiratory diseases. However, associations between asthma and polymorphism in other (non-X chromosome) genes encoding apoptosis regulatory proteins, such as Survivin (encoded by *BIRC5*) (301), and *DAP3* (210) have been reported.

Table 1.3: Studies examining *BIRC4* variations for association with disease

Pathology (study size)	Major findings	Ref.
Wilson Disease (n=98)	SNP frequencies did not significantly deviate from previous reports except rs5956583 which associated with altered lymphocyte abundance.	(302)
Periodic fever (n=133; controls, n=182)	Missense SNP 1268A>C (rs5956583), Q423P substitution in exon 6 was associated with idiopathic periodic fever ($P < 0.007$), possibly by modulating monocyte function.	(296)
Lung cancer (n=582; controls, n=582)	Twelve novel *BIRC4* SNPs were identified; four selected for frequency analysis showed no association with susceptibility to lung cancer.	(303)
Common variable immunodeficiency (n=30)	No *BIRC4* SNP-associated phenotype was detected.	(304)
X-linked lymphoproliferative syndrome (n=7)	Reduced expression and truncated XIAP protein in peripheral blood mononuclear cells of four subjects.	(305)
X-linked lymphoproliferative syndrome (n=1)	Nonsense SNP 840C >T (R238X) and decreased XIAP expression was identified.	(306)
X-linked lymphoproliferative syndrome (three families)	Reduced XIAP expression, increased lymphocyte death with apoptotic stimuli, and reduced natural killer T-cell counts in two families due to several identified *BIRC4* SNPs and deletion mutations.	(294)
Allelic expression screen (n=80)	Six *BIRC4* SNPs have a high allelic mRNA expression difference and are each represented within the top 5% for allelic expression imbalance compared to 2968 SNP in 1380 genes examined.	(297)

Abbreviations: SNP, single nucleotide polymorphism.

The *BIRC2* and *BIRC3* genes, are positioned consecutively approximately 9 kilo bases apart on chromosome 11, and may have arisen from a gene duplication event (307). There are no published studies which examine BIRC2 or BIRC3 polymorphism and disease susceptibility, however, the long arm of chromosome 11 (Chr11q) has consistently been associated with polymorphisms in asthma susceptibility genes for example, the *IL-6 receptor* gene (308), and the *High-affinity IgE receptor-β chain* (309). Moreover, a number of polymorphisms in the gene coding IL-18, which resides with in the same chromosomal sub-band as *BIRC2* and *BIRC3*, have been associated with asthma and other atopic diseases (e.g. (310)). Genetic studies of *BIRC2* and *BIRC3* are largely confined to identification of somatic DNA changes observed in various forms of cancer cells (e.g. (311, 312)), and the well described cIAP2-MALT fusion protein generated by the t(11;18)(q21;q21) translocation event in lymphoma (313).

1.5.7 IAPs in Disease

IAPs are strongly implicated in the pathogenesis of cancer (reviewed in (314, 315)). IAP expression is increased in a range of cell lines and tumour biopsy samples (273, 316, 317), although expression levels may be quite variable (318). This increase in IAP expression is thought to maintain the tumour cell apoptotic threshold, thereby prolonging survival (319). Consistent with this, leukaemia patients with reduced XIAP protein have a significantly extended period of survival (320). As previously mentioned, *BIRC3* (which encodes cIAP2) has been implicated in the MALT-cIAP2 fusion translocation event in β-cell lymphoma, and amplification of the 11q21-q23 region, which contains the cIAPs, is observed in several human malignancies (315). IAPs are therefore a therapeutic target of great interest in cancer, and a large number of reports indicate that inhibition of IAP protein expression, or activity, sensitises cancer cells to chemotherapeutic drugs. For example, antisense oligonucleotide reduction of XIAP abundance in bladder cancer promotes apoptosis and contributed to doxorubicin-mediated cytotoxicity (321). While most IAP research has come from oncology, less is known about their involvement in other diseases.

There are few studies linking IAPs to other diseases, and the majority so far have focussed on XIAP. Rigaud *et al.* reported that loss-of-function mutations in *BIRC4* are associated with X-linked lymphoproliferative syndrome (a rare immunodeficiency disorder) as a result of decreased natural killer T-cell survival (294). Some XIAP knockout mice exhibit clinical symptoms. For example, Olayioye *et al.* produced $XIAP^{-/-}$ mice which have a reduction in mammary alveolar generation. (322), while XIAP knockout mice produced by Burstein *et al.* exhibited toxic accumulation of copper due to altered regulation of COMM domain-containing protein-1 (previously designated MURR1) (323). Other studies, in rat and mouse models, have identified the potential for XIAP as a therapeutic target in a range of diseases. For example, adenovirus-mediated transduction of XIAP prevents neural apoptosis in animal models of neural injury (Table 1.4). Similarly, Trapp *et al.* (2003) observed elevated XIAP levels in neurons after ischemic brain injury in rats, and transgenic upregulation of XIAP (prior to injury) was protective of widespread cell death (324). Mouse and human models of islet transplantation have demonstrated that amplification of XIAP can prevent β-cell apoptosis in diabetes (reviewed by (325)). Further, Zehntner *et al.* reported that XIAP levels are increased in a mouse model of autoimmune encephalomyelitis, and that XIAP-antisense oligonucleotides reversed disease progression by permitting autoimmune T-cells to undergo apoptosis (326). Less is known about the involvement of the IAPs in inflammatory conditions, in which aberrations in apoptosis contribute to the disease (218).

Table 1.4: XIAP in non-malignant pathologies

Condition	Major findings	Ref.
Neural conditions		
Brain hypoxia-ischemia	IAP antagonists inhibited XIAP leading to caspase-mediated apoptosis; XIAP over-expression reduced apoptosis	(327)
Transient focal cerebral ischemia	Middle cerebral artery occlusion/re-profusion lead to a biphasic change in XIAP expression in regions of the brain	(328)
Traumatic brain injury	XIAP cleavage in TBI mice was elevated in females, and ovary excised females treated with exogenous oestrogen	(329)
Parkinson's disease	Transduction of XIAP prevented dopaminergic neuronal apoptosis	(330)
Axotomised optic nerve	Transduction of XIAP prevented neuronal cell death after optic nerve lesion	(331)
Glaucoma	Transduction of XIAP promoted optic nerve survival after chromic intraocular pressure injury	(332)
Retinitis pigmentosa	Transduction of XIAP promoted structural and functional stability of rat photoreceptors	(333)
Autoimmune diseases		
Rheumatoid arthritis	XIAP was elevated in the synovium of active rheumatoid arthritis sufferers and it inhibited caspase-3 activity	(334)
Multiple sclerosis	Systemic administration of antisense XIAP prevented symptom relapse, and T-cell count reduced by 88-93%.	(326)
XLP	Mutations in XIAP reduced its expression and enhanced apoptosis of lymphocytes, particularly natural killer T-cells	(294)
Sojgren's syndrome	XIAP expression in salivary gland cells was increased by IL-1β, TGF-1β or IL-10, and decreased by TNFα	(335)
Cardiovascular disease		
Myocardial infarction	XIAP mimetic inhibited apoptosis of coronary artery myocyte after occlusion/reperfusion injury	(336)
Atherosclerosis	XIAP protein was increased in atherosclerotic plaques of symptomatic patients with carotid stenosis	(337)
Cardiac atherosclerosis	Advanced atherosclerotic plaques exhibit increased XIAP and XIAP was increased in infiltrating macrophages	(338)
Copper toxicity	XIAP maintained copper homeostasis by interacting with the COMMD1 protein and potentiating its degradation by the proteasome	(323)
Other		
Presbycusis (hearing loss)	Trans-mice over expressing XIAP demonstrated reduced hair cell (inner ear sensory receptors) loss, and develop hearing loss at a slower rate	(339)
Pancreatitis	XIAP was completely degraded in the rat model of pancreatitis, but remained unchanged in the mouse model	(340)

Abbreviations: COMMD1, COMM domain-containing protein-1; XLP, X-linked proliferative syndrome.

1.5.8 IAPs and Asthma

XIAP and cIAPs have recieved significant attention for their involvement in a number of aspects of the immune response. These IAPs regulate the formation of the inflammasome, and are indispensable in the signalling pathways which activate the proinflammatory transcription factor NF-κB (260, 341-343). The inflammasome is a large multiprotein molecular platform assembled around NALP (nucleotide-binding, leucine-rich repeat, pyrin domain containing) scaffolds which facilitates processing of inflammatory cytokines through activation of caspase-1. XIAP is known to associate with the NALP1 inflammasome where its cleavage permits inflammasome activation, possibly by lowering the threshold of caspase-1 activation (344-346). Conversely, Labba *et al.* found cIAP1 and cIAP2 incorporation and polyubiquitination activity within the inflammasome was a requirement for its efficient assembly, and caspase-1 activation (343). One investigation of the inflammasome revealed an 8-fold upregulation in *NAIP* mRNA in whole lung preparations from OVA-challenged mice (347), which is consistent with NAIP being able to substitute for NALP3 and other components of the inflammasome (348). XIAP's capacity to modulate apoptosis has been implicated in a variety of inflammatory diseases including Sjogren's syndrome (335), rheumatoid arthritis (349), inflammatory breast cancer (350), periodic fever (296), and type 1 diabetes (179). The IAPs are also known to prolong the survival of neutrophils, macrophages and eosinophils (351-353), which is relevant for diseases such as asthma because sustained activity of inflammatory cells is a characteristic of inflammation at the airways.

Relatively little is known about contributions of IAPs to inflammatory conditions such as asthma. The corticosteroid dexamethasone, an inhibitor of apoptosis in the A549 alveolar cancer cell line, induced expression of *cIAP2* transcripts (protein was not tested); there were also smaller increases in cIAP1, but no increase in XIAP was observed (214). The authors suggested that induction of cIAP2 in epithelial cells by corticosteroids may be one mechanism by which they suppress the inflammatory response. Similarly, Webster *et al.* found dexamethasone acts through a glucocorticoid response element in the cIAP2 *BIRC3* promoter, which in combination with induction of NF-κB (the *cIAP2* promoter also contains two NF-κB sites), strongly increased cIAP2 expression in A549 cells (354).

Tumes *et al.* investigated the role of Survivin and apoptosis in infiltrating lung eosinophils in OVA-challenged mice (355). Mouse strains susceptible to the development of asthma (BALB/c and C57BL/6 mice) demonstrated a reduced rate of eosinophil apoptosis due to increased Survivin expression. In contrast, mice resistant to the development of asthma (CBA/Ca mice) expressed a lower level of Survivin and exhibited increased eosinophil turn-over. Taken together, these investigations suggest an imbalance in the expression of IAPs in the AE and in inflammatory cells may be an important factor for the development of asthma.

At this stage we do not know whether in asthma there is a consistent change in expression, protein levels or function in any of the IAPs, except for some recent data regarding *BIRC5* (the gene for Survivin). One study has reported increased levels of *Survivin* mRNA (other IAPs were not studied) in induced sputum samples of patients with bronchial asthma compared with healthy subjects (301). *Survivin* mRNA abundance positively correlated with sputum eosinophilia. The same group examined whether *BIRC5* polymorphisms contribute to asthma susceptibility by comparing SNP allele frequencies between controls and asthmatics. The minor alleles of two *BIRC5* SNPs, both located in the gene-5′ region, were found to be significantly associated with asthma, especially for non-allergic female asthmatics. These results suggest there may be a genetic basis for the other IAPs in the pathogenesis of asthma.

1.5.9 IAPs and TNFα

As TNFα is a major proapoptotic and proinflammatory cytokine elevated in the lungs of asthmatics, it was chosen to assess AEC apoptosis in asthma. As shown in Table 1.5, the involvement of the IAPs in TNFα signalling has been well described. A mutual relationship between TNFα signalling and IAP expression exists, where by cIAPs are important members of the TNF-R signalling complex, and TNF-R signal transduction upregulates the expression of XIAP, cIAP1 and cIAP2. Consequently, the apoptotic influence imparted by TNFα signals is countered, at least in part, by upregulation of the IAPs. The cIAPs operate at the TNF-R signalling complex by associating with the TRAFs (shown in Figure 1.9). There they increase the activation threshold of caspase-8 (233, 356), potentiate NF-κB signal transduction (236), and modulate the inflammatory and survival

signals of the receptor-associated kinase RIP1 (357, 358). The cIAPs are also able to influence the activity of MAP kinase and JNK when situated in the TNF-R complex (359, 360). In line with this, inhibition of NF-κB or the IAPs in cells exposed to TNFα can elicit apoptosis due to the activation of caspase-8 (e.g. (361, 362)). Although XIAP may not interact with the TNF-R complex through the TRAFs (363), it is also an influential participant in NF-κB, MAPK, and JNK signalling pathways, and is upregulated by TNFα signalling events (360, 364, 365).

Consequently, whether a cell exposed to TNFα survive or undergo apoptosis, depends on signal transduction by signalling effectors, such as RIP, JNK, and IκB kinase (IκB kinase leads to NF-κB nuclear translocation), and the expression of the IAPs (366). Central to this, the E3 ubiquitin ligase activity of the cIAPs are responsible for regulating many aspects of the TFN-R signalling complex (356). There is less information regarding the effects of the IAPs and TNFα signalling in AEC. However, one study by Pryhuber *et al.* showed A549 alveolar cancer cells exposed to TNFα also form cIAP-TNF-R complexes to promote NF-κB-dependent inflammation and prosurvival pathways (367). This supports observations for other cells, in which the IAPs influence their own upregulation and promote cell survival when associated with the TNF-R signalling complex. Similarly, Kuwano and Hara, suggest cIAP2 may have a central role in inflammatory lung disease, due to its interaction with TRAF1, and its role in potentiating cell survival (163).

Table 1.5: The relationship between TNFα and the IAPs in diverse cell types

Cell type	Major findings	Ref.
Mouse retina	TNFα null mice produced less XIAP exhibit less NF-κB activation, and increased markers of apoptosis	(368)
Keratinocytes	TNFα induced the expression of XIAP and the cIAPs through NF-κB, which inhibited TNFα-related apoptosis	(369)
Macrophages	XIAP knockout mouse macrophages were sensitive to TNFα induced apoptosis	(370)
Human dermal fibroblasts	Suppression of NF-κB inhibited cIAP1, cIAP2 and XIAP expression, sensitising cells to TNFα-induced apoptosis	(361)
Pancreatic cancer cells	Suppression of NF-κB reduced TNFα-mediated XIAP, cIAP, Bcl2, and survivin expression	(153)
Ovarian cancer cell lines	IAP antagonism enabled TNFα-induced apoptosis, and was reversed by NF-κB blocking	(371)
Eosinophils	TNFα increased XIAP expression through NF-κB, which protect from Fas ligand-mediated apoptosis	(372)
Cancer cell lines	cIAPs negatively regulated TNFα induced apoptosis, and XIAP positively modulated TNFα signalling	(373)
Human endothelial cells	IFNγ induced cIAPs through MAP kinase and JNK; blocking kinases lead to TNFα induced apoptosis	(360)
Myeloid leukaemia cells	Suppression of NF-κB inhibited TNFα-mediated upregulation of XIAP and cIAP2	(374)
Breast cancer cell line (MCF7)	TNFα decreased the abundance of XIAP and the cIAPs and induced apoptosis	(375)
A549 alveolar cancer cell line	TNFα induced the expression of TRAFs, which enabled cIAPs to associate with the TNF-R signalling complex	(367)
Endothelial cells	TNFα upregulated XIAP through NF-κB, and inhibiting NF-κB led to endothelial cell death	(375)
Glioma cell line	cIAP2 was upregulated specifically by the p65 subunit of NF-κB, and was needed to confer resistance to TNFα	(376)

Abbreviations: JNK, c-Jun N-terminal kinase; Mitogen-activated protein kinase, MAPK; TRAF, TNF-receptor associated factor.

1.5.10 IAPs and IFNγ

Similar to TNFα, IFNγ is a potent proinflammatory cytokine that is able to elicit apoptosis in a variety of cell types. However, there is less known about the relationship between IFNγ and the IAPs. Various types of cells stimulated with IFNγ respond differently to with regards to the regulation of the IAPs, and may reflect the relative influence of signalling pathways leading to IFNγ-mediated gene expression. For example, cIAP2 is upregulated in neutrophils (377), but is unchanged in most other cell types stimulated with IFNγ (Table 1.6). This is interesting, given that cIAP2 contains an IFNγ regulatory element in its promoter region (378). For AECs, XIAP, cIAP1 and cIAP2 expression was unchanged with IFNγ in the A549 alveolar cancer cell line examined by Park *et al.* (379). Indeed, there are few reports examining the IAPs in AECs exposed to IFNγ. However, A549 AECs are cancer cells, and there are no reports for the regulation of IAPs by IFNγ in primary AECs. Hence, given the important disease potentiating role for IFNγ in the airways of asthmatics, further understanding of this cytokine in the context of the IAPs in AECs is warranted.

Table 1.6: The relationship between IFNγ and the IAPs in different cell types

Cells type	Major findings	Ref.
A549 alveolar cancer cell line	IFNγ led to increased caspase-8 activity in A549, but did not alter the expression of XIAP, cIAP1, cIAP2	(379)
RAW 264.7 mouse macrophages	IFNγ caused the downregulation of XIAP, cIAP1 and cIAP2,	(380)
Neutrophils	IFNγ-mediated induction of cIAP2 increased neutrophils survival through the JAK2-STAT3, and may involve NF-κB signalling	(377)
HT-29 Colon cancer cell line	IFNγ did not alter cIAP2 abundance	(378)
HeLa	IFNγ did not alter XIAP, cIAP1 or cIAP2 abundance	(381)
HeLa	IFNγ reduced the expression (or destabilises) of cIAP2 after Chlamydia trachomatis infection	(382)
Primary rat hepatocytes	IFNγ did not alter cIAP2 abundance	(383)
Mouse insulinoma cell line	IFNγ reduced NF-κB-induced upregulation of XIAP, sensitising cells to apoptosis	(384)
Mouse β-islet cells	IFNγ inhibited XIAP protein through STAT1	(385)
INS-1E line and rat β-islet cells	IFNγ did not alter the expression of XIAP or cIAP2	(386)
Human β-islet cell line	TNFα-mediated upregulated of XIAP was inhibited by IFNγ through (STAT1)	(387)
Human salivary gland epithelial cells	IFNγ (with TNFα) led to XIAP cleavage (inactivation) and induction of the intrinsic apoptosis pathway	(388)

Abbreviations: INS-1E, Rat insulinoma cell line-1E; STAT1, signal transducer and activator of transcription-1.

1.5.11 IAPs as Therapeutic Targets

As the IAPs inhibit apoptosis and are overexpressed in many malignancies they are a promising candidate for cancer management (315). Indeed, all IAP-based therapeutics currently under development are for the management of cancer (Table 1.7). Generally these therapies elicit cell death in cells overexpressing IAPs, or lower their apoptotic threshold to increase the efficacy of current treatment modes (e.g. doxorubicin) (314). The main strategies being investigated to inhibit the IAPs involve antisense technology, and membrane-permeable small molecular inhibitors which target IAP caspase binding sites. Antisense oligonucleotides which bind and reduce *XIAP* mRNA can directly induce apoptosis and sensitise cells to chemotherapy and irradiation (389). Of note, Hu *et al.*, investigating XIAP antisense oligonucleotides in breast cancer cell lines and in mice, found XIAP depletion sensitised cancer cells to doxorubicin, Paclitaxel (Taxol®), Vinorelbine (Navelbine®), and etoposide (390). The most clinically advanced IAP therapeutic is the antisense molecule AEG 35156/GEM® 640 (Aegera Therapeutics), an oligonucleotide in phase I/II clinical trials for end stage cancer patients (391, 392). Small molecular peptides and non-peptidyl inhibitors which mimic Smac-IAP interactions are being tested as lead compounds by several cancer groups, and some are able to induce apoptosis directly (316, 319, 321).

The challenge for pharmaceutical groups will be to develop therapies which target disease-contributing cells which are resistant to apoptosis. As the IAPs have several activities besides inhibition of apoptosis, IAP-based therapeutics may unintentionally target cells which upregulate IAPs to perform normal cellular functions. In line with this, a cIAP specific antagonist is under development, which has been shown to degrade both cIAP1 and cIAP2 without affecting the abundance of other IAPs (393). Targeting specific IAPs may counter unintended side effects brought about by blocking the activity of several family members. Another issue is that new properties are assigned to the IAPs on a regular basis. For example it was understood Smac can also target the cIAPs after preclinical trials of some XIAP-specific Smac mimetics were initiated (220).

Although IAPs have been studied extensively in the context of cancer, we still know very little about their roles in other diseases, especially conditions such as asthma. However, given their role in potentiating inflammation, the IAPs may be ideal targets for inflammatory conditions in which aberrations in apoptosis also contributes to the disease. However, a better understanding of the IAP's role in regulating apoptosis of AEC is needed.

Table 1.7: IAP therapeutics in development

Agent	Company/Group	Stage
Antisense		
AEG 35156/GEM® 640	Aegera Therapeutics	Phase I/II
Peptidometic		
Tripeptide	Abbott Laboratories	Preclinical
Tripeptide	University of Michigan	Preclinical
N-terminal Smac peptide	Mayo Clinic	Preclinical
Omi peptide	Mayo Clinic	Preclinical
MV1	Genentech	Preclinical
SM‑164	University of Michigan	Preclinical
LBW242	Novartis	Preclinical
CS3	Genentech	Preclinical
TL32711	Tetralogic Pharmaceuticals	Phase I
Natural Product		
Embelin	University of Michigan	Preclinical
Synthetic		
Tetrazoyl thioether C_2-symmetric diyne	University of Texas Southwest Medical Center	Preclinical
Aryl sulfonamide	Novartis	Preclinical
Di/tri-polyphenylruea	The Burnham Institute/TPIMS	Preclinical
AEG40730	Aegera Therapeutics	Preclinical

Sources: (314, 394, 395)

1.6 Summary

The respiratory system relies on the AE to provide a protective interface between the environment and the underlying pulmonary tissues. However, the AE displays disease-related modifications in the lungs of asthmatics, and AECs exhibit an injury susceptible phenotype and apoptotic changes. Aberrant apoptosis of AECs correlates with the pathogenesis of asthma (reviewed in (170)). Apoptosis of AECs is potentiated by the inflammation, and may be related to intrinsic defects in the mechanisms which regulate cell survival (e.g. (396)). In addition, variations in the genome of asthmatics are strongly associated with the pathogenesis of asthma, and can cause disease-related phenotypes. The IAPs are potent inhibitors of the caspases and participate in a number of cell survival activities. However, the IAPs have not been examined for defects in AE of asthmatics, and there are relatively few studies of the *BIRC* genes in disease. Hence, the primary objective of this thesis was to determine the role of the IAPs in AECs exposed to the inflammation related to asthma, and to identify polymorphisms in the *BIRC* genes that associate with the disease.

1.7 Central Hypothesis and Statement of Aims

Specific hypotheses and aims of this thesis are:

Hypotheses:

1. IAPs regulate caspase activation and apoptosis in AEC in both normal and asthmatic individuals
2. IAPs exhibit intrinsic defects or genetic variations which promote apoptosis of AEC and contribute to the pathogenesis of asthma

Aims:

1. To characterise the IAP and apoptotic response in *ex vivo* cultured primary AEC treated with the proinflammatory cytokines TNFα and IFNγ. The expression of XIAP and the cIAPs, and their antagonists Smac and XAF1, are characterised at the mRNA and protein level in primary AECs treated with TNFα and IFNγ. Markers of apoptotic and necrotic changes will be measured and correlated with IAP expression profiles (Hypothesis 1)
2. To use siRNA knockdown to determine the effect of individual IAPs of interest on AEC apoptosis in *ex vivo* cultures treated with TNFα and IFNγ (Hypothesis 1).
3. To compare the IAP gene expression levels in primary AEC isolated from both asthma patients and controls (Hypothesis 2)
4. To compare the IAP and apoptotic response in *ex vivo* cultured primary AEC, isolated from both asthma patients and controls, following treatment with TNFα and IFNγ (Hypothesis 2)
5. To examine the association between asthma and polymorphism in the genes encoding XIAP (*BIRC4*), cIAP1 (*BIRC2*) and cIAP2 (*BIRC3*). In addition to SNP polymorphism, copy number variation in *BIRC4* will also be assessed for association with asthma. Genetic studies will be performed using cases and controls derived from a community based cohort (the North West Adelaide Health study) (Hypothesis 2)

Chapter 2: Methods and Materials

All experiments described in this thesis conform to the rules set out by the Queen Elizabeth Hospital Committee on Human Ethics and The Adelaide University Human Research Ethics Committee.

2.1 Mammalian Cell Culture

2.1.1 The 16HBE14o- Bronchial Epithelial Cell Line

2.1.1.1 Routine culture of 16HBE14o- cells

Consent to use the 16HBE14o- bronchial airway epithelial cell line was kindly granted by Dr Dieter Gruenert (397). 16HBE14o- cells were routinely cultured in plastic culture vessels using HBE growth media in a humidified, 37°C incubator, with atmosphere composed of 95% medical grade air plus 5% CO_2. HBE growth media was aspirated from the cell monolayer, washed twice with PBS and fresh growth media added every 48 h. When cell growth reached approximately 80% density, media was aspirated and the monolayer washed twice with PBS, and cells detached by adding 0.25% trypsin/EDTA and incubating at 37°C for 5-10 min. Detached cells were transferred into a centrifuge by suspending in HBE growth media, and centrifuged at 282 g for 7 min at 10°C. The cell pellet was re-suspended in HBE growth media and transferred at the required numbers for experimentation using trypan blue- haemocytometry, or split 1:4 for sub-culture into the next passage. HBE cells doubled approximately every 18 h and reached 80-100% confluence in approximately 4 days after sub-culturing.

2.1.1.2 Cryopreservation and thawing of 16HBE14o- cells

Cryogenic preservation was used to maintain a continual supply of passage 5-8 cells for experimentation. Cells from one T75 flask at 80-90% density were detached and suspended at 2 million cells per ml in cold CryoStor®CS5 solution, and pipetted into cryo-tubes standing in ice. Tubes were placed into a 5100 Cryo 1°C freezing container for 1°C per minute cooling to -80°C, then either left in -80°C for short term storage (1-3 month) or transferred to -140°C vapour phase nitrogen for long term storage. One cryo-tube of cells

was thawed by immersing in a 37°C water bath until the frozen cell suspension was mobile enough to be transferred into a centrifuge tube containing 10 ml of HBE growth media pre-warmed to 37°C. The tube was centrifuged at 282 g for 7 min at 10°C, and the cell pellet re-suspended in 10 ml of HBE growth media, then transferred to a T75 flask for propagation.

2.1.2 Normal Human Bronchial Epithelial Cells

2.1.2.1 Routine culture of NHBE cells

Normal Human Bronchial Epithelial (NHBE) cells have a finite life span, commercially available cell line (Lonza Group Pty Ltd, Walkersville, MD, USA) received in a frozen state. NHBE cells were propagated as per the manufacturers' directions in Bronchial Epithelial Growth Media (BEGM^{TM}). Briefly, cells were thawed as previously described for HBE cells and the cell suspension transferred into 20 ml of BEGM^{TM}. Cells were then immediately seeded into two T75 culture containers (10 ml per flask) for passage one growth. Once cells reached 90% density they were detached using 0.25% trypsin/EDTA as previously described, and subcultured into six T75 flasks for growth into passage two. At the end of passage two NHBEs were either expanded for experimentation at passage three or four, or frozen for later use.

2.1.2.2 Cryopreservation and thawing of NHBE cells

If not needed immediately NHBEs were cryopreserved at passage two. Cells were detached using 0.25% trypsin/EDTA as previously described and re-suspended in Cryostor® CS5 primary cell cryopreservation. Cells were transferred into cryotubes (2 million cells per tube), then placed into a 5100 Cryo 1°C freezing container, to allow 1°C per min cooling in a -80°C freezer. After two hours in -80°C the cells were transferred to -140°C vapour phase nitrogen for long term cryopreservation. Cells were thawed as previously described and allowed to grow for a further passage before use.

2.1.3 Primary Human Airway Epithelial Cells

2.1.3.1 Collection and plating

Informed consent was obtained from subjects prior to collection of primary human nasal airway epithelial cells (AEC). All participants included did not exhibit sinus conditions on the day of AEC collection, and reported a history free of chronic sinus conditions such as rhinosinusitis. Cytology brushes were inserted 5-6 cm into the nasal cavity (three consecutively per nostril) and rotated 2-3 times to collect AECs. Brushes were immediately immersed in 15 ml AEC collection media and transferred to the laboratory on ice. Cells were dislodged from each brush by gentle vortexing, and centrifuged at 565 g at 10°C to form a cell pellet. Washing was repeated 4-5 times in PBS to clear as much mucous as possible. The final cell pellet was suspended in 10 ml of AEC growth media and passed five times through a 25 gauge syringe to disperse cell clumps. To deplete monocytes the cell suspension was added to a 10 cm culture plate coated with anti-CD68 solution, and then placed in 37°C incubator for 20 min. The monocyte-depleted cell suspension was divided into two pools of 5 ml and transferred to T25 collagen coated flasks (n=2) and incubated at 37°C, in humidified 95% medical grade air with 5% CO_2.

2.1.3.2 Anti-CD68 coating of culture dishes

5 ml of anti-CD68 coating solution was added to a 10 cm culture dish and allowed to incubate at 37°C for 20 min to allow antibodies to attach to the plastic surface. Excess solution was aspirated and the coated plate used immediately for monocyte depletion of extracted AEC suspended in AEC growth media.

2.1.3.3 Routine culture

Once AECs in T25 culture flasks approached 80% density they were washed twice with PBS and detached using 0.25% trypsin/EDTA, as previously described. The detached cells were suspended in AEC growth media and centrifuged 282 g for 7 min at 10°C to pellet cells. The cell pellet was gently broken up and re-suspended in 20 ml of AEC growth media. The cell suspension was transferred in 10 ml volumes to collagen coated T75 flasks

(n=2) for passage two growth. When passage two cells reached 80-90% density they were either frozen for later use, or propagated for use at passage three.

2.1.3.4 Cryopreservation and thawing

If not needed immediately AECs were cryopreserved at passage 2. Cells were detached using 0.25% trypsin/EDTA as previously described and re-suspended in Cryostor® CS5. 1-2 million cells per cryotube were placed into a 5100 Cryo 1°C freezing container, to allow 1°C per min cooling in a -80°C freezer. After 2 h the cells were transferred to -140°C vapour phase nitrogen for long term cryopreservation. Cells were thawed as previously described and allowed to grow for a further passage before use.

2.2 Microscopy and Immunocytochemistry Procedures

2.2.1 Preparation of Cells for Microscopy

2.2.1.1 Cytospin preparations

Cells were re-suspended at $5.0x10^5$ cells per ml in PBS, and 100 µl ($5.0x10^4$ cells) added to a cytospin assembly composed of a funnel, super-frost microscopy slide, and retaining clip. The cytospin assembly was entered into a cytospin centrifuge and spun at 1,000 rpm for 10 min. The slide was removed and the cells allowed to air dry overnight, and either placed into 4°C without fixation, or fixed in 2.5% neutral buffered formalin (outlined below) until required. Cytospin preparations were used for the optimisation of antibody concentrations (using NHBE and 16HBE14o- cells) for immunocytochemistry, or for cell lineage analysis of AEC.

2.2.1.2 Cells grown in 8-chamber culture slides

Cells were seeded into 8-chamber slides wells at $4.0x10^4$ cells per well. Cells were allowed to grow at 37°C in humidified 95% medical grade air plus 5% CO_2 overnight ready for treatment the following day. After treatment the slide was removed from the chamber and fixed in 2.5% neutral buffered formalin.

2.2.1.3 Cells grown on transwells

Cells cultured on polyethylene terephthalate transwell membranes were washed for 1min in 0.1% DTT in PBS to remove mucus, followed by 2x rinses with warm PBS. Cells were chilled at 4°C for at least 3 h, and then rinse by transferring the transwell sequentially to 5 beakers of ice cold PBS. Transwells were then transferred to a 12-well plate containing -20°C methanol, and incubated at -20°C overnight. Methanol was aspirated and 350 µl of -20°C acetone added to the transwells for one min. Acetone was aspirated and the transwells air-dried for approximately 1-2 h in a fume hood. Cells fixed on transwells were stored at 4°C until needed for immunofluorescence.

2.2.2 Fluorescence Immunocytochemistry

2.2.2.1 Fluorescence microscopy of cells grown on chamber slides

Immuno-fluorescence was used to determine the localisation of cIAP2 and XAF1 in cells cultured in 8-chamber slides. Cells were washed 2x in PBS then fixed in 2.5% neutral buffered formalin for 20 min at RT, then washed a further 5x in PBS and allowed to air dry for 2 h. Cells were permeabilised using 1% sodium dodecyl sulphate (SDS) in PBS for 10 min, and then washed 4x 3 min with tris-buffered saline with 0.05% Tween-20 (TBST). Serum free blocker (SFB) was added to the cells for 1 h at RT, followed by a cocktail of 6.6 µg/ml cIAP2 anti-human-goat polyclonal antibody, and 5 µg/ml XAF1 anti-human rabbit polyclonal antibody, diluted in 10% SFB/PBS solution. Goat (6.6 µg/ml) and rabbit (5 µg/ml) mock IgG proteins were used to control for non-specific binding of immunoglobulin to cells. Primary antibodies were incubated with the cells in a humidified chamber overnight at 4°C.

The following day unbound primary antibody was removed with 5x 7 min TBST washes. A cocktail of anti-goat Alexa-488 and anti-rabbit Alexa-594 conjugated secondary antibodies, diluted in 10% SFB/PBS solution at 1/100 and 1/200 respectively, were added to the cells for 1 h at RT, in dark conditions. The cells were then washed 5x 7 min using TBST to remove unbound secondary antibodies. After the third wash 200 ng/ml of the nuclear counter-stain 4',6-diamidino-2-phenylindole (DAPI) was added to resolve cell nuclei. Fluorescence mounting media was then applied to the cells, followed immediately

by a cover-slip. Slides were stored at 4°C in dark conditions until visualised using Fluorescence microscopy.

2.2.2.2 Fluorescence microscopy of cells grown on transwells

AECs grown on transwells were assessed for the formation of tight junction (TJ) complexes by staining for Zona occludens-1 (ZO-1) protein. Transwell filters with a monolayer of fixed epithelial cells were cut away and transferred to a 24-well plate, hydrated with TBST, then immersed in 10 drops of SFB for 45 min, at RT. Mouse monoclonal anti-human ZO-1, diluted to 5 µg/ml in TBST-10%SFB, was added to the excised transwell filters and allowed to incubate overnight at 4°C, with shaking at 60 rpm. The transwells filters were rinsed once, and then wash 5x 7 min in 500 µl of TBST, with shaking at 100 rpm. Anti-mouse Alexa-594 conjugated secondary antibody was diluted to 2 µg/ml in TBST-10%SFB, and incubated for 2 h at RT with the transwell membrane. The filters were rinsed once, and then wash 5x 7 min in 500 µl TBST, shaking at 100 rpm. After the third wash 200 ng/ml of the nuclear counter-stain DAPI was added to resolve cell nuclei. Filters were rinsed once with UPW, and 95% ice cold ethanol added for 1 h at 4°C. Filters were then transferred to a glass slide and a drop of anti-fade mounting medium added before cover-slipping. Mounting media was allowed to cure overnight, and then slides were stored at 4°C in dark conditions until visualised using Fluorescence microscopy.

2.3 Lineage Analysis of Primary Airway Epithelial Cells

2.3.1 Diff-Quick (Romanosky) Morphological Analysis

Diff-Quick analysis was used to determine the per cent composition of epithelial vs. contaminating cells in nasal brushing isolates, using cytospin preparations. Diff-Quick is a commercial version of Romanosky staining used for rapid lineage analysis of biological smears, producing similar results to Wright-Giemsa staining. Cells differentially stain:

Epithelial cells	- Nuclei dark blue
	- Nucleoli dark purple almost black
	- Cytoplasm varying light blue shades
Erythrocytes	- Pink or yellow-red
Platelets	- Violet to purple granules
Leucocytes:	
Neutrophils	- Nuclei dark blue
	- Cytoplasm blue
	- Granules reddish lilac
Eosinophils	- Nucleus blue
	- Cytoplasm blue
	- Granules red to red-orange
Basophils	- Nucleus purple or dark blue
	- Granules dark purple almost black
Monocytes	- Nucleus (lobulated) violet
	- Cytoplasm sky blue

Air dried cytospin preparations of cells immediately isolated from asthmatic individuals (n=2) and non-asthmatic individuals (n=2), were compared to cytospin preparations of cells cultured to passage 3 from the same subjects, from the same extraction (i.e. a portion of extracted cells were also kept for culture). The aim was to examine the comparative purity of AECs isolated from asthmatics vs. non-asthmatics with regard to inflammatory cell contamination, and to determine the percentage of AECs present in culture at passage three, when cells were routinely used. Cells were sent to the Institute for Medical and Veterinary Science (IMVS) Cytology Department (The Queen Elizabeth Hospital) for staining, cover-slipping and professional lineage analysis.

Imaging of Diff-Quick stained cells was performed using bright-field microscopy at 20x objective magnification. Counts of 100 cells were performed using three fields of view from replicate slides (n=2), for both direct-extraction and passage three cell preparations. Counts were averaged and expressed as a percentage of AEC and inflammatory cells (Table 2.1).

2.3.2 Immmunohistochemical Analysis

To complement Diff-Quick lineage analysis of AECs, immunocytochemistry was also performed to differentiate epithelial cells and leukocytes. Cytospin preparations were prepared using the same asthmatic (n=2) and non-asthmatic (n=2) subjects analysed in section 2.3.1. Here cells were fixed for 20 min in 2.5% neutral buffered formalin, washed 5x with PBS and allowed to dry for 3-5 h before storage at 4°C. Cells were sent to the IMVS cytology department (The Queen Elizabeth Hospital) for colorimetric immune-staining, and cover-slipping. Cells were labelled with monoclonal antibodies anti-AE1/3 (anti-PAN cytokeratin) to resolve AECs (brown) and anti-CD45 to resolve leukocytes (blue). Bright-field microscopy imaging and counting was performed by the Cytology staff at 20x objective magnification. Scoring was performed as per the counting system used for the Diff-Quick cell typing analysis. Any cell with brown staining was considered epithelial. Counts were averaged and expressed as a percentage of AEC and inflammatory cells (Table 2.2).

Table 2.1: Lineage analysis of primary airway cells via morphological characteristics

Cells taken from each donor (n=2 control, and n=2 asthmatic) were examined after extraction and after culture to passage 3. Morphological characteristics were used to define epithelial and inflammatory cells. Values represent an average of three counts of 100 cells, and are expressed as a percentage of total cells. Although a significant amount of cells from asthmatics are of an inflammatory origin on extraction, the culture process favours the growth of cells of an epithelial lineage.

	Extracted cells		Cultured cells	
	Epithelial (%)	Inflammatory (%)	Epithelial (%)	Inflammatory (%)
Asthmatic 1	53	47	100	0
Asthmatic 2	45	55	100	0
Control 1	96	4	100	0
Control 2	98	2	100	0

Table 2.2: Lineage analysis of primary airway cells via immunohistochemistry

Cells taken from each donor (n=2 control, and n=2 asthmatic) were examined after extraction and after culture to passage 3. Reactivity to primary antibodies directed to cytokeratin (an epithelial marker) and CD45 (a leukocyte marker) were used to define epithelial and inflammatory cells, respectively. Values represent an average of three counts of 100 cells, and are expressed as a percentage of total cells. Similar to the morphological analysis, results indicate the culture process favours the growth of epithelial cells.

	Extracted cells		Cultured cells	
	Epithelial (%)	Inflammatory (%)	Epithelial (%)	Inflammatory (%)
Asthmatic 1	88	12	100	0
Asthmatic 2	93	7	100	0
Control 1	100	0	100	0
Control 2	98	2	100	0

2.3.3 Primary Cells Grown at an Ari-Liquid Interface Exhibit Characteristics of Airway Epithelial Cells

Primary AEC were seeded on to transparent polyethylene terephthalate transwells, to enable them to be cultured at an air-liquid interface (ALI; Figure 2.1.A). Passage 2 AECs were seeded at a density of $1.0x10^5$ cells per well onto the transwells, and cultured to confluence for five days using AEC growth media (500 µl in the apical chamber and 1.5 ml in the basal chamber). AEC growth media was then removed from the apical chamber, exposing the apical cell surface to the atmosphere, and the cells left for at least five days at an ALI. Unlike inflammatory cells, epithelial cells are able to survive at an ALI. Cells cultured in this manner exhibit characteristic of epithelial cells including a polygonal shape, contact growth inhibition and defined margins (Figure 2.1.B).

The formation of TJ complexes at the apicolateral margin of plasma membranes between adjacent cells is a feature of the airway epithelium. Primary AECs from three control donors grown at an ALI, exhibit an interconnecting pattern of ZO-1 expression (Figure 2.1.C). ZO-1 is an essential protein in TJs (e.g. (398)), hence this expression pattern is indicative of cell-to-cell interactions through TJ complexes. Cell cultures from an additional two control donors were grown at an ALI and examined for the production of cilium. PneumaCult® cilium growth supplements (100x) were added to AEC growth media (1x final concentration), and transferred into the basal chamber of ALI cultures for 15-20 days. Scanning electron microscopy performed by the Adelaide Microscopy Unit (University of Adelaide, North Medical School), confirmed the presence of cilium at the apical margins of cells monolayers (Figure 2.1.D).

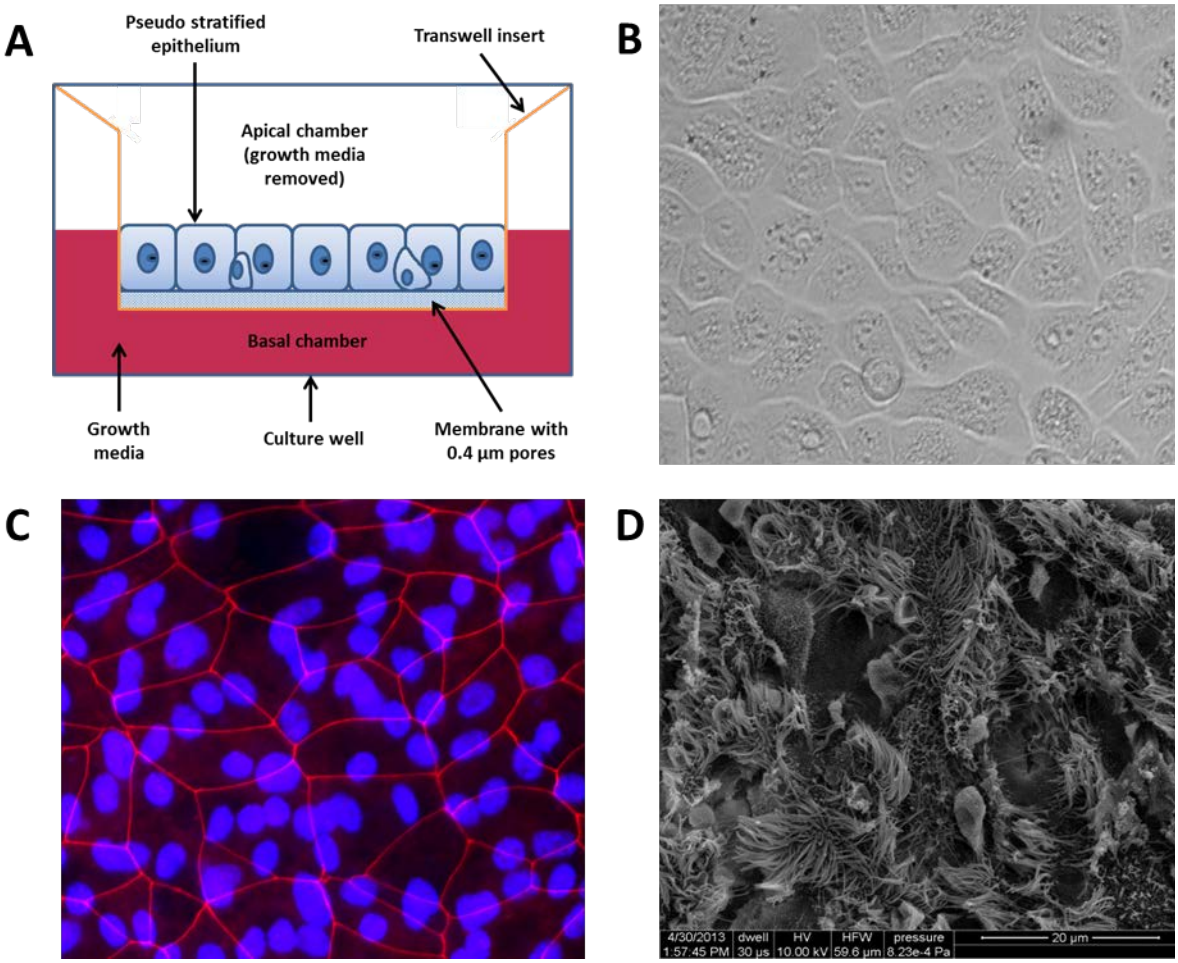

Figure 2.1: Airway biopsies propagated *ex vivo* exhibit characteristics of AECs

Cells derived from healthy donors were maintained at an air-liquid interface (ALI) and assessed for traits consistent with airway-epithelial cells. **A**: A schematic representation of cells grown at an ALI. Removing growth media from the apical chamber exposes cells to the atmosphere. Nutrient in the basal chamber passage a membrane perforated with 0.4 µm pores. A psuedostratifed layer of epithelial cells forms, and exhibit significant mucus production after approximately 7 days at an ALI, indicative of the presence of goblet cells. **B**: Cells grown at an ALI demonstrate contact inhibition, polygonal shape, and defined margins common to cells of an epithelial lineage. **C**: Transwells with cells grown at an ALI and stain for the presence of ZO-1 protein (red), which is localised between interfacing cells, indicating the formation of tight junction complexes. The DNA counter-stain DAPI was used to resolve nuclei (blue), and shows more nuclei than cell boundaries, suggesting the formation of a three dimensional, pseudostratified cell layer. **D**: Scanning electron microscopy shows cells grown at an ALI can be stimulated to produce cilia.

2.4 Western Analysis

2.4.1 Protein Extraction

Culture plates were placed on ice and media aspirated from the cell monolayer, which were washed twice with ice cold PBS. Cell monolayers were covered with M-Per® mammalian protein extraction buffer, containing 1x Halt® protease inhibitors, and 1 mM phenylmethanesulfonyl fluoride. Cells were collected using a cell scraper and the resulting homogenate transferred to -80°C to fracture any cells still intact. Before quantifying, the protein solution was thawed and centrifuged at 1,600 g at 4°C for 5 min to remove insoluble cellular debris.

2.4.2 Quantification of Protein Concentration

Protein samples were quantified using the DC™ protein assay kit (a modified version of the Lowry protein assay), as per the manufacturers' instructions, to allow equal sample loading during electrophoresis. Briefly, 5 µl of protein samples were added in triplicate to a 96-well microplate, followed by 25 µl of solution A (alkaline copper tartrate), and 200 µl of solution B (Folin reagent), and allowed to react for 30 min. Protein absorbance (750 nm) was then read using a microplate reader and concentration determined in relation to a bovine serum albumin (BSA) protein standard, via linear regression.

2.4.3 Denaturing Gel Electrophoresis

2.4.3.1 Sample preparation

Typically 10 µg of protein to a total volume of 10 µl in sample buffer were run in electrophoresis, using reducing conditions. To denature protein, samples in electrophoresis sample buffer were heated to 72°C for 10 min before loading,

2.4.3.2 Electrophoresis

The XCell SureLock ™ electrophoresis tank system was used in conjunction with 4-12% Bis-Tris gradient gels for electrophoresis, as per the manufacturers' instructions. Protein samples were added to the gel, and an electric potential was applied across the gel of 200V for 2 h. Electrophoresis running buffer was supplemented with antioxidant to prevent

oxidation of amino acids such as methionine. In each case a protein molecular weight marker was included to the outer most wells.

2.4.4 Western transfer

The XCell SureLock^{TM} mini cell transfer apparatus was used to perform protein transfer from Bis-Tris gels to nitrocellulose membranes, as per the manufacturers' instructions. An electrical potential difference was applied through the gel/nitrocellulose membrane assembly at 40 V for 2 h, while bathed in western transfer buffer. After transfer the membrane was washed in TBST to remove residual gel, and either probed immediately with antibodies or stored in TBST at 4°C until needed.

2.4.4.1 Antibody labelling

Membranes were blocked for 1 h at RT with gentle shaking, in the appropriate blocking reagent for the primary antibody being used, as outlined in Table 2.3. Primary antibodies were diluted in 10 ml of the appropriate blocking reagent, either 5% skim milk or 5% BSA, suspended in TBST. The primary antibody solution was added to the membrane and allowed to incubate overnight at 4°C with gentle rotation using an orbital shaker. The following day, the membranes were washed 5x 20 min in TBST. The appropriate concentration of anti-mouse or anti-rabbit horseradish peroxidase (HRP) labelled secondary antibody was diluted in 5% skim milk in TBST, and incubated with the membrane for 1 h at RT, with gentle shaking. Membranes were then washed 5x 25 min in TBST before imaging.

Table 2.3: Detection antibodies specification for western analysis

Designation	Molecular weight (kDa)	Host	Primary antibody dilution	Secondary antibody dilution	Manufacturer	Product number	Blocking and primary antibody incubation (w/v)
B-actin	42	Mouse	1:10,000	1:20,000	Sigma	A1978	**5% non-fat dry milk**
Bcl2	27	Rabbit	1:500	1:2000	Santa Cruz	sc-492	**5% non-fat dry milk**
PARP	116/89	Rabbit	1:2000	1:5000	Cell Signalling	9542	**5% non-fat dry milk**
Caspase-3	35, 19, 17	Mouse	1:3000	1:5000	Cell Signalling	9668	**5% non-fat dry milk**
cIAP1	72	Mouse	1:2000	1:4000	R&D Systems	MAB818	**5% non-fat dry milk**
cIAP2	70	Rabbit	1:1000	1:2000	Cell Signalling	3130	**5% BSA**
XAF1	32 (35 predicted)	Rabbit	1:4000	1:5000	AbCAM	Ab17204	**5% non-fat dry milk**
XIAP	57	Mouse	1:10,000	1:10,000	BD Biosciences	610762	**5% non-fat dry milk**

2.4.4.5 Imaging and quantification

Membranes were incubated for 5 min in dark conditions with ECL^{TM} detection reagent. ECL^{TM} reagent contains luminal to allow the production of a chemiluminescent signal through its oxidation by HRP bound to the secondary antibody. After incubation the membrane was entered into a LAS-3000 imager (Fujifilm Corp.) for chemiluminescent signal detection. Images were quantified using an analysis of histogram data using Multi Gauge software (V3.0, Fujifilm Science Lab), and results average for three repeat experiments.

2.5 Real Time Polymerase Chain Reaction

2.5.1 RNA Extraction

RNA was extracted from cells cultured in 6-well plates using the RNeasy® isolation method as per the manufacturers' instructions. Briefly, RLT cell lysis buffer was supplemented with B-mercaptoethanol (10 µL per ml of RLT buffer) and 600 µl added to cell monolayers to release and stabilize RNA. The cell lysate was collected and passed through a QIAshredder® filter column by centrifugation at 1,600 g for 2 min at RT to retain cellular debris, and then 600 ul of 70% ethanol added to the filtrate. The RNA filtrate-ethanol mix was added to an RNeasy® filter column to bind RNA by centrifugation at 1,600 g, for 30 seconds. RNA bound to the filter was further washed of protein and salt impurities using RNA wash buffers provided in the RNeasy® isolation kit. Finally, RNA was eluted into a collection tube using 50 µl of nuclease free water, and stored at -80°C.

2.5.2 Nucleic Acid Quantification and Quality Assessment

RNA concentration was quantified using spectrophotometry to allow equal amounts to be loaded into reverse transcription reactions. RNA preparations were accepted when 260/280 ratios of 1.8 – 2.0, and 260/230 ratios of 1.8 or greater were achieved. A quality control RNA sample of known concentration was included during spectrophotometry.

2.5.3 Complementary DNA Synthesis

RNA was reverse transcribed into complementary DNA (cDNA) to enable examination of transcript abundance using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Reverse transcription was routinely performed using M-MLV reverse transcriptase and random hexamers, using 2 µg of RNA in 20 ul reactions, as outlined below. Samples were incubated using a PTC Peltier 200 thermal cycler for 1 h at 37°C to maintain enzyme activity for cDNA synthesis, followed by a 72°C enzyme inactivation step. A control lacking reverse transcriptase was included to test for genomic DNA (gDNA) contamination, and a control lacking RNA was included to test for RNA contamination during cDNA synthesis. Each control was entered into subsequent qRT-PCR experiments with cDNA samples. cDNA samples were maintained at 4°C for short term storage (weeks) or -80°C for long term storage.

Reverse transcription reaction chemistry:

	µl	Final Concentration
First Strand Buffer (5x)	4	1x
Deoxyribonucleotides (10 mM)	1	0.5 mM
DTT (0.1 M)	2	10 mM
Random hexamers (250 ng/µl)	2	25 ng/µl
MMLV reverse transcriptase (200 U/µl)	1	10 U/µl
RNA (2 µg)	X	100 ng/µl
PCR grade ultra-pure water	to 20 µl	

2.5.4 Quantitative Polymerase Chain Reaction

qRT-CPR was performed using a Corbett Life Sciences (Pty Ltd) Rotor-Gene^{TM} 6000 thermal cycler in 20 µl reactions (outlined below), using 100 ng of cDNA template. HotStar® Taq DNA polymerase was used in conjunction with Syto®-9 green Fluorescence DNA staining solution (absorption 485 nm and emission 498 nm), for amplicon generation and measurement, respectively. Cycling conditions included a single 15 min 95°C enzyme activation step, followed by 35 cycles of a 15 sec 95°C DNA melting step, and a 1 min 65°C duel annealing/extension step. Syto®-9 fluorescence was acquired during the annealing/extension step on the FAM channel. Amplification threshold was maintained above background noise and gain set within 3-4 for all runs. An amplicon melt analysis was performed after each run to determine the number of PCR products being generated. Reaction chemistry (shown below) was optimized to allow primer sets to be used interchangeably in the same PCR conditions. Primers were designed in house as described below. In each run, a reaction devoid of template nucleotides (no template control) was incorporated to assess for DNA contamination of the reaction mixture.

PCR reaction chemistry:

	1x (µl)	Final concentration
Reaction buffer (10x)	2	1x
Deoxyribonucleotides (10 mM)	0.4	0.2 mM
$MgCl_2$ (25mM)	0.2	0.25 mM
Forward primer (50 µM)	0.8	2 µM
Reverse primer (50 µM)	0.8	2 uM
Syto®-9 (0.1 mM)	1.0	5 nM
HS polymerase (5 U/µl)	0.1	0.025 U/µl
Template cDNA (100 ng/µl)	1	5 ng/µl
Ultra-pure water	to 20 ul	

2.5.5 PCR Product Purification

The QIAquick® PCR product purification kit was used to clean PCR reaction products for subsequent analysis, following the manufactures' instructions. Briefly, 100 µl of PCR product from a single run was added to binding buffer and transferred into a DNA binding column. The column was centrifuged at 1,600 g for 60 sec to bind DNA to the filter, and the flow through was discarded. The DNA was cleaned of impurities by centrifuging wash buffer through the filter with at 1,600 g for 60 sec. DNA was then eluted using TE buffer (pH 8) into a clean tube.

2.5.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to examine PCR reactions, to determine amplicon size and test for the presence of gDNA amplification. Agarose gels were prepared by adding 2 g of agarose I to 100 ml of 0.5x TBE (2% agarose w/v) buffer and boiled in a microwave oven for 2 min. GelRed^{TM} nucleic acid stain (10,000x stock) was added to the agarose gel for a final concentration of 1x. The agarose gel solution was then poured into a gel setting frame and a 20 well comb inserted. Once solid (approximately half an hour) the agarose gel was placed into an electrophoresis tub and 0.5x TBE buffer added until the gel was completely immersed. Typically 5 µl of PCR reaction product was added to 1 µl of 6x loading buffer and the sample added to wells. In addition, 5 µl of pUC19 DNA ladder was added to each of the outer wells to allow amplicon sizes to be determined. Electrophoresis was performed for 2 h at 100 volts and then imaged with 300 nm transillumination using a gel dock system. Gel photos were taken and digital images acquired for examination.

2.6 qRT-PCR Primer Design

2.6.1 Primer Design

Target sequences were obtained from the National Center for Biotechnology Information (NCBI) Nucleotide data base (http://www.ncbi.nlm.nih.gov/nuccore). To generate mRNA specific oligonucleotide primers which avoid amplification of gDNA, at least one primer was designed which spanned an exon-exon boundary, which did not share sequence similarity with gDNA in its 3` bases (described in Figure 2.2.A). One exception was the primers for the reference gene *Hypoxanthine phosphoribosyltransferase-1* (*HPRT1*). The nature of *HPRT1's* nucleotide sequence was not amenable to this method of excluding gDNA amplification. Instead primers for *HPRT1* were designed to span an 18, 059 base pair (bp) intron (predicted amplicon size = 18,287 bp) located in its genomic sequence, to favour the amplification of the significantly shorter 228 bp product, predicted for its mRNA sequence. To prevent cross reaction of IAP primers with other IAP family members, an alignment of *BIRC2*, *BIRC3* and *BIRC4* transcripts was performed (Clustal W Multiple Sequence Alignment (399)), to identify and exploit dissimilarities in their respective mRNA nucleotide sequences (described in Figure 2.2.B).

Primer sequences were further examined using the online application Oligoproperties Calculator (400), which was used to exclude primer oligonucleotides predicted to exhibit self-complementarity or produced primer dimers. Oligoproperties Calculator was also used to customize primer melt temperatures (between 67-68°C; salt adjusted), to allow all primer sets to share identical cycling conditions during qRT-PCR. Finally, NCBI's Primer-BLAST (401) was used to ensure primer sequences were predicted to bind to their intended mRNA target, and exclude gDNA (with the exception of *HPRT1* primers), unintended mRNA nucleotides, and pseudogenes.

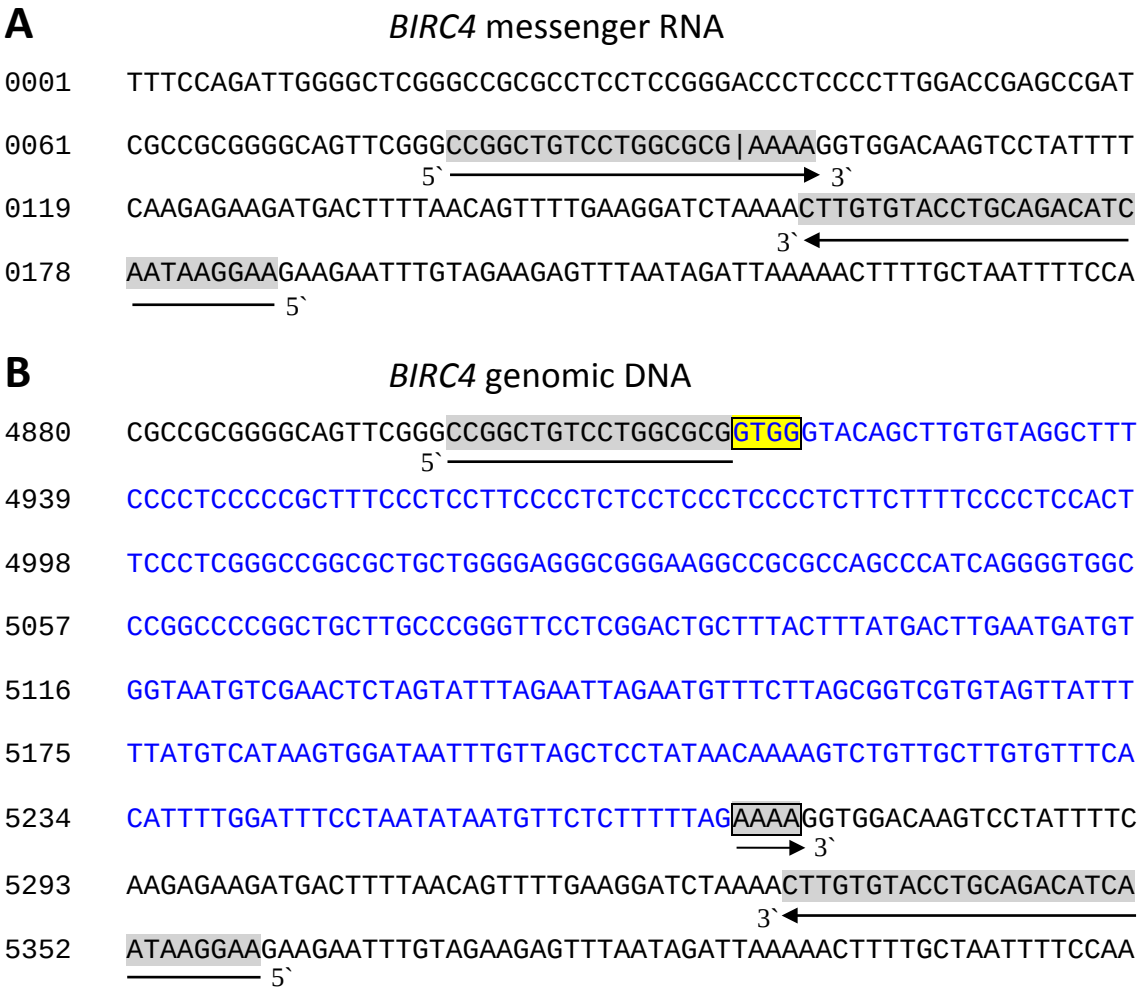

Figure 2.2: Design of mRNA specific qRT-PCR primers

Primers for qRT-PCR were designed to bind specifically to cDNA (i.e. reverse transcribed mRNA; black text), and prevent amplification of gDNA (blue text). **A**: For example, primers (grey highlight) directed to *BIRC4* (XIAP) transcripts, are predicted to bind faithfully to its mRNA sequence (accession number NM_001167.2) and produce a 109 bp amplicon using cDNA template. The forward primer spans the boundary of exons one and two (delineated by |). **B**: However, in the *BIRC4* genomic sequence (accession number NG_007264.1), primers span intron 2 (blue text), and are predicted to produce a larger 469 bp amplicon. To further prevent the amplification of gDNA, the four 3` bases of the forward primer (box, grey highlight) were selected to prevent complementary base pairing with the first four bases of intron 2 (box, yellow highlight). The presence of a 3` primer-gDNA mismatch, has the effect of preventing the binding of the polymerase enzyme, thereby stopping the amplification of the gDNA sequence. Forward primers are denoted by "→" arrows, and reverse primers by "←" arrows. Numbers in left margin denote bases in the mRNA or gDNA sequences of *BIRC4*.

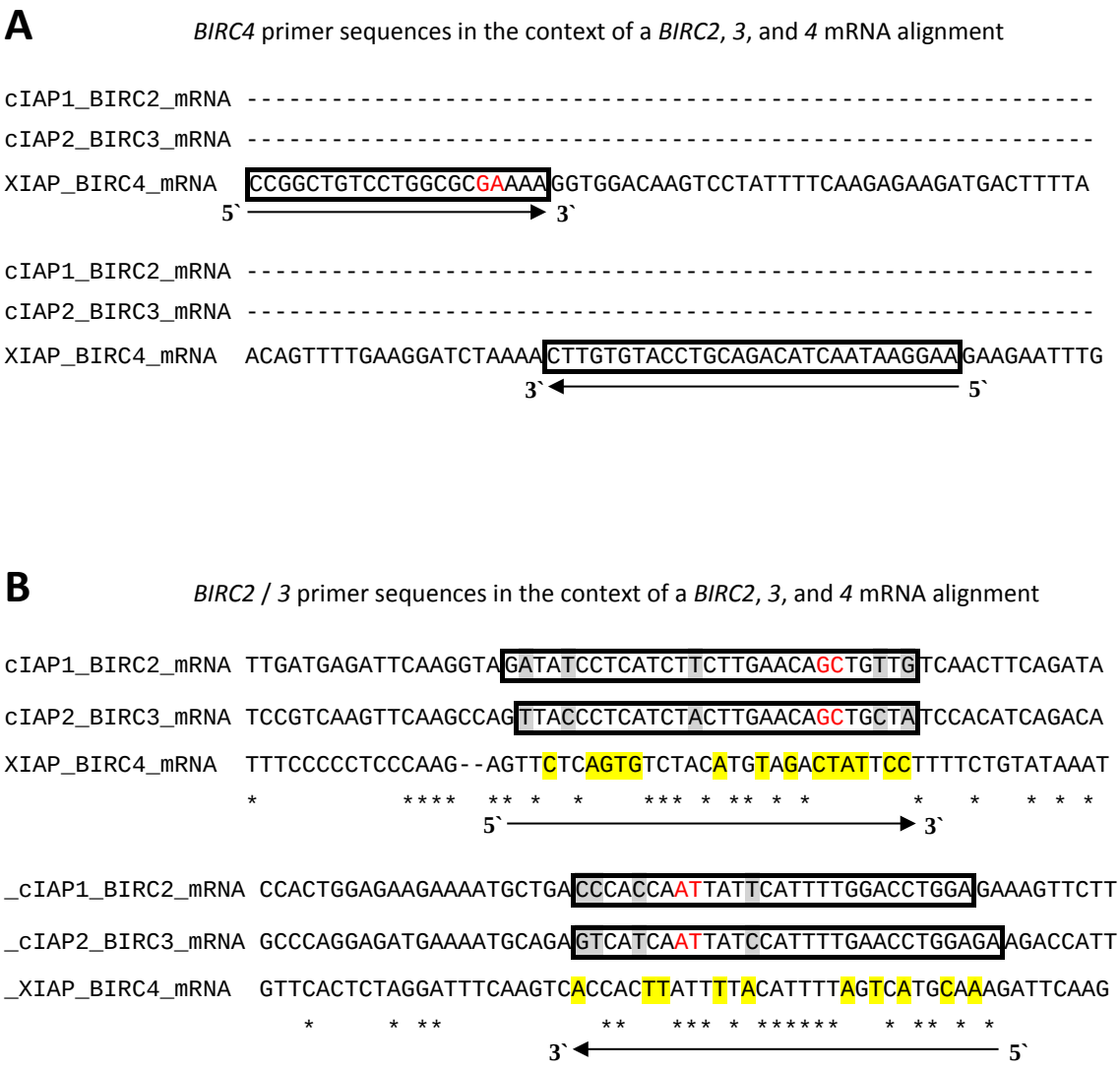

Figure 2.3: Design of IAP specific qRT-PCR primers

An alignment of *BIRC2*, *BIRC3*, and *BIRC4* transcripts shows primer sequences (box) exploit base mismatches to permit specific amplification of targeted IAP family members. **A**: Primers for *BIRC4* were chosen to exploit its mRNA's extended 3` untranslated region, which is not present in *BIRC2* or *BIRC3*. **B**: *BIRC2*, *BIRC3*, and *BIRC4* transcript sequences are highly similar around exon-exon boundaries. Hence, priming regions chosen for *BIRC2* and *BIRC3* exploit base mismatches (grey highlight) in their respective transcripts, which correspond with the 3` region of both the forward and revers primer. Similarly, the region of the *BIRC4* transcript corresponding to the *BIRC2* and *BIRC3* primer sequences exhibit significant dissimilarity (yellow highlight). Forward primers are denoted by "→", and reverse primers by "←." Exon boundaries are indicated by red text. Bases not present in a transcript covered by the alignment are shown as a dash (-). Bases shared by each transcript are denoted by an asterisk (*).

2.6.2 Primer Validation

Primer oligonucleotides designed in silico were synthesised (GeneWorks Pty Ltd, Adelaide, Australia), for empirical validation. To determine whether primers exhibited reaction efficiencies between 90-110%, qRT-PCR reactions were performed with a 10-fold template DNA dilution series, to generate standard curves for the primer sets (Figure 2.4.A). Template DNA for efficiency experiments was made by amplifying cDNA generated with the 16HBE14o- cell line, using the primers to be assessed. The resulting PCR product was then re-amplified, using the same primers, in a dilution series. The cycle threshold (Ct) was set to produce Ct values above background signals. Final reaction efficiency values (shown in Table 2.4), represent an average of five replicate standard curve experiments in which regression analysis produced R^2 values exceeding 0.990. qRT-PCR reaction graphics, linear regressions, R^2 values and reaction efficiency values were determined using the Rotor-Gene™ 6000 series software (V1.7, build 87).

To determine whether reactions using the primers produced a single amplicon, amplification product melt analysis (APMA) was performed following reactions, using cDNA generated from both 16HBE14o- and NHBE airway cell lines (Figure 2.4.B). APMA operates by progressively increasing the temperature of completed PCR reactions. DNA amplicons melt (dissociate into single stands), thereby releasing the DNA dye (here Syto® -9), which can be subsequently read by the Rotor-Gene™ 6000 photo-detector. Amplicons of different size and GC content melt at specific temperatures, enabling the detection of multiple PCR products in a single reaction. The presence of a single melt peak was observed for all PCR primers (ultimately used in this investigation), indicating targeted sequences were amplified, and not gDNA or unintended mRNA nucleotide sequences. Melt curve analysis was performed using the Rotor-Gene® 6000 series software. To verify APMA results, qRT-PCR product generated using HNBE-derived cDNA, was assessed using agarose gel electrophoresis (Figure 2.4.C). Single bands of amplified product are observed, and confirm the production of a single amplicon when using the respective primer sets, and reaction conditions. Primers exhibiting efficiency below 90%, or which produced more than two amplicons were redesigned. Table 2.4 summarises the specifications of the validated primers sets.

Figure 2.4: qRT-PCR primer validation.

Primers were tested for reaction efficiency, and specificity for target transcripts. **A**: Standard curves for each primer pair (*BIRC3* is shown here) were generated using four to five 10-fold dilutions of template. Amplification curves (orange lines) represent the increase in amplicon generation (fluorescence) for successive cycles. Cycle threshold (Ct) was set to produce Ct values above background noise. **B**: Amplification product melt curve analysis of reactions using *BIRC3* primers produces a single peak, indicating a single amplicon is generated. Template cDNA was derived from 16HBE14o- (blue) and NHBE (orange) airway epithelial cell mRNA. A reaction lacking cDNA template (black) serves as a 'no template control.' For the y-axis, dF/dT refers to the derivative fluorescence vs. temperature. **C**: qRT-PCR products generated using cDNA derived from NHBE mRNA, were examined using agarose gel electrophoresis. A single product is detected for each primer set, and the no-template control (right, adjacent lane) did not amplify. PCR product sizes here are consistent with those predicted during in silico primer design. Marker is pUC19 ladder. "bp" base pair. The gel graphic was altered (separation) to exclude amplicons generated with primers that were not used in later investigations.

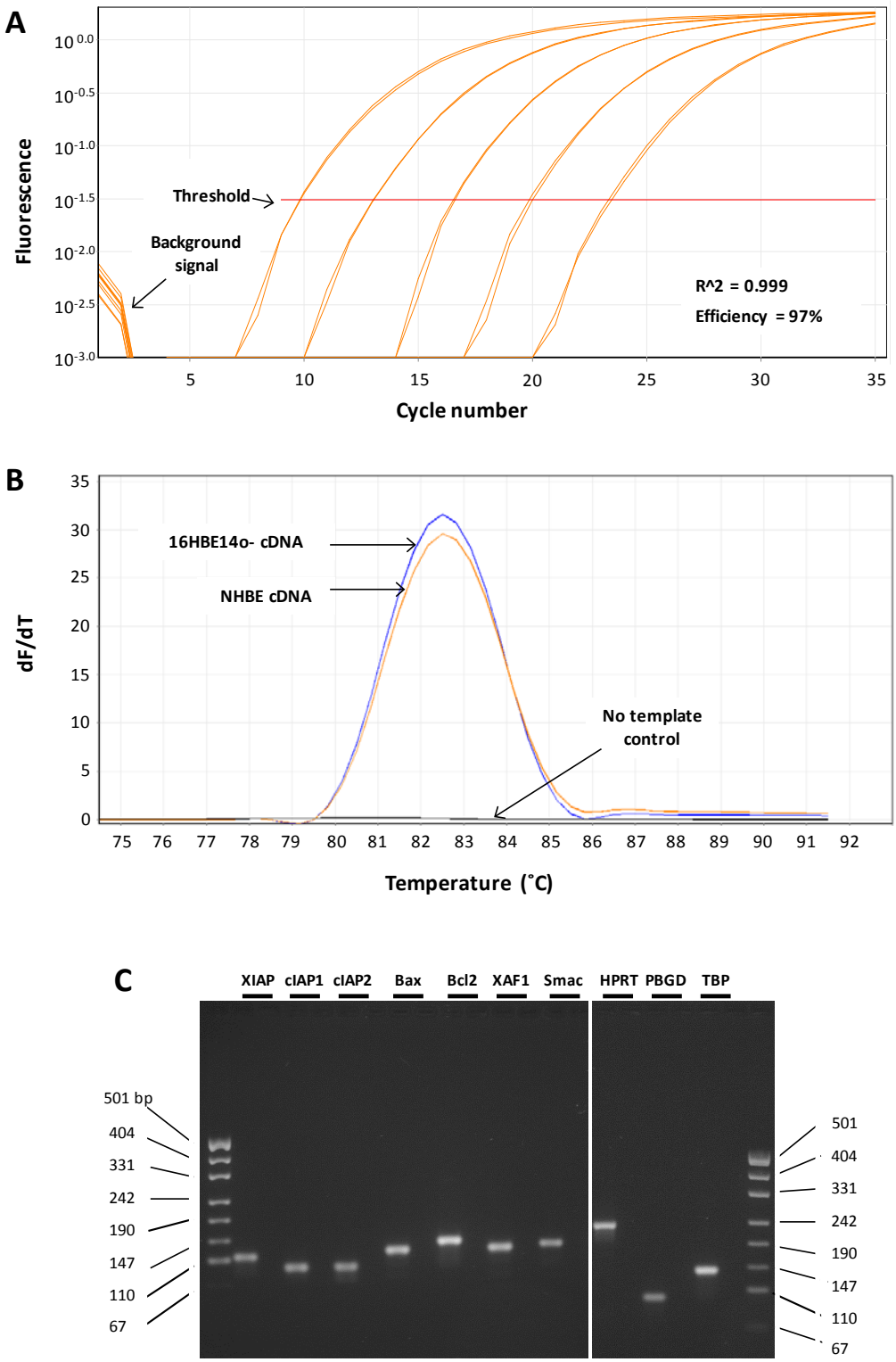
A
Fluorescence
Threshold
Background signal
R^2 = 0.999
Efficiency = 97%
Cycle number
B
dF/dT
16HBE14o- cDNA
NHBE cDNA
No template control
Temperature (˚C)
C
XIAP cIAP1 cIAP2 Bax Bcl2 XAF1 Smac HPRT PBGD TBP
501 bp
404
331
242
190
147
110
67

Table 2.4: qRT-PCR primer specifications

Gene	Accession	Primer sequence (5` → 3`)	Direction	Spans exons	Melt temp. (˚C)	GC content (%)	Length (bp)	Efficiency (%, +/- SD)	Amplicon length (bp)
BIRC4 (XIAP)	NM_001167.2	CCG GCT GTC CTG GCG CGA AAA	Forward	Y	67	67	21	93 (4.09)	109
		TTC CTT ATT GAT GTC TGC AGG TAC ACA AG	Reverse	N	67	41	29		
BIRC2 (cIAP1)	NM_001166.3	GAT ATC CTC ATC TTC TTG AAC AGC TGT TG	Forward	Y	67	41	29	100 (3.37)	92
		TCC AGG TCC AAA ATG AAT AAT TGG TGG G	Reverse	Y	67	43	28		
BICR3 (cIAP2)	NM_001165.3	TTA CCC TCA TCT ACT TGA ACA GCT GCT A	Forward	Y	67	43	28	100 (3.66)	92
		TCT CCA GGT TCA AAA TGG ATA ATT GAT GAC	Reverse	Y	67	37	30		
Smac	NM_019887.3	TCA TAG GAG CCA GAG CTG AGA TGA C	Forward	Y	67	52	25	99 (4.63)	129
		CCT GAT CTG CGC CAG TTT GAT ATG C	Reverse	N	67	52	25		
XAF1	NM_017523.2	GCC CAG CTC GGG AAA GGG GAA A	Forward	Y	68	64	22	98 (4.38)	124
		CTG AGT CTG GAC AAC ATT TAC CCA TAT G	Reverse	Y	67	43	28		
Bcl2	NM_000633.2	GTC ATG TGT GTG GAG AGC GTC AAC	Forward	N	67	54	24	101 (4.13)	137
		AGT TCC ACA AAG GCA TCC CAG CC	Reverse	Y	67	57	23		
BAX	NM_138761.2	CAG TAA CAT GGA GCT GCA GAG GAT GA	Forward	Y	68	50	26	102 (3.83)	117
		ACC CGG CCC CAG TTG AAG TTG C	Reverse	N	68	64	22		
HPRT1	NM_000194.2	GGC TAT AAA TTC TTT GCT GAC CTG CTG	Forward	N	67	44	27	105 (4.16)	228
		CAA AGT CTG CAT TGT TTT GCC AGT GTC	Reverse	N	67	44	27		
TBP	NM_003194.4	CGA AAC GCC GAA TAT AAT CCC AAG CG	Forward	Y	68	50	26	101 (2.34)	136
		CCA GTC TGG ACT GTT CTT CAC TCT TG	Reverse	N	68	50	26		
PBGD	NM_000190.3	CGC ATC TGG AGT TCA GGA GTA TTC G	Forward	Y	67	52	25	99 (2.49)	92
		TGC CAG GAT GAT GGC ACT GAA CTC	Reverse	N	67	53	24		

2.7 Cell Viability, Apoptosis and Necrosis Assaying

2.7.1 Lactate Dehydrogenase Cytotoxicity Assay

Cytotoxicity was determined via lactate dehydrogenase (LDH) release from compromised cells using a cytotoxicity detection kit as per the manufacturers' instructions. LDH is a highly stable enzyme which is released from compromised cells, from the cytosol into the media. After treatment media was taken from cell monolayers and stored at -20°C for future cytotoxicity assays. In addition to media, a single well of cells was lysed using 2% Triton-X 100 detergent dissolved in growth media to provide a maximum LDH control, and growth media alone was kept to provide a LDH background control. Samples of media or control (100 µl) were mixed with reaction buffers to produce a red colour reaction, and absorbance measured at 490 nm in triplicate. To determine percentage cytotoxicity the equation below was applied.

$$Cytotoxicity\ (\%) = (experimental\ value - low\ control) \div (high\ control - low\ control)$$

Experimental value = treated cells

Low control = untreated cell control

High control = LDH maximum made by complete cell lysis with Triton-X 100

2.7.2 Microscopy for Caspase-3/7 Activity and Necrosis

Caspase-3/7 activity was detected via Fluorescence microscopy using the FAM-FLICA™ caspase activity detection kit. Analysis of specific caspases is possible as caspase enzymes react with particular protein sequences, here for caspase-3/7 the DEVD region of protein substrates is recognised. FAM-FLICA™ fluorescence is prolonged when covalently bound to the DEVD binding regions of activated caspases-3 and -7. After treatment, adherent cells growing on 8-chamber culture slides were washed 3x with PBS. Then 250 µl of growth media containing 50 µl of 30x FAM-FLICA™ reagent, 6 µg/ml Hoechst stain, and 7.5 µg/ml propidium iodide stain was added to cells. Hoechst is a nuclear stain which was used to localise cells. Propidium iodide is also a nuclear stain, but is excluded from viable cells, and incorporated into the DNA of necrotic cells. The cells were returned to the

culture cabinet to incubate for 1 h, and then washed 4x with growth media. Cells were fixed using 2.5% neutral buffered formalin in PBS, as previously described. Fluorescence imaging was used to resolve cleaved FAM-FLICA^{TM} (excitation 488 nm and emission 530 nm), Hoechst (excitation 365 nm and emission 480 nm) and propidium iodide (excitation 490 nm and emission 520 nm) at 40x magnification, using a Nikon 90i microscope.

2.7.3 Western Analysis for Caspase Activity and Apoptosis Detection

Western analysis was used to demonstrate caspase activity by resolving cleavage products of caspase-3. Caspases are converted from inactive zymogens by cleavage events leading to the production of caspase heterotetramers. In their activated state, caspases are able to cleavage cellular substrates. Apoptosis was detecting through the cleavage of Poly(ADP-Ribose) Polymerase (PARP), which is mediated by active caspase-3, and demonstrates the cells entry into programmed cell death.

2.7.4 Determination of the *Bax*:*Bcl2* Transcript Ratio

qRT-PCR was used to determine the modulation of *Bax* and *Bcl2* transcript levels. The relative abundance of Bax which potentiates cytochrome-*c* release from the mitochondria, and Bcl2, an inhibitor of cytochrome-*c* release, is an early indicator of apoptotic change (402). Results were expressed as a ratio of *Bax* vs. *Bcl2* transcript abundance.

2.8 Gene Expression Knockdown with Small Interfering RNA

Small interfering RNA (siRNA) was used to deplete levels of *cIAP2* transcripts, and hence protein, in AEC cultures. A list of siRNA oligonucleotides used is shown in Table 2.5. $1.2x10^5$ cells were plated one day before transfection in a 12-well plate. Growth media was removed and replaced with 500 µl of fresh media and cells returned to the incubator. Transfection complex was made up to 100 µl in supplement free growth media, by adding 6 µl of 20 µM stock siRNA preparations with 9 µl of HiPerFect^{TM} transfection reagent. The transfection complex mix was then vortexed for 10 sec and allowed to incubate at RT for 10 min. Cells were retrieved form incubation, and transfection complex added drop wise to the appropriate wells, and then returned to incubate for 3 h. After 3 h a further 1 ml of media was added to each well and allowed to incubate for a further 24 h for a final siRNA concentration of 75 nM. These silencing (transfection) conditions were derived

after optimising both transfection reagent and siRNA concentration. A Fluorescence siRNA probe was used during optimisation, which could be examined microscopically to track its entry into the cells. A transfection reagent control and a scrambled siRNA control were included in all experiments to rule out knockdown by the transfection conditions alone. Knockdown was confirmed using western analysis.

Table 2.5: siRNA molecules used in knockdown procedures

Designation	Gene Accession	Function
Hs_BIRC3_7	NM_001165	cIAP2 silencing
Alexa 488 siRNA control	n/a	siRNA optimisation
All Stars_1 scrambled siRNA	n/a	Scrambled siRNA control

2.9 Statistics

Quantitative PCR (qPCR) data was analysed by the delta-delta Ct (403), method and results expressed as fold change after double normalisation to both endogenous control genes and control samples.

Densitometry data from western blots (section 2.4.4.5) were (natural) logarithm transformed prior to analysis, normalised to the control protein data, and then exponentiated to derive relative abundance values.

Analysis was performed using a linear regression approach, and p-values were derived from the regression coefficients and their standard errors (Wald test).

Statistical analysis methods for the genotyping data are described in the manuscripts in the relevant sections.

Chapter 3: Cellular inhibitor of apoptosis-2 is a critical regulator of apoptosis in airway epithelial cells treated with asthma related inflammatory cytokines

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Chapter 3: Synopsis

The current focus on the epithelium of asthmatics has led to the discovery that its disease-potentiating properties manifest in response to the inflammation, and intrinsic defects in airway epithelial cells (AEC) which render them susceptible to injury. However, this has not furthered the understanding of why AECs of asthmatics undergo apoptotic changes, or whether anomalies exist in the endogenous mechanisms which regulate apoptosis. This is demonstrated by the paucity of research scrutinising the role of the potent prosurvival family of proteins, called the Inhibitors of Apoptosis (IAP).

IAPs are influential regulators of caspases, death receptor complexes, and multiprotein death-inducing platforms. Further to this, the IAPs exhibit other cell survival functions, and are important in the mechanisms which regulate inflammation. X-linked inhibitor of apoptosis (XIAP), and the Cellular inhibitors of apoptosis (cIAP1 and cIAP2), inhibit multiple apoptosis signalling pathways, and are expressed in most mammalian cells. Consequently, dysfunction in the IAPs may render AECs of asthmatics susceptible to proapoptotic changes.

In Chapter 3, XIAP, cIAP1 and cIAP2, were initially examined for a role in regulating apoptosis in an *ex vivo* model of cytokine-treated primary nasal AEC from controls and asthmatics. The IAP-antagonist, Second mitochondrial activator of caspases (Smac) and XIAP-associated factor-1 (XAF1) were also assessed. Apoptotic changes were characterised in primary airway epithelial cells exposed to the inflammation associated with asthma which is known to activate the caspase-cascade. The abundance of IAPs (and their antagonists) in AECs from asthmatic and control participants were compared, to determine whether intrinsic defects in IAP expression rendered AECs of asthmatics susceptible to apoptosis. Functional analyses were performed by depleting IAP transcripts in primary AECs, to determine whether they are important regulators in epithelial cells subject to the inflammation associated with asthma.

As it turned out, treated AEC in this model underwent early changes in apoptosis but did not proceed to full apoptotic cell death. The results suggest that the IAPs are influential in protecting AECs exposed to proinflammatory cytokines. AECs were maintained in a proapoptotic state by the activity of the IAPs. It was concluded that IAPs may potentiate AEC survival in the presence of chronic inflammatory stimuli, such as asthmatic airways.

Statement of Authorship

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Author Contributions

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Name of Principal Author (Candidate)	Eugene Roscioli		
Contribution to the Paper	Conceptualisation of work, laboratory work, manuscript production, data analysis and interpretation, manuscript evaluation, corresponding author		
Signature		Date	28/10/13

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Contribution to the Paper	Evaluation of manuscript, data interpretation		
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ORIGINAL RESEARCH

Cellular inhibitor of apoptosis-2 is a critical regulator of apoptosis in airway epithelial cells treated with asthma-related inflammatory cytokines

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Abstract

Aberrant apoptosis of airway epithelial cells (AECs) is a disease contributing feature in the airways of asthmatics. The proinflammatory cytokines tumor necrosis factor α (TNFα) and interferon γ (IFNγ) are increased in asthma and have been shown to contribute to apoptosis at the airways. In the present study, we investigated the role of the inhibitor of apoptosis protein (IAP) family in primary AECs exposed to TNFα and IFNγ. IAPs are potent regulators of caspase activity elicited by the intrinsic and extrinsic apoptosis pathways. However, while caspase-mediated apoptosis was observed in AECs exposed to doxorubicin, it was not observed after cytokine treatment. Instead, AECs exhibited proapoptotic changes evidenced by an increased *Bax*:*Bcl2* transcript ratio and partial processing of procaspase-3. Examination by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western analysis showed that proapoptotic changes were associated with a time- and dose-dependent induction of cellular IAP-2 (cIAP2), potentiated primarily by IFNγ. The abundance of the IAP antagonists X-linked IAP-associated factor 1 (XAF1) and second mitochondria-derived activator of caspases did not change, although a moderate nuclear redistribution was observed for XAF1, which was also observed for cIAP2. Small interfering RNA (siRNA)-mediated depletion of cIAP2 from AECs leads to caspase-3 activation and poly (ADP-ribose) polymerase cleavage, but this required extended cytokine exposure to produce a concomitant decrease in cIAP1 and Bcl2. These results indicate that AECs possess endogenous mechanisms making them highly resistant to apoptosis due to asthma-related inflammatory cytokines, and the activity of cIAP2 plays an important role in this protection.

Introduction

The primary function of the airway epithelium (AE) is to maintain the airways for efficient ventilation. Central to this, the AE possess a remarkable regenerative capacity, with most airway epithelial cells (AECs) able to function as stem cells (Crystal et al. 2008). In asthma, the AE exhibits a fragile phenotype and inefficient repair after injury, a phenomenon hypothesized to sustain focused areas of AEC activation (Holgate 2011). Prolonged activation of AECs disrupts the epithelial barrier and leads to inappropriate secretion of immunomodulatory cytokines and growth factors (Holgate 2011; Lambrecht and Hammad 2012). The concept of a chronically injured epithelium has been convincingly linked with the unifying signs of asthma, such as Th2-related bronchial hyperreactivity, infiltration of inflammatory cells, and airway remodeling (Davies 2009; Bartemes and Kita 2012; Lambrecht and Hammad 2012). Consequently, dysregulation of the mechanisms regulating AEC apoptosis may significantly impact epithelial fragility and repair, and contribute to the disease.

The airways of asthmatics exhibit an elevated rate of epithelial apoptosis (Zhou et al. 2011), a phenomenon which increases with disease severity (Cohen et al. 2007).

Conversely, infiltrating inflammatory cells are resistant to death in asthmatics (de Souza and Lindsay 2005), prolonging the release of factors such as transforming growth factor β (TGF-β), tumor necrosis factor α (TNFα), Fas ligand, and interleukin 1β (IL-1β), which can elicit apoptosis of AECs (Trautmann et al. 2002; Nakamura et al. 2004; Makinde et al. 2007; White 2011). However, apoptosis of AECs in asthmatics has been observed in the absence of prolonged inflammation, and demonstrate abnormalities for the production of Bcl2 and activation of caspases (Cohen et al. 2007; Holgate 2011; Zhou et al. 2011). Other factors shown to promote apoptosis of the AE include dysregulated zinc homeostasis (Roscioli et al. 2013), decreased production of E-cadherin (Trautmann et al. 2005), and heightened sensitivity to disease-related agents such as Fas ligand (White 2011). Whether elevated AEC apoptosis potentiates the fragile AE phenotype, or is a distinct phenomenon, remains unclear (White 2011). Further to this, less is known about the function of endogenous suppressors of the caspase cascade in the inflamed airways, and whether they exhibit deficits which may explain the aberrant apoptosis.

Members of the inhibitor of apoptosis protein (IAP) family are best known for their capacity to inhibit caspases; however, they also participate in other prosurvival activities (Roscioli et al. 2013). Of the IAPs, X-linked IAP (XIAP), cellular IAP-1 (cIAP1), and cIAP2, have been examined most rigorously due to their ubiquitous expression and association with cancer (Fulda and Vucic 2012). XIAP in particular is noted to inhibit caspase-3, -7, and -9, while some contention exists whether cIAP1 and cIAP2 inhibit caspases directly (Eckelman and Salvesen 2006). A more likely scenario is that multiple IAPs are required to maintain the apoptotic threshold (Moulin et al. 2012), and employ overlapping mechanisms to inhibit caspase activity. XIAP and the cIAPs have also gained significant attention through their involvement in a number of aspects of the immune response, including the regulation of the inflammasome and nuclear factor-kappa beta (NF-κB) signaling (Gyrd-Hansen and Meier 2010; Beug et al. 2012). Given the fragile nature of the AE in asthmatics, and the significant apoptotic pressure posed by the inflammation, dysfunction of the IAPs may have significant consequences for the integrity of the AE.

Here, we use primary AEC cultures stimulated with TNFα and interferon γ (IFNγ) to determine whether dysregulation of XIAP, cIAP1, and cIAP2 contributes to apoptosis observed in asthma-related inflammation. Although TNFα and IFNγ are pleiotropic cytokines which can influence several downstream pathways, their elevation in the airways of asthmatics has been shown to potentiate apoptosis of AECs (e.g., Trautmann et al. 2002, 2005). We hypothesize that apoptosis of AECs, at least in part, occurs through the reduction in IAP expression and function, or the upregulation of the IAP antagonists second mitochondrial-derived activator of caspases (Smac) and XIAP-associated factor 1 (XAF1).

Experimental Procedures

Human samples

Asthmatic (n = 10, five females, median age 50 years) and control subjects (n = 10, five females, median age 34 years) were selected from individuals attending clinics at the Queen Elizabeth Hospital and Lyell McEwin Hospital (Adelaide, Australia). Asthma status was based on self-report and previous diagnosis of asthma by a clinician. Asthmatic subjects exhibited mild-to-moderate, persistent form of the disease, and either did not require asthma medication, or used β2-receptor agonists (60%). Control volunteers were selected with no previous history of asthma and other respiratory diseases. Participants were free of conditions of the nasal cavity, and did not report a history of allergic rhinitis. This study was approved by The Queen Elizabeth Hospital and Lyell McEwin Hospital Ethics of Human Research Committee, and was conducted in accordance with the Declaration of Helsinki.

Primary AEC culture

Informed consent was obtained prior to collection of AEC via nasal brushing. Nasal AECs were used as they are easily accessed, and do not require the donor to be sedated. In addition, nasal AECs exhibit comparable morphology to bronchial epithelial cells, and respond similarly in the context airway inflammation (McDougall et al. 2008). AECs were suspended in Bronchial Epithelial Growth Media (BEGM, Lonza, Walkersville, MD), and subject to monocyte depletion using anti-CD68 (Dako, Glostrup, Denmark) coated flasks for 20 min, in routine cell culture conditions (37°C, humidified, 5% CO_2). Cells were expanded using type I collagen-coated flasks (Thermo Fisher, Waltham, MA), in BEGM. TNFα, IFNγ, and doxorubicin (all Sigma-Aldrich, St. Louis, MO) were used as treatment agents in AEC cultures. Cell cultures were confirmed to be of epithelial linage by professional cytologists (IMVS Cytology Department, The Queen Elizabeth Hospital, Adelaide, Australia), via reactivity to PAN-cytokeratin versus CD45 antibodies, and morphological examination via Diff-Quick analysis.

Quantitative RT polymerase chain reaction

RNA was extracted from AEC cultures using the RNeasy RNA isolation method (Qiagen Valencia, CA). Comple-

mentary DNA (cDNA) was generated using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer RNA primers (Qiagen), and quantitative RT polymerase chain reaction (qRT-PCR) was performed using HotStar Taq polymerase kit (Qiagen) and the syto-9 DNA stain (Life Technologies, Carlsbad, CA). qRT-PCR was performed using the Corbett Rotor-Gene 6000 thermocycler (Qiagen), and results normalized to two endogenous control genes. Primers sequences, shown in Table 1, were designed in-house and synthesized by GeneWorks (Adelaide, Australia). All primer pairs were assessed for the production of a single PCR product, and reaction efficiencies exceeding 90%. Relative quantification was performed using the ΔΔCt method.

Western analysis

Protein was extracted from AEC cultures in situ, and Western blotting performed using the XCell SureLock Mini Cell System (Invitrogen). Membranes were probed with antibodies directed to β-actin (Sigma), Bcl2 (Santa Cruz, Dallas, TX), poly (ADP-ribose) polymerase (PARP), caspase-3, cIAP2 (both Cell Signaling, Danvers, MA), cIAP1 (R&D Systems, Minneapolis, NE), XAF1 (AbCAM, Cambridge, U.K.), and XIAP (BD Biosciences, San Diego, CA). Luminescence was detected using the LAS-4000 Imager (Fugifilm, Tokyo, Japan), and densitometry analyses performed using Multi-Gauge software (V3.0, Fugifilm Science Lab). Log-transformed density scores were normalized to both β-actin and the biological control.

Table 1. Primers used for qRT-PCR.

Gene name	Oligonucleotide sequences
XIAP	5′-CCGGCTGTCCTGGCGCGAAAA-3′
	5′-TTCCTTATTGATGTCTGCAGGTACACAAG-3′
cIAP1	5′-GATATCCTCATCTTCTTGAACAGCTGTTG-3′
	5′-TCCAGGTCCAAAATGAATAATTGGTGGG-3′
cIAP2	5′-TTACCCTCATCTACTTGAACAGCTGCTA-3′
	5′-TCTCCAGGTTCAAAATGGATAATTGATGAC-3′
Smac	5′-TCATAGGAGCCAGAGCTGAGATGAC-3′
	5′-CCTGATCTGCGCCAGTTTGATATGC-3′
XAF1	5′-GCCCAGCTCGGGAAAGGGGAAA-3′
	5′-CTGAGTCTGGACAACATTTACCCATATG-3′
Bax	5′-CAGTAACATGGAGCTGCAGAGGATGA-3′
	5′-ACCCGGCCCCAGTTGAAGTTGC-3′
Bcl2	5′-GTCATGTGTGTGGAGAGCGTCAAC-3′
	5′-AGTTCCACAAAGGCATCCCAGCC-3′
Hypoxanthine phosphoribosyl-transferase-1[1]	5′-GGCTATAAATTCTTTGCTGACCTGCTG-3′
	5′-CAAAGTCTGCATTGTTTTGCCAGTGTC-3′
Tata-binding protein[1]	5′-CGAAACGCCGAATATAATCCCAAGCG-3′
	5′-CCAGTCTGGACTGTTCTTCACTCTTG-3′

[1]Endogenous control gene.

Fluorescent immunocytochemistry

AECs fixed in 2.5% neutral buffered formalin were simultaneously probed with anti-cIAP2 goat (R&D Sytems) and anti-XAF1 rabbit (Abcam) antibodies. Goat and rabbit mock immunoglobulin G (IgG) proteins were used to control for nonspecific binding of the respective primary antibodies. The nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was used to resolve nuclei. Microscopy was performed using a Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan).

Apoptosis and necrosis assays

The processing of procaspase-3 and cleavage of PARP are events associated with apoptosis. Hence, western analysis antibodies were selected which detect both the unprocessed and cleaved forms of procaspase-3 and PARP. In addition, caspase-3/7 activity was detected using the FAM-FLICA fluorophore (ImmunoChemistry Technologies, Bloomington, MN) which binds to the catalytic site of these caspases. Microscopy was used to image fluorescence generate by caspase-bound fluorophores. Finally, qRT-PCR was used to determine modulation of *Bax* and *Bcl2* transcript levels. The relative abundance of Bax (potentiates cytochrome-*c* release from mitochondria) and Bcl2 (an inhibitor of cytochrome-*c* release) is an indicator of apoptotic changes (Salakou et al. 2007). Lactate dehydrogenase (LDH) released from compromised cells was used to quantify necrosis in cultures, according to the manufacturer's instructions (Cytotoxicity Detection Kit; Roche, Penzberg, Germany). In addition, cells grown in chamber slides were stained with propidium iodide to assess for necrotic cells, and Hoechst was used to resolve nuclei (both ImmunoChemistry Technologies).

Small interfering RNA knockdown of cIAP2

Prevalidated small interfering RNA (siRNA) was used to deplete *cIAP2* transcripts in primary AEC cultures. cIAP2-specific siRNA (Qiagen, siRNA id: Hs_BIRC3_7) was transfected into AECs using HiPerFect transfection reagent (Qiagen). Scrambled siRNA oligonucleotide (AllStars Negative control siRNA; Qiagen) was used to control for nonspecific gene silencing as a result of the transfection process.

Results

Primary airway epithelia cells treated with TNFα and IFNγ demonstrate proapoptotic changes

We first examined the apoptosis in primary AECs treated with TNFα and IFNγ. AEC from healthy donors treated

with cytokines exhibit a time- and dose-dependent increase in *Bax*/*Bcl2* transcripts (Fig. 1A). Assessment of cytotoxicity in these cultures shows cell necrosis remained unchanged over the treatment regime (Fig. 1B). Supporting a proapoptotic outcome, cytokine treatment is sufficient to potentiate the generation of a 19 kDa caspase-3 subunit, however, the 17 kDa subunit was not detected (Fig. 1C). Consequently, PARP cleavage is not observed, as a second processing step needed to generate the caspase-3 heterodimer was inhibited or not elicited. Interestingly, AECs treated with cytokines exhibit punctuate regions of active caspase-3/7 within the cytoplasm, whereas doxorubicin produces a nuclear and cytosolic distribution (Fig. 1D). In agreement with results for cytotoxicity, propidium iodide is excluded from AECs treated with the cytokines. These results indicate primary AEC resist apoptotic stimuli brought about by TNFα and IFNγ, and may employ endogenous mechanisms to arrest the caspase cascade.

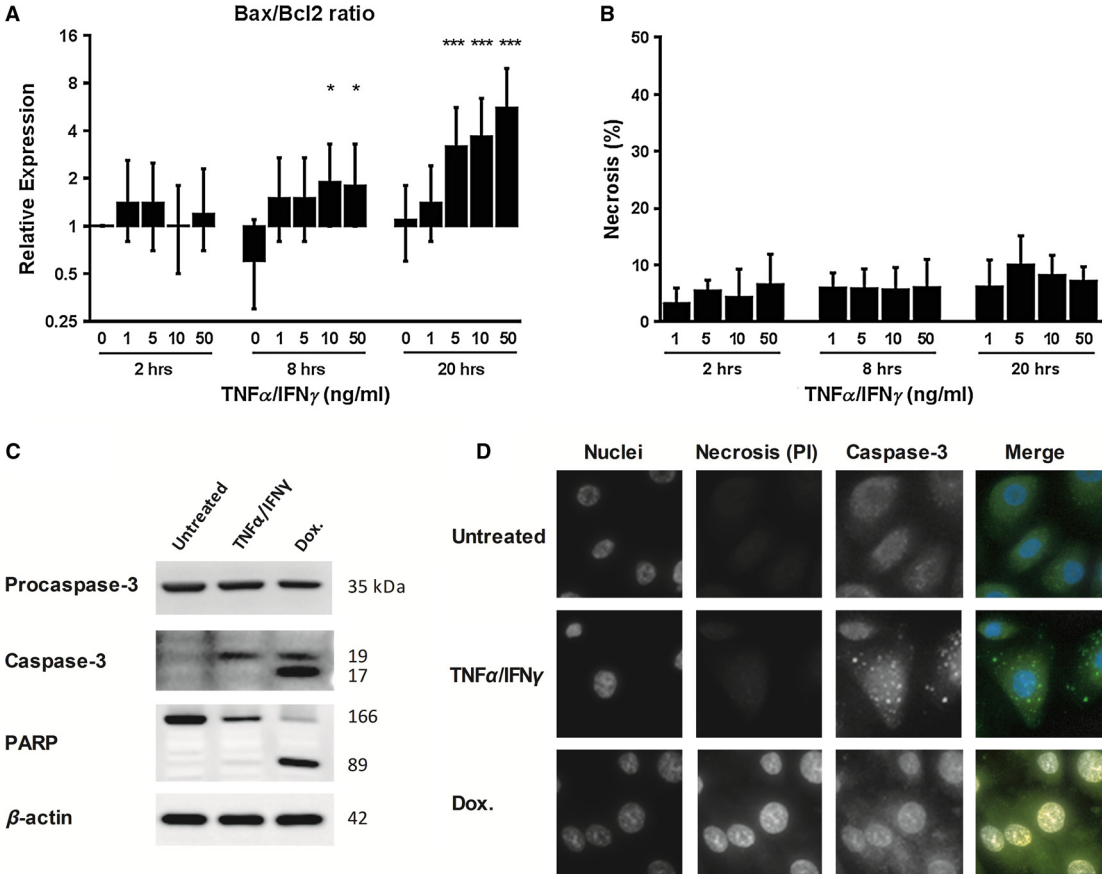

Figure 1. TNFα and IFNγ produce proapoptotic changes in primary airway epithelial cells (AECs). Cultured primary AECs from healthy donors were treated with TNFα and IFNγ, and assessed for apoptosis and necrosis. (A) Time- and dose-dependent elevation in the *Bax*/*Bcl2* transcript ratio is detected, particularly after 20 h of cytokine exposure ($n = 5$ donor AECs). Gene expression data were baselined to the 2-h untreated sample, and normalized to hypoxanthine phosphoribosyltransferase-1 and TATA box-binding protein reference genes. $^{*}P < 0.05$; $^{***}P < 0.001$. Error bars represent 95% confidence intervals. (B) Cultures assessed for relative *Bax*/*Bcl2* transcript abundance were also examined for cytotoxicity, by measuring lactate dehydrogenase release into culture media. Error bars represent 95% confidence intervals. (C) Western analysis demonstrates procaspase-3 is partially processed in AECs treated with 50 ng/mL cytokines (single 19 kDa product). In contrast, doxorubicin (Dox; a control for caspase-mediated apoptosis) -treated cells (1 μmol/L for 20 h) exhibit complete procaspase-3 processing (both 19 and 17 kDa products), which is associated with cleavage of poly (ADP-ribose) polymerase (PARP). Results represent experiments using three AEC donors. (D) AECs treated with TNFα and IFNγ (50 ng/mL, 20 h) were assessed via immunofluorescence for necrosis using propidium iodide (PI), and caspase-3 localization. PI incorporation was not detected for AECs treated with proinflammatory cytokines, versus doxorubicin-treated (1 μmol/L) cells, which appear to exhibit secondary necrosis. Cells treated with proinflammatory cytokines demonstrate punctate regions of caspase-3 and/or -7 in the cytosol versus a more generalized and nuclear localization in doxorubicin-treated cells. Hoeschst staining was used to resolve cell nuclei. Results are representative of experiments performed using three AEC donors.

TNFα and IFNγ elevate *cIAP2* and *XAF1* transcription in AECs from asthmatic and control donors

Next, we determined whether the expression of cIAP1, cIAP2, and XIAP, or their antagonists XAF1 and Smac, modulate in a manner consistent with resistance to cytokine-induced apoptosis. The increased *Bax:Bcl2* transcript ratio previously observed in nonasthmatics is similarly elevated in AECs from asthmatic donors (Fig. 2). In line with this observation, the expression of each transcript was found to be comparable between AECs derived from control and asthmatic participants. Although, XIAP, cIAP1, and Smac demonstrate marginal elevation in gene expression after 20-h cytokine treatment, cIAP2 exhibited a considerably time- and dose-dependent increase. Interestingly, an escalation in XAF1 transcription may represent a response to counter increasing levels of cIAP2.

Induction of cIAP2 by inflammatory cytokines is associated with partial processing of procaspase-3

We next determined whether IAP protein abundance reflects changes observed in gene expression. As for its transcript, XIAP protein expression remained stable over the treatment regimen (Fig. 3A). cIAP1 protein also remain consistent, although a nonsignificant reduction with both cytokines (2.75-fold, $P = 0.062$) was observed. Basal levels of cIAP2 were often undetected in untreated AEC. However, exposure to IFNγ or both cytokines lead to a dose- and time-dependent induction in cIAP2, matching XIAP and cIAP1 (Fig. 3A and B). Interestingly, induction of cIAP2 coincides with the generation of a single 19 kDa caspase-3 product, which is for the most part potentiated by IFNγ. Conversely, the previously observed increase in *XAF1* transcript did not produce an associated elevation in protein abundance. Bcl2 protein levels also remained stable, with the exception of a nonsignificant reduction with 50 ng/mL of both cytokines (1.5-fold, $P = 0.19$). Immunofluorescent analysis shows that XAF1 and cIAP2 share a uniform distribution throughout the cytosol, and a moderate increase in nuclear localization as a result of IFNγ and combination treatment (Fig. 3C).

Doxorubicin inhibits cytokine-mediated cIAP2 induction, and is associated with apoptosis of AECs

While using doxorubicin to provide a positive control for caspase-mediated apoptosis, we observed that a reduction in cIAP1 and Bcl2 protein expression was consistent with apoptosis (e.g., Fig 3A). Hence, to determine whether doxorubicin also arrests cIAP2 induction, AECs were treated with doxorubicin in the presence or absence of TNFα and IFNγ. Approximately 1 μmol/L of doxorubicin induces apoptosis in AEC after 20 h (Fig. 4). Interestingly, this concentration also inhibits cytokine-mediated cIAP2 induction, reducing its protein abundance to basal levels. Inhibition of cIAP2 correlates with complete procaspase-3 processing, and apoptosis as evidence by PARP cleavage. Of note, the proapoptotic influence of TNFα and IFNγ did not sensitize AECs to apoptosis using doxorubicin below 1 μmol/L, where cIAP2 expression remains elevated. Consequently, basal level cIAP2 with concomitant reduction in cIAP1 and Bcl2 may produce conditions suitable for apoptosis in AECs.

Downregulation of cIAP1 and Bcl2 is associated with cytokine-induced apoptosis in cIAP2-depleted AECs

As cIAP2 upregulation by TNFα and IFNγ correlates with AEC survival, and this induction is inhibited by doxorubicin, may indicate cIAP2 has a role in preventing apoptosis. To examine this, siRNA was used to knockout *cIAP2* transcripts in AECs stimulated with cytokines. AECs depleted of cIAP2 and treated for 20 or 48 h exhibited the previously observed pattern of incomplete procaspase-3 processing, and full-length PARP (not shown). However, extending cytokine exposure to 72 h potentiated conditions permissive to caspase-3 activation and PARP cleavage (Fig. 5A). In the absence of cIAP2, procaspase-3 processing favors the generation of the 19 kDa caspase-3 subunit. This may have limited the formation of caspase-3 heterodimers, as incomplete cleavage of PARP was a consistent observation. Unlike earlier time points, extending cytokine exposure to 72 h caused a significant reduction in cIAP1 and Bcl2 protein abundance (Fig 5B and C). This suggests that apoptosis as a result of cIAP2 depletion may depend on reductions in cIAP1 and Bcl2, which is in agreement with finding for doxorubicin-induced AEC apoptosis.

Discussion

In this study, we report that AECs exhibit a proapoptotic phenotype when exposed to asthma-related proinflammatory cytokines, and cIAP2 in partnership with cIAP1 and Bcl2, may be primary factors blocking commitment to apoptosis. In comparison to other reports (e.g., Trautmann et al. 2002, 2005; Zheng et al. 2005; Stout et al. 2007; Basinski et al. 2009), we were unable to elicit apoptosis exclusively through exposure to TNFα and IFNγ, a situation which may reflect differences in cell culture (White 2011). For example, unlike AECs cultured directly

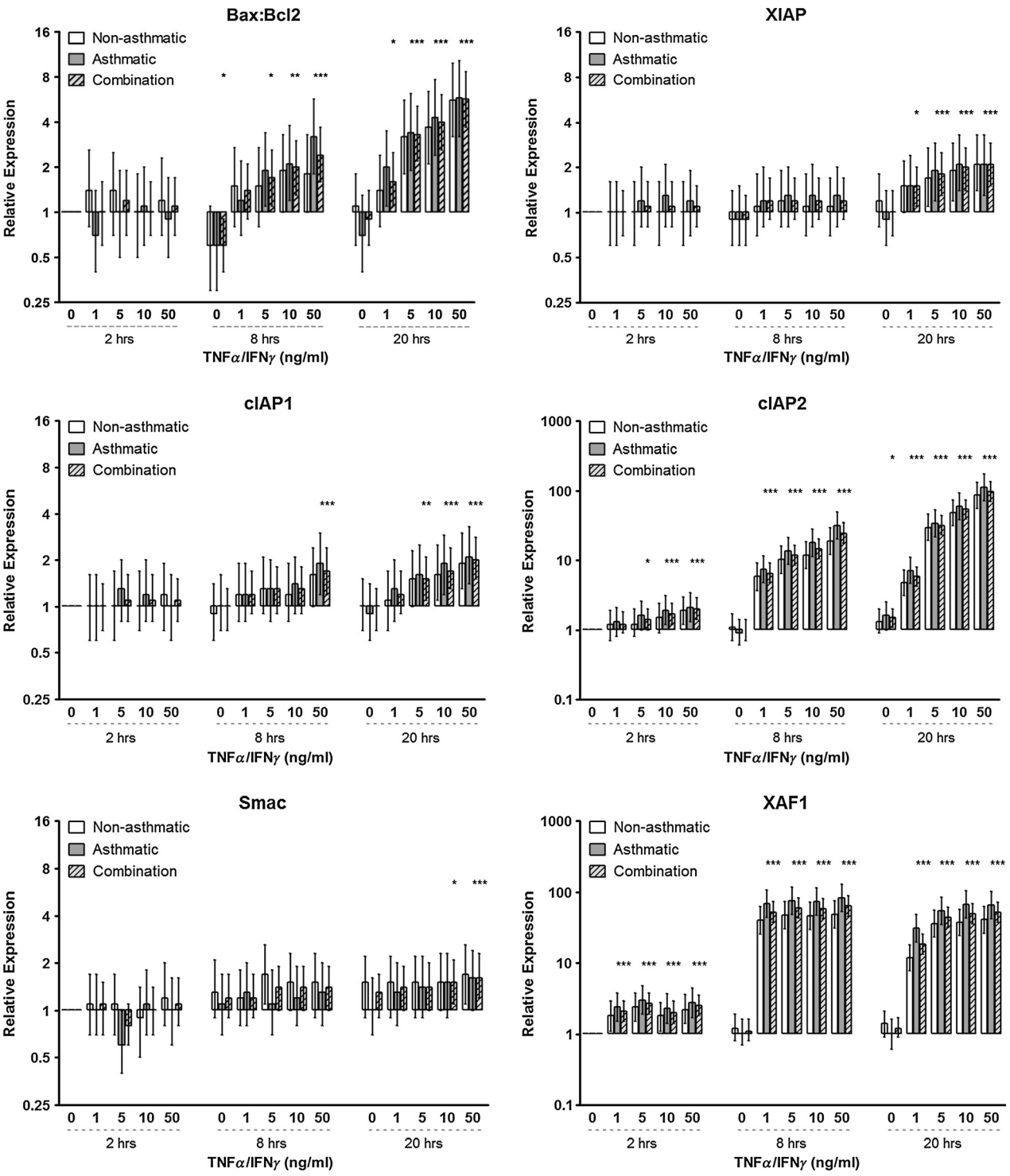

Figure 2. Airway epithelial cells (AECs) from asthmatic and healthy donors exposed to proinflammatory cytokines, exhibit corresponding levels of inhibitor of apoptosis protein (IAP) transcript abundance. Cultured primary AECs from asthmatic (n = 5) and nonasthmatic (n = 5) donors were cultured in the presence of TNFα and IFNγ, and assessed for *Bax/Bcl2*, IAP, and IAP-antagonists transcript abundance. Gene expression data were baselined to the 2-h untreated sample, and normalized to hypoxanthine phosphoribosyltransferase-1 and TATA box-binding protein reference genes. *P < 0.05, **P < 0.01, and ***P < 0.001, and pertain to the combined expression data. Error bars represent 95% confidence intervals.

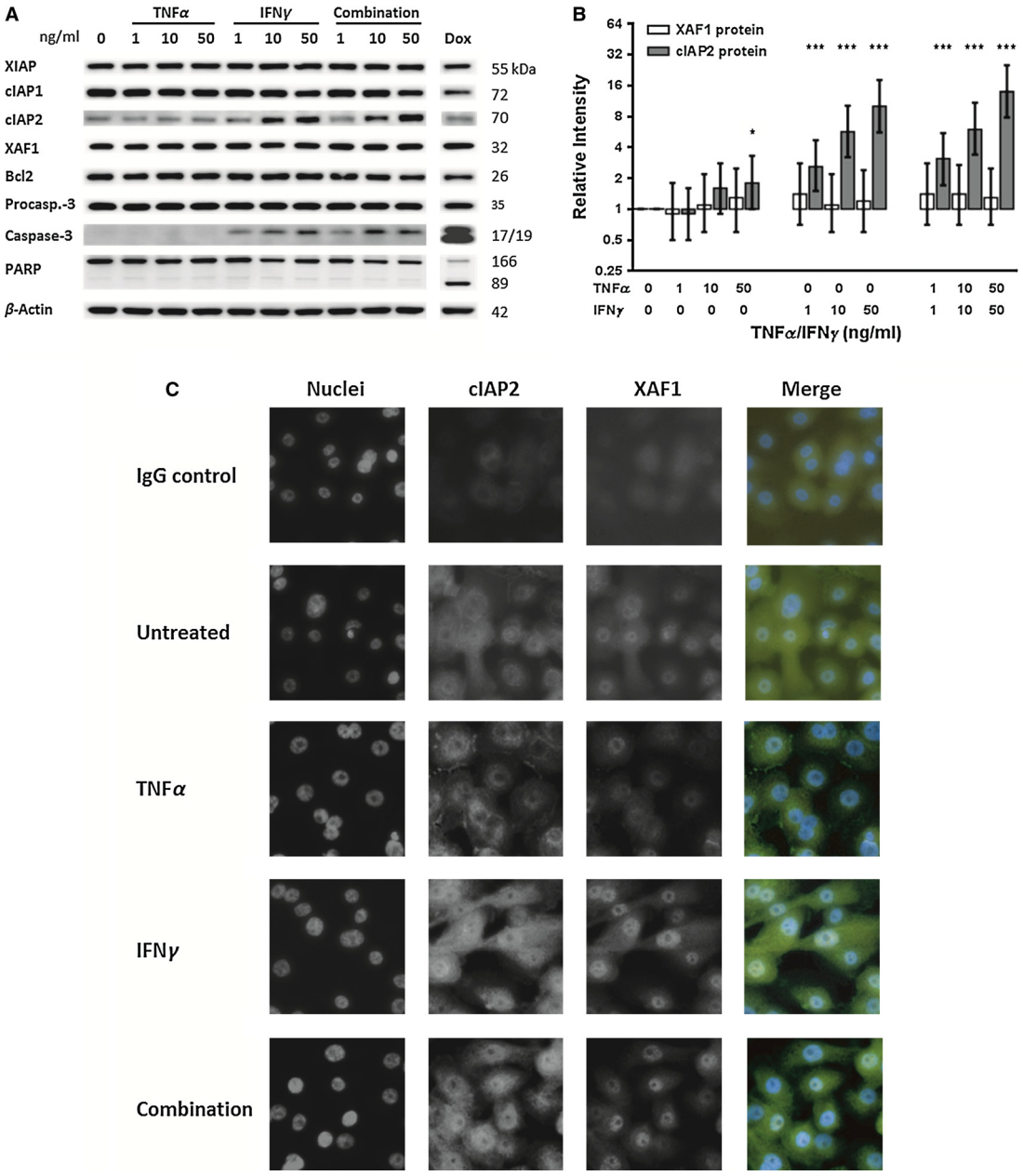

Figure 3. Cellular inhibitor of apoptosis-2 protein is upregulated in response to IFNγ in primary airway epithelial cells (AECs). Cultured primary AECs from nonasthmatic donors (n = 3) were treated with TNFα and/or IFNγ for 20 h, and protein expression assessed via Western analysis and immunocytochemistry. (A) An increase in cIAP2 protein abundance in AECs treated with IFNγ correlates with the production of a single 19 kDa cleaved caspase-3 band, but not cleavage of poly (ADP-ribose) polymerase (PARP). Doxorubicin (Dox) is used as a positive control for caspase-mediated apoptosis. (B) Densitometry analyses of Western blot results confirm a dose- and time-dependent increase in cIAP2 protein abundance, particularly in response to IFNγ. In contrast, XAF1 protein does not alter with cytokine treatment. Protein expression was baselined to the abundance in the untreated sample, and normalized to the expression of β-actin. *P < 0.05, **P < 0.01, and ***P < 0.001, and pertain to the cIAP2 expression data. Error bars represent 95% confidence intervals. (C) XAF1 and cIAP2 have a uniform distribution throughout the cell, with a moderate nuclear relocalization in response to IFNγ. IgG cont. refers to an isotype control antibody. 4′,6-diamidino-2-phenylindole (DAPI) was used to resolve cell nuclei.

from donors, the commonly used normal human bronchial epithelial (NHBE) cell line can grow on a plastic substratum, and are often examined at subconfluence. Primary cells are more resistant to cell death, particularly on surfaces such as collagen at densities over 85% (Aoshiba et al. 1997; Shi et al. 2002; Leverkus et al.

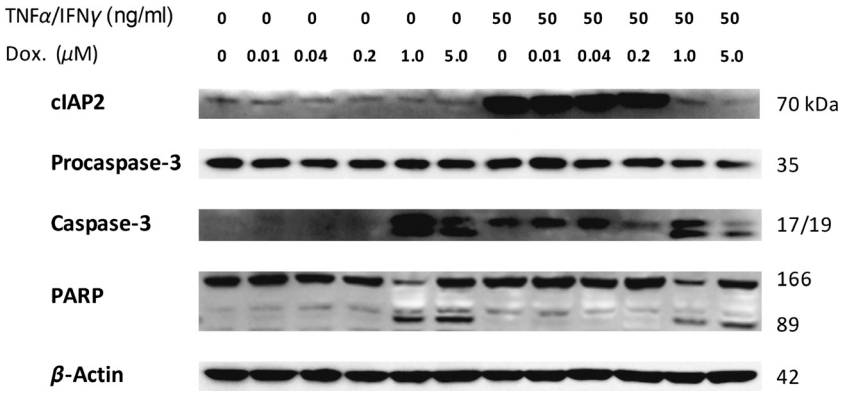

Figure 4. Doxorubicin-induced downregulation of cIAP2 correlates with airway epithelial cells (AECs) apoptosis. Cultured primary AECs were treated for 20 h with increasing concentrations of doxorubicin (Dox), in the presence or absence of 50 ng/mL TNFα and IFNγ. TNFα and IFNγ potentiate the induction of cIAP2 until doxorubicin concentration reaches 1 μmol/L, after which cIAP2 expression is inhibited and apoptosis is detected. Results are representative of experiments using three AEC donors.

2003). Hence, NHBE shedding often ascribed to TNFα- and IFNγ-related apoptosis may mask other important cell survival mechanisms. In line with this, recently Liao et al. (2011) found that NHBE cells are resistant to IFNγ, and targeted downregulation of apolipoprotein was required to produce caspase-dependent apoptosis. Here, AECs demonstrated proapoptotic changes, evidence by an increased *Bax*:*Bcl2* transcript ratio, and partially processed procaspase-3 in the absence of PARP cleavage (Fig. 1A and C). Interestingly, immunofluorescence revealed localized regions of caspase activation within the cytosol of cytokine-treated cells (Fig. 1D). As active caspases were not detected during western analysis, this observation may represent caspases incorporated into protein complexes, or an artifact of the fluorophore associating with partly processed procaspases. The presence of partially cleaved procaspases without progression into apoptosis indicates regulation by the IAPs.

The significance of IAP expression in the inflamed airway is yet to be explored. We found that basal levels of XIAP and cIAP1 were high in AEC, implying a sentinel function. Conversely, basal level cIAP2 was often below detection, but was strongly induced by IFNγ (Fig. 3A). This suggests that the cIAPs are differentially regulated, and cIAP2 may provide a cytoprotective function. However, investigations using the A549 AEC line demonstrated that IFNγ did not influence IAP expression, and inhibited TNF-related apoptosis-inducing ligand-mediated upregulation of cIAP2 (Wen et al. 1997; Park et al. 2002, 2004). Interestingly, similar IAP expression is found in neutrophils stimulated with IFNγ, which demonstrate a moderate upregulation in cIAP2 via activation of the JAK-STAT pathway (Sakamoto et al. 2005). In addition, the cIAP2 promoter contains a putative binding element for interferon regulatory transcription factor 1, which is known to regulate several proteins involved in apoptosis (Hong et al. 2000; Maher et al. 2007). Rat hepatocytes treated with a TNFα and IFNγ also upregulate cIAP2, but likely as a result of TNFα/NFkB signaling (Schoemaker et al. 2002; Peng et al. 2012). Although the cIAP2 promoter contains NF-κB-responsive elements (e.g., Jin et al. 2009), TNFα potentiated a modest increase in cIAP2 in AECs examined here. Of the IAP antagonists, a vast increase in XAF1 transcription did not translate into elevated protein abundance, suggesting influential posttranslational regulation. However, XAF1 and cIAP2 were observed to exhibit a partial relocalization from the cytosol to the nucleus after stimulation with IFNγ (Fig. 3D). XAF1 is known to complex with XIAP and sequester it to the nucleus (Liston et al. 2001). cIAP2 can also localize to the nucleus (Mekhail et al. 2007) and is known to bind XAF1 (Arora et al. 2007), but whether this occurs as a proapoptotic mechanism through XAF1–cIAP2 complexes requires further investigation. We were unable to detect altered gene expression in AECs derived from asthmatics versus controls (Fig. 2), which may suggest there is no intrinsic defect in IAP regulation.

The cIAP2 induction correlated with partial processing of procaspase-3, pointed toward cIAP2 preventing progression into apoptosis. During our use of doxorubicin, we found that downregulation of cIAP1 and Bcl2, with basal level cIAP2, was consistent with apoptosis (e.g., Fig. 3A). Further to this, induction of cIAP2 by proinflammatory cytokines was abolished by doxorubicin (Fig. 4), suggesting that basal levels of cIAP2 may be a requirement for caspase activation in primary AECs. Similarly, targeted depletion of cIAP2 using siRNA did not result in caspase-3 activation, unless cytokine exposure was extended to

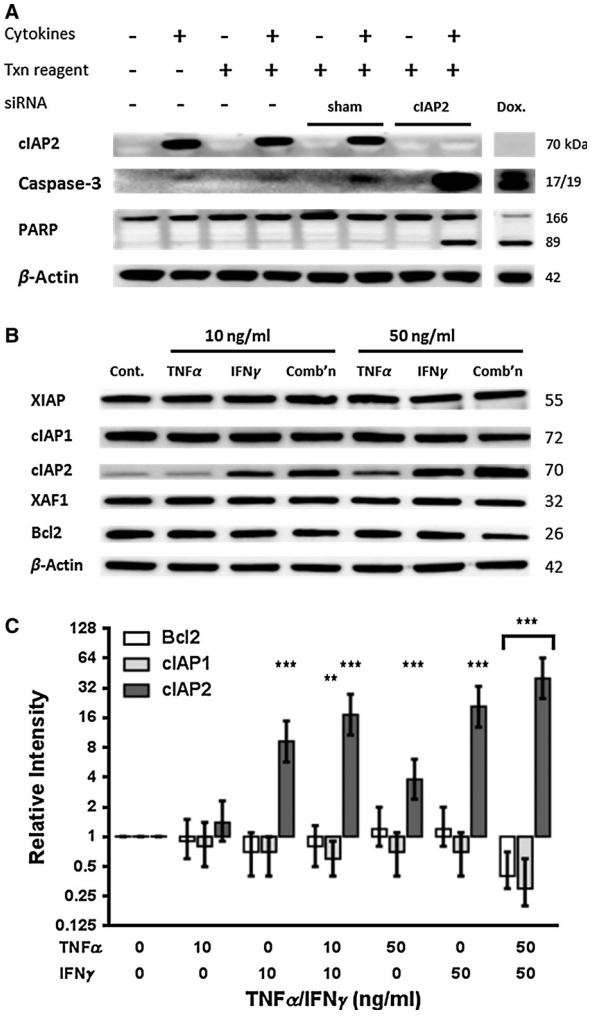

Figure 5. Apoptosis of cIAP2-depleted airway epithelial cells (AECs) by TNFα and IFNγ correlates with cIAP1 and Bcl2 downregulation. (A) AECs from nonasthmatic donors were transfected with cIAP2-specific siRNA, and cultured for 72 h in the presence of TNFα and IFNγ (50 ng/mL). A large 19 kDa caspase-3 product is produced, with a relatively small 17 kDa product. Transfection (Txn) reagent and scrambled siRNA (sham) oligonucleotides were included to control for unintended cIAP2 silencing, and apoptosis as a result of transfection conditions. Doxorubicin (Dox) was used as a positive control for caspase-mediated apoptosis. These results are representative of experiments performed using three AEC donors. (B) Primary AECs treated with TNFα and IFNγ for 72 h exhibit downregulation of cIAP1 and Bcl2 protein abundance, while XIAP and XAF1 levels remain stable. (C) Densitometry analysis of Western blots ($n = 3$) shows cIAP1 and Bcl2 downregulation becomes significant when AECs are treated with 50 ng/mL of TNFα and IFNγ. Results are representative of three experiments using different AEC donors. Protein expression was baselined to the abundance in the untreated sample, and normalized to the expression of β-actin. $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars represent 95% confidence intervals.

produce a significant reduction in cIAP1 and Bcl2 (Fig. 5). This may mean cIAP2 plays a distinct prosurvival role that becomes apparent after the overlapping activities of cIAP1 and Bcl2 decline. These findings are consistent with other reports which found apoptosis through reduced IAP surveillance requires the depletion of more than one IAP (Rumble et al. 2008; Moulin et al. 2012). Sensitization to apoptosis by depleting the cIAPs, without a reduction in XIAP, has been observed in a number of investigations (e.g., Ruemmele et al. 2002; Kim et al. 2012). Here, cIAP2 knockout strongly favored the generation of a single caspase-3 product (Fig. 5A), suggesting that cIAP2 regulates the initial step of procaspases-3 maturation, performed by initiator caspases. The imbalance in caspase-3 products may have limited the formation of active caspase-3 heterodimers. However, XIAP is a potent inhibitor of caspase-3 enzymatic activity, and can therefore inhibit activated caspases which perform the final cleavage for procaspases-3 maturation (Zorn et al. 2012). Hence, abundant XIAP in cytokine-treated AECs was likely responsible for the imbalance in procaspases-3 processing observed after cIAP2 knockdown. Indeed, convincing evidence exists that the cIAPs do not possess the necessary residues to physically interact with caspases in vivo (Eckelman and Salvesen 2006), and they primarily effect cell survival through alternative mechanisms (summarized in Roscioli et al. [2013]). Hence, cIAP2 is no doubt an influential prosurvival factor, but may not be sufficient to protect against apoptosis in isolation.

Here, AECs from asthmatic and control donors produced similar IAP expression profiles when treated with proinflammatory cytokines. Given the fragile nature of AECs derived from asthmatics, we hypothesized that IAP expression may be downregulated after exposure to TNFα and IFNγ. The use of air–liquid interface culture models have been shown to emphasize asthma-associated phenotypes exhibited by AECs during in vitro investigations (e.g., Hackett et al. 2011, 2013). Consequently, we are now examining the possibility of whether an air–liquid culture system will define differences in IAP expression, in AECs derived from asthmatics.

Our findings demonstrate that cIAP2 is a critical factor providing resistance to AECs exposed to asthma-related inflammation. Contemporary asthma therapies which passage the AE to target the mediators of inflammation and bronchial constriction overlook disease-causing factors presented by AECs. Further to this, there is strong evidence that some asthma medications contribute to AE apoptosis (Dorscheid et al. 2006). In the future, therapeutics with the duel objective of preventing inflammation and maintaining the viability of the AE may provide significant progress toward countering the perpetuating factors associated with asthma.

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Conflict of Interest

None declared.

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Chapter 4: IAP genetics in asthma

Chapter 4: Synopsis

Asthma exhibits a strong genetic component, with approximately 50% of asthmatics possessing heritable alterations in their genome which can be associated with the disease. The genetics of asthma is highly complex. A single asthma-related phenotype can be potentiated by network of genes (epistasis). Conversely, different genetic mechanisms can produce seemingly identical disease-related traits (genetic heterogeneity). Consequently, although a number of genetic polymorphisms have been associated with a heightened susceptibility for asthma, the search for a definitive genetic basis underlying the pathogenesis of asthma is ongoing.

In Chapter 4, the *Baculoviral IAP repeat containing-4* (*BIRC4*), *BIRC2*, and *BIRC3* genes, which encode XIAP, cIAP1 and cIAP2 respectively, were assessed for genetic polymorphisms which associate with the development of asthma. Genomic DNA from a community based cohort of Caucasian participants enrolled in the North-Western Adelaide Health Study was assessed for an association between IAP polymorphisms and asthma. The frequency of *BIRC* single nucleotide polymorphism (SNP) and haplotypes were compared in asthmatic and control participants. Alterations in the copy number of the *BIRC4* gene were also assessed for an association with asthma.

This was the first investigation assessing *BIRC2, BIRC3 and BIRC4* polymorphisms for a relationship with asthma. Observations here suggest that *BIRC4* SNPs, haplotypes, and copy number variation do not associate with a heightened susceptibility for asthma. Observation for cIAP1 and cIAP2 in Chapter 3 indicate the cIAPs may have an influential regulatory role in AECs exposed to the inflammation associated with asthma. Consistent with these findings *BIRC2* SNPs, rs4385869 and rs10895290, were significantly decreased in asthma cases requiring steroid therapy. In addition, a haplotype tagged by both *BIRC2* SNPs was also significantly protective of steroid use in asthma cases. These observations suggest *BIRC2* polymorphisms modulate the severity of asthma.

Chapter 4: Part A

X-linked inhibitor of apoptosis single nucleotide polymorphisms and copy number variation are not risk factors for asthma

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Chapter 4: Part B

BIRC2 single nucleotide polymorphisms modulate the severity of asthma in a Caucasian community-based cohort

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***BIRC2* single nucleotide polymorphisms modulate the severity of asthma in a Caucasian community-based cohort**

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ABSTRACT

Dysregulation of apoptosis is potentially a unifying disease susceptibility mechanism for asthma. Fragility of the airway epithelium in asthmatics and prolonged survival of inflammatory cells, contributes to pathogenesis and prolongation of the disease. The Cellular Inhibitor of Apoptosis Proteins (cIAP) ablate apoptosis, and participate in the innate immune response. In this study, single nucleotide polymorphisms (SNP) in the *BIRC2* (codes cIAP1) and *BIRC3* (cIAP2) genes were evaluated for association with asthma. Caucasian asthmatic (n=203) and control (n=198) subjects were selected from participants in the North West Adelaide Health Study. Asthmatics individuals reported a history of doctor diagnose asthma, and exhibited increased FEV1 measures after inhalation of a β2-adrenergic agonist. SNPs (n=9) spanning the consecutively positioned *BIRC2 and BIRC3* genes were selected using a haplotype tagging approach. Genotype analysis of genomic DNA was performed with the SEQUENOM MassARRAY® iPLEX® Gold. Alleles and haplotype associations were analysed by logistic regression, assuming an additive genetic model, and adjusted for gender and atopy. Preparatory results showed genotyping success rate was > 99%, and all SNPs were in Hardy Weinberg equilibrium for both case and control groups. There was no association between the selected SNPs or haplotypes, and asthma susceptibility. However, the minor alleles of two *BIRC2* SNPs, rs4385869 and rs10895290, were both significantly decreased in asthma cases requiring steroid therapy. Further, a haplotype tagged by both of these alleles was significantly protective of steroid use in asthma cases (OR 0.43, 95% CI 0.19, 0.95, p = 0.039), suggesting that *BIRC2* polymorphism may modulate asthma severity. These findings indicate that further genetic association studies between *BIRC2* and asthma, and indeed other genes encoding apoptosis regulatory proteins, are warranted.

INTRODUCTION

Asthma is maintained in genetically susceptible individuals who are exposed to environmental and lifestyle-associated risk factors, such as air bourn allergens (March *et al.,* 2011). A range of susceptibility genes have been associated with the pathogenesis of asthma (Poon *et al.*, 2011), particularly those related to known asthma phenotypes including airway remodelling and chronic inflammation (March *et al.*, 2011; Marsh *et al.,* 2009). However the intricacies of gene-environment interactions which potentiate asthma provide a significant challenge for determining gene networks responsible for its pathogenesis (McLeish & Turner, 2007). Consequently, asthma is now recognised as complex genetic disease (Holloway *et al.,* 2010), and the identification of underlying genetic susceptibility is ongoing.

To better elucidate asthma susceptibility genes the scope of genetic studies has expanded in areas of innate immunity, including the airway epithelium which, for example, produces potent pro-inflammatory cytokines such as thymic stromal lymphopoietin (Bartemes & Kita, 2012; March *et al.,* 2011; Ober & Yao, 2011). Airway epithelial cells of asthmatics also exhibit a fragile phenotype, and undergo premature apoptosis, leading to sustained inflammation (Davies *et al.*, 2003; Holgate, 2011; Trautmann *et al.,* 2005). Conversely, a reduced rate of apoptosis observed for eosinophils, T-lymphocytes, neutrophils and macrophages, further potentiates epithelial destruction and airway remodelling (Tumes *et al.,* 2007; Vignola *et al.,* 1999; Vignola *et al.*, 2000). Consequently, the expression, function, and latency of endogenous inhibitors of caspases may be central to asthma pathogenesis.

Cellular Inhibitor of Apoptosis Protein-1 (cIAP1) and cIAP2, encoded by *BIRC2* and *BIRC3* respectively, are influential cell survival proteins. Although the capacity of the cIAPs to directly bind caspases is contentious (Choi *et al.*, 2009; Eckelman & Salvesen, 2006; Herman *et al.*, 2009), a number of investigations have shown they ablate apoptosis via caspase inhibition (e.g. Burke *et al.*, 2010; Durkop *et al.,* 2006; Roy *et al.*, 1997, Weber *et al.*, 2010). The cIAPs also specialise in the negative regulation of multiprotein death

inducing signalling platforms including the necrosome (McComb *et al.*, 2012), the inflammasome (Vince *et al.*, 2012), and the ripoptosome (Tenev *et al.,* 2011). In line with these observations, deletion of *BIRC2* and *BICR3* in mice is embryonically lethal (Moulin *et al.,* 2012). Important for asthma, the cIAPs have been shown to prolong the survival of neutrophils, macrophages and eosinophils, thereby prolonging the inflammation (Bureau *et al.,* 2002; Conte *et al.*, 2006; O'Neill *et al.,* 2004). Coupled to this, the cIAPs and are also key regulators of, and are themselves regulated by, Nuclear Factor-Kappaβ (NF-κβ), and also participate in other inflammatory signalling pathways (Damgaard & Gyrd-Hansen, 2011; Gyrd-Hansen & Meier, 2010).

To our knowledge there are currently no investigations which examine *BIRC2* or *BIRC3* single nucleotide polymorphisms (SNP) with susceptibility to disease. Genetic analyses of *BIRC2* and *BIRC3* are largely confined to investigations demonstrating their amplification in various forms of cancer (e.g. Cheng *et al.*, 2010; Choschzick *et al.,* 2012), and the well described cIAP2-MALT fusion protein generated by the t(11;18) (q21;q21) translocation event in lymphoma (Rosebeck *et al.,* 2011). Given the apparent dysregulation of apoptosis in the airway epithelium, and the role of the cIAPs in the regulation of apoptosis and inflammation, we hypothesized that SNPs in *BIRC2* or *BIRC3* may be associated with susceptibility to asthma. This was examined in an Australian Caucasian cohort of asthma cases, recruited from a population setting.

METHODS

Study population

Asthma cases were identified from participants in the North West Adelaide Health Study (Grant *et al.,* 2006; Grant *et al.,* 2009), which is a community based, longitudinal investigation of chronic health conditions in randomly selected adult individuals from the north-western suburbs of Adelaide. Asthma status was defined using a self-report of doctor diagnosed asthma, in conjunction with spirometry and a positive bronchodilator response (>15% increase in FEV1 or >12% and >200 ml increase in FEV1), as previously described (Grant *et al.,* 2006; Grant *et al.,* 2009). Positive atopic status was determined

using skin prick testing, with a >2 mm reaction to at least one of the following allergens: alternaria, cat dander, cockroach, feather, house dust mite, or rye grass.

Two hundred and three asthma cases, with Caucasian parents, were identified from this cohort; and a further 198 age and gender matched Caucasian individuals, without asthma, were also selected from this cohort as controls.

This study was approved by The Queen Elizabeth Hospital and Lyell McEwin Hospital Ethics of Human Research Committee, and was conducted in accordance with the Declaration of Helsinki.

***BIRC2 and BIRC3* single nucleotide polymorphism selection**

SNPs were chosen to provide maximal coverage of polymorphism across the *BIRC2 and BIRC3* genes, which are positioned in tandem approximately 9 kB apart on chromosome 11q22. This was achieved by analysis of the linkage disequilibrium structure across the relevant genomic region, and subsequent selection of tagging SNPs. Briefly, HapMap data (2003) for a 79 kB genomic region, spanning both genes (Chr11: 10168900 – 10176800, HapMap Data PhaseIII/Release#2, Feb 2009, NCBI Build 36 assembly, db SNP build 126) was analysed for linkage disequilibrium patterns in Caucasian individuals (Utah residents with ancestry from northern and western Europe, CEU) using Haploview software (Barett *et al.*, 2005). SNPs (n=30), with a minor allele frequency > 5%, exhibited strong linkage disequilibrium structure across the genomic region containing *BIRC2* and *BIRC3* (Figure S1). Nine tagging SNPs, which captured the remaining SNPs (r^2> 0.995), were selected for inclusion in the study (Table S1). The locations of the tagging SNPs in relation to the *BIRC2* and *BIRC3* gene structure are depicted in Figure 1.

***BIRC2 and BIRC3* SNP genotyping**

SNP genotyping was performed with the SEQUENOM MassARRAY® iPLEX® Gold platform (San Diego, CA, USA) at the Australian Genome Research Facility Ltd (Sydney, NSW,

Australia), using genomic DNA in a single multiplex reaction. The genotyping success rate was over 99%.

Statistical analysis

Association analyses were performed by logistic regression, assuming an additive genetic model. All analyses were stratified by gender and the presence of atopy (because some controls were also atopic). Effect sizes were reported as odds ratios with 95% confidence intervals. Allelic association analyses were performed using R 2.15.2 (R Development Core Team, 2011), and libraries SNPAssoc (González *et al.,* 2012), and haplo.stats (Sinnwell and Schaid, 2012).

RESULTS

Participant demographics

The characteristics of the asthma cases included in the study are reported in Table 1. The median age of the asthma cases was 52 vs, (range 18-79), 64% were female, and the majority (58%) were also atopic. Approximately two thirds of the asthma cases were taking regular medication for their asthma, and approximately two thirds of participants using asthma medication regularly used a combination of inhaled steroid and long-acting β2-agonists, in addition to short-acting β2-agonists bronchodilators. The asthma cases and controls were well matched for gender, however the asthma cases were slightly older (median 58 vs. 52 years, p = 0.017, Wilcoxon rank sum test).

***BIRC2 and BIRC3* SNPs and haplotypes associate with the non-asthmatic phenotype**

All nine SNPs included in the study were in Hardy Weinberg equilibrium, in both cases and controls. Hence, genotype frequencies were reliably predicted from allele frequencies, and consequently, only allele frequencies were subjected to further analysis.

SNP allele frequencies were comparable between asthma and control cases (Table 2). One exception was the *BIRC3* SNP rs3460 which was reduced in asthmatics; however this relationship was lost after adjustment for atopy. The nine bi-allelic SNPs formed seven

common haplotypes, with an individual frequency of > 5%, and a collective frequency of 88.4% (Table 2). However, similar to the SNP frequencies, haplotype frequencies were comparable between asthma cases and controls.

The use of steroid therapy may be considered a surrogate marker for asthma severity. In an exploratory analysis within asthma cases, there was some evidence that *BIRC2* SNPs may influence the requirement for steroid use in asthma cases. In an atopy adjusted analysis, the minor alleles of two SNPs, rs4385869 and rs10895290, were decreased in frequency for asthma cases requiring steroid treatment ($p = 0.033$, 0.035 respectively, Table 3), suggesting that these alleles may be protective of severe forms of the disease. Interestingly, the minor alleles of these two SNPs collectively tag Haplotype 2, which is the second most frequent haplotype in both asthma cases and controls (Table 2), and also demonstrated a reduction in frequency in asthma cases requiring steroids (OR 0.43, 95% CI 0.19, 0.95, $p = 0.039$ (Table 3). These results suggest that while there is no evidence that *BIRC2* and *BIRC3* polymorphism influences susceptibility to asthma, *BIRC2* polymorphisms are more frequently exhibited in Caucasian asthmatics who do not require steroid treatment to manage their symptoms.

DISCUSSION

Asthma is a complex genetic pathology which exhibits a number of overlapping phenotypes (Wenzel, 2012). Consequently, genetic association studies are an important tool for understanding the basis of this disease, particularly for genes encoding proteins with several distinct functions. As factors affecting the function, expression or latency of anti-apoptotic proteins may be central to asthma pathogenesis, we hypothesized that polymorphism in *BIRC2* or *BIRC3* may contribute to asthma susceptibility. In addition, inflammation of the airways is a central characteristic of asthma, and cIAP1 and cIAP2 are known to participate co-operatively in inflammatory signalling pathways (Mayer *et al.,* 2011). This was the first study to investigate a specific association between *BIRC2* or *BIRC3* polymorphisms and asthma susceptibility, and to our knowledge, is the first for any disease.

There was a strong basis to undertake a candidate gene study of *BIRC2* and *BIRC3* in asthma. The long arm of chromosome 11 (Chr11q) has been consistently associated with polymorphisms in asthma susceptibility genes (Los *et al.*, 1999), for example, the interleukin-6 receptor gene (Ferreira *et al.,* 2011), and the β-chain of the high affinity receptor for IgE (Cookson & Moffatt, 2000). A number of polymorphisms in the gene coding interleukin-18, which resides with in the same chromosomal sub-band as the *BIRC2* and *BIRC3*, have been associated with asthma and other atopic diseases (e.g. Shin *et al.*, 2005). In addition, associations between asthma and polymorphism in other (non-chromosome 11) genes encoding apoptosis regulatory proteins, such as Survivin (encoded by *BIRC5*; Ungvari *et al.,* 2012), and *DAP3* (Hirota *et al.,* 2004), have also been reported. Hence, as the cIAPs are also regulators of innate immunity, they are ideal candidates to investigate association with inflammatory diseases in which dysregulated apoptosis is also contributes to pathogenesis.

We observed no association between *BIRC2* and *BIRC3* SNPs and/or haplotypes with susceptibility to asthma. However, there was some evidence that *BIRC2* polymorphism may modulate disease severity. The minor alleles of SNPs rs4385869 and rs10895290, and a common haplotype which is tagged by these two alleles, were decreased in frequency in asthma cases requiring steroid therapy (Table 3). Hence, these polymorphisms may protect from severe asthma, where suppression of cells of the inflammatory response with steroids is required for symptomatic relief. Further to this, these two SNPs are both located in intron 3 of *BIRC2*, approximately 1.5 kb apart, and do not tag any other Hapmap SNPs (Table S1). Interestingly, SNPs rs4385869 and rs10895290 “bookend” an interrupted, long interspersed nuclear element (LINE) in intron 3. Retroelement insertions into both introns and exons of genes are known to interfere with gene expression, and are associated with a variety of genetic diseases (Kaer & Speek, 2013). It is possible that these SNPs may tag variation in this retroelement associate with differential *BIRC2* expression, which may limit unscheduled airway epithelial cell death, lower the apoptotic threshold of inflammatory cells, or reduce the pro-inflammatory potential of the cIAPs.

However, differential expression of *BIRC2* in relation to these SNPs remains to be demonstrated.

A limitation of this study is the relatively small sample size, which may result in an inflated type II error. The power of a genetic study is determined by both the allele frequencies and the sample size. Therefore the best assessment of uncertainty associated with the conclusion of this study is examination of the 95% confidence intervals for the effect sizes, which are reported in Tables 2 and 3. In general, this study was not adequately powered to detect very small associations between *BIRC2 and BIRC3* polymorphism and asthma.

Hence, asthma is undoubtedly a complex, heterogeneous disorder, yet dysregulation of apoptosis is potentially a unifying disease susceptibility mechanism. Our finding that *BIRC2* polymorphism may modulate disease severity indicates that further genetic association studies between *BIRC2* and asthma, and indeed other genes encoding apoptosis regulatory proteins, are warranted.

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Table 1: Participant Demographics

Characteristic	Asthma Cases (n = 203)	Controls (n = 198)
Age (yrs.), median (range)	52 (18,79)	58 (19,87)
Female, n (%)	128/203 (63%)	124/198 (63%)
Atopic, n (%)	118/203 (58%)	27/198 (14%)
Regular asthma medication use, n (%)	104/160 (65%)	
SABA, n (%)	40/104 (38%)	
SABA + ICS/LABA, n (%)	64/104 (62%)	

SABA = short acting beta agonist; ICS = inhaled corticosteroid; LABA = long-acting beta-agonist

Table 2: *BIRC2 and BIRC3* allele and haplotype frequencies were comparable between asthma cases (N = 203) and controls (N = 198).

Effect sizes (unadjusted, and atopy adjusted) were derived from an additive logistic regression model, and are reported as odds ratios with 95% confidence intervals, OR (95% CI). The nine bi-allelic *BIRC2* and *BIRC3* SNPs form seven common haplotypes, with an individual frequency of > 5%, and a collective frequency of 88.4%. Haplotype odds ratios were calculated relative to the most frequent haplotype (Haplotype 1).

	SNP	Minor Allele	Frequency		Unadjusted		Atopy Adjusted	
			Controls	Asthma	OR (95% CI)	p-value	OR (95% CI)	p-value
1.	rs17882441	A	0.10	0.10	1.0 (0.6, 1.6)	0.98	1.1 (0.7, 1.9)	0.73
2.	rs7928663	C	0.37	0.36	0.9 (0.7, 1.2)	0.61	1.0 (0.7, 1.4)	0.89
3.	rs7127583	T	0.42	0.37	0.8 (0.6, 1.1)	0.15	0.9 (0.6, 1.2)	0.42
4.	rs3460	C	0.14	0.10	0.7 (0.4, 1.0)	0.046*	0.7 (0.5, 1.2)	0.17
5.	rs34740624	G	0.16	0.13	0.8 (0.5, 1.1)	0.17	0.8 (0.5, 1.3)	0.37
6.	rs4385869	A	0.29	0.28	1.0 (0.7, 1.3)	0.79	1.1 (0.8, 1.6)	0.54
7.	rs10895290	A	0.16	0.18	1.1 (0.8, 1.6)	0.55	1.2 (0.8, 1.9)	0.33
8.	rs1943781	T	0.14	0.12	0.8 (0.5, 1.2)	0.34	0.9 (0.6, 1.4)	0.59
9.	rs12271457	T	0.07	0.08	1.1 (0.6, 1.8)	1.00	1.2 (0.6, 2.1)	0.65
	ID	Haplotype[1]	Controls	Asthma	OR (95% CI)	p-value	OR (95% CI)	p-value
	Haplotype 1	GTCGAGGCC	0.20	0.22	1	0.36	1	0.84
	Haplotype 2	GTCGAAACC	0.15	0.18	1.0 (0.6, 1.6)	0.98	1.5 (0.9, 2.6)	0.17
	Haplotype 3	GCTGAGGCC	0.10	0.14	1.3 (0.8, 2.2)	0.35	1.6 (0.9, 3.0)	0.10
	Haplotype 4	GCTCAGGCC	0.14	0.10	0.6 (0.4, 1.0)	0.07	0.9 (0.5, 1.6)	0.73
	Haplotype 5	GTCGAAGCC	0.13	0.10	0.7 (0.4, 1.2)	0.17	1.1 (0.6, 1.9)	0.85
	Haplotype 6	ATCGAGGCC	0.09	0.09	0.8 (0.5, 1.4)	0.44	1.2 (0.6, 2.2)	0.59
	Haplotype 7	GCTGGGGTT	0.07	0.06	0.9 (0.5, 1.7)	0.67	1.2 (0.6, 2.5)	0.63
	rare	*********	0.12	0.11	0.8 (0.5, 1.3)	0.37	1.1 (0.6, 2.0)	0.64

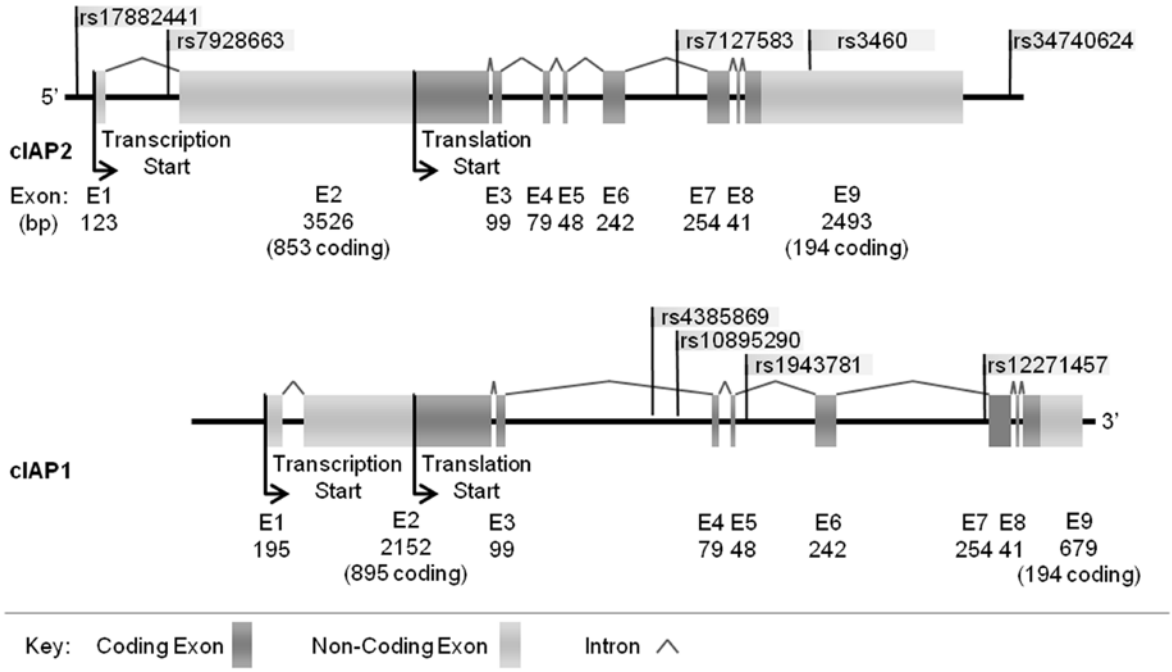
[1]The sequence of alleles on the haplotype follows the order SNP 1…9.

Table 3: *BIRC2* and *BIRC3* allele and haplotype frequencies influence steroid use within asthmatics

Effect sizes (gender and atopy adjusted) were derived from an additive logistic regression model, and are reported as odds ratios with 95% confidence intervals, OR (95% CI). Haplotype odds ratios were calculated relative to the most frequent haplotype (Haplotype 1). The minor alleles of two SNPS, rs4385869 and rs10895290 were decreased in frequency in asthmatics using steroids, and Haplotype 2, which specifically carries both of these minor alleles, was protective of steroid use in asthma patients.

	SNP	Minor Allele	Frequency		Unadjusted		Atopy adjusted	
			No Steroid	Steroid	OR (95% CI)	p-value	OR (95% CI)	p-value
1.	rs17882441	A	0.07	0.11	1.8 (0.8, 4.1)	0.18	1.8 (0.8, 4.1)	0.17
2.	rs7928663	C	0.34	0.36	1.1 (0.7, 1.7)	0.78	1.1 (0.7, 1.7)	0.73
3.	rs7127583	T	0.36	0.37	1.1 (0.7, 1,7)	0.80	1.1 (0.7, 1.7)	0.76
4.	rs3460	C	0.10	0.10	0.9 (0.4, 2.0)	0.87	1.0 (0.5, 2.0)	0.91
5.	rs34740624	G	0.12	0.12	1.0 (0.5, 1.9)	0.89	1.0 (0.5, 1.9)	0.92
6.	rs4385869	A	0.35	0.23	0.6 (0.3, 1.0)	0.028*	0.6 (0.3, 1.0)	0.033*
7.	rs10895290	A	0.22	0.13	0.5 (0.3, 1.0)	0.076	0.5 (0.3, 1.0))	0.035*
8.	rs1943781	T	0.11	0.08	0.7 (0.30 1.5)	0.72	0.7 (0.3, 1.5)	0.34
9.	rs12271457	T	0.064	0.07	1.1 (0.5, 2.7)	0.52	1.1 (0.5, 2.6)	0.85
	ID	Haplotype[1]	No steroid	Steroid	OR (95% CI)	p-value	OR (95% CI)	p-value
	Haplotype 1	GTCGAGGCC	0.22	0.27	1	0.77	1	0.98
	Haplotype 2	GTCGAAACC	0.22	0.13	0.4 (0.2, 0.9)	0.033	0.4 (0.2, 1.0)	0.039*
	Haplotype 3	GCTGAGGCC	0.13	0.17	1.0 (0.5, 2.1)	0.99	1.0 (0.5, 2.2)	0.99
	Haplotype 4	GCTCAGGCC	0.10	0.10	0.7 (0.3, 1.7)	0.44	0.7 (0.3, 1.8)	0.49
	Haplotype 5	GTCGAAGCC	0.11	0.10	0.7 (0.3, 1.6)	0.34	0.7 (0.3 1.7)	0.40
	Haplotype 6	ATCGAGGCC	0.06	0.11	1.4 (0.6, 3.7)	0.47	1.5 (0.6, 3.8)	0.45
	Haplotype 7	GCTGGGGTT	0.06	0.05	0.9 (0.3, 2.5)	0.80	0.9 (0.3, 2.5)	0.82
	rare	*********	0.10	0.07	0.6 (0.2, 1.4)	0.22	0.6 (0.2, 1.5)	0.27
	1The sequence of alleles on the haplotype follows the order SNP 1…9.				LRT: chi2 = 8.81, df = 7, p = 0.27		LRT: chi2 = 8.28, df = 7, p = 0.31	

Figure 1: A schematic representation of the *BIRC2* (cIAP1) and *BIRC3* (cIAP2) genes showing intron and exon structure and tagging SNPs included in the study. *BIRC3* and *BIRC2* are positioned consecutively in the q arm of chromosome 11.



SUPPLEMENTARY INFORMATION

Table S1: *BIRC2 and BIRC3* SNPs included in the study.

The nine *BIRC2* and *BIRC3* SNPs included in the study were selected on the basis of a haplotype tagging approach from HapMap data, as described in the Methods. The additional SNPs captured by this approach are listed in the final column. Nine tagging SNPs were selected which captured a total of 30 SNPs with $r^2 > 0.995$.

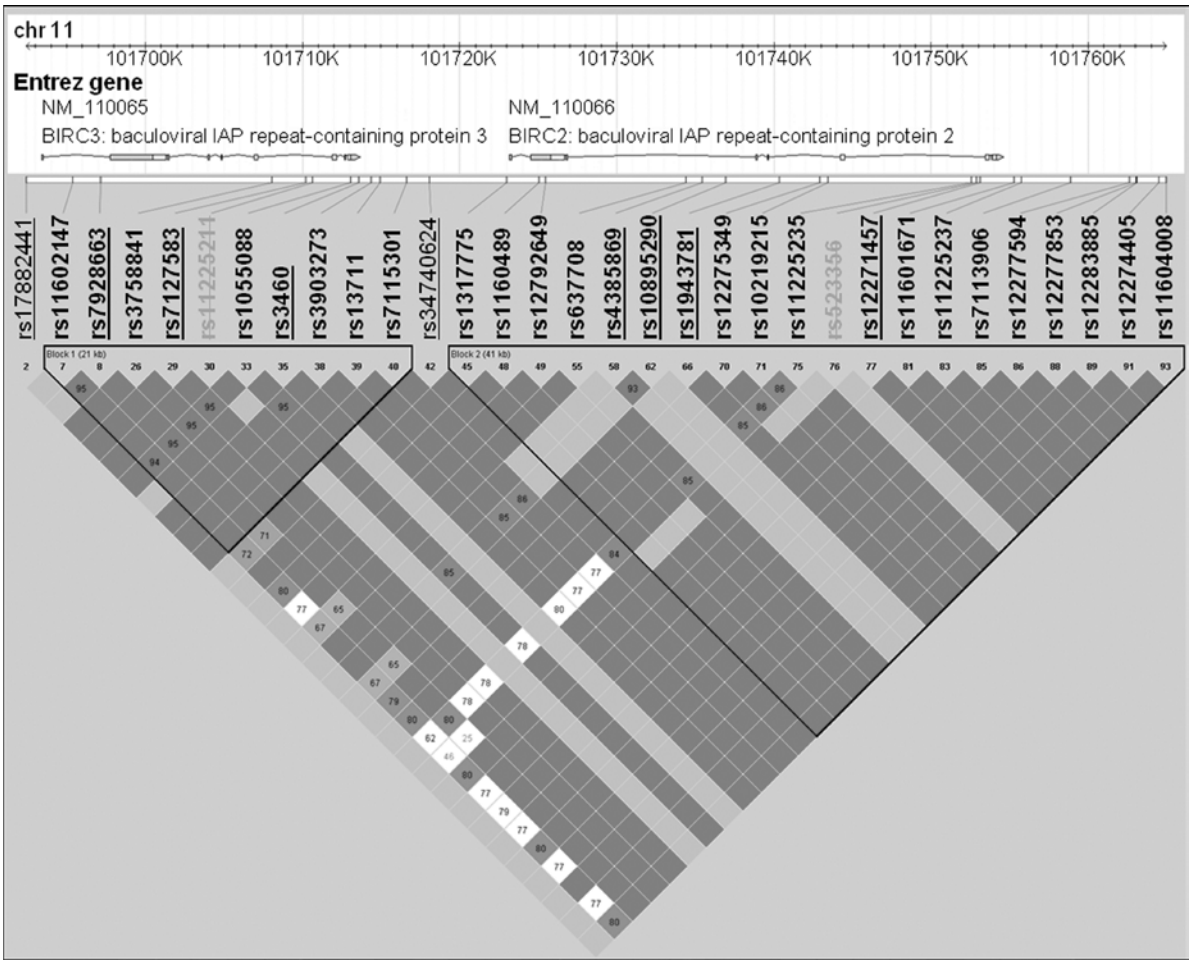
SNP	Chr 23 Position[1]	Gene	Alleles	Region	Additional SNPs captured
rs17882441	102,187,213	5` of *BIRC3*	A/G	near gene 5`	
rs7928663	102,191,967	*BIRC3*	C/T	Intron 1	rs7928663, rs11602147
rs7127583	102,205,037	*BIRC3*	C/T	Intron 6	rs7115301, rs7127583, rs3903273, rs1055088, rs13711, rs3758841
rs3460	102,208,390	*BIRC3*	G/C	3' UTR	
rs34740624	102,212,880	Intergenic[2]	G/A	Intergenic	
rs4385869	102,230,255	*BIRC2*	G/A	Intron 3	
rs10895290	102,231,726	*BIRC2*	G/A	Intron 3	
rs1943781	102,235,141	*BIRC2*	C/T	Intron 5	rs1943781, rs637708
rs12271457	102,247,928	*BIRC2*	C/T	Intron 6	rs7113906, rs11225237, rs11601671, rs11225235, rs12275349, rs12792649, rs12277594, rs12274405, rs10219215, rs11604008, rs12271457, rs11604189, rs12277853, rs1317775, rs12283885

[1] Genome build 104.0, Assembly GRCh37.p10

[2] Between *BIRC2* and *BIRC3* gene sequences

Figure S1: *BIRC2 and BIRC3* linkage disequilibrium heat map

Linkage disequilibrium (LD) analysis was performed over a 79kb region spanning the *BIRC2 and BIRC3* genes (Chr 11), using Hapmap data (Chr11: 10168900 – 10176800, HapMap Data PhaseIII/Release#2, Feb 2009, NCBI Build 36 assembly, db SNP build 126) from Utah residents with Northern and Western European (CEU) ancestry. The genomic region analysed is depicted in the top panel, and the heatmap depicts LD (r^2) values for all pair-wise combinations of SNPs in the HapMap dataset, with the intensity of shading proportional to the r^2 values. Strong linkage disequilibrium relationships were exhibited between the 30 Hapmap SNPs (with a minor allele frequency > 5%) in this region. SNPs not included (n=2) are indicated with strikethrough.



Chapter 5: Discussion

The Gap in the Literature this Investigation Aimed to Address

Recently there has been significant attention paid to the structural elements of the lungs, to identify unifying factors involved in the pathogenesis of asthma (66). The role of the airway epithelium (AE) has come under particular scrutiny (e.g. (75)). Airway epithelial cells (AEC) are hypothesised to exhibit a fragile and an activated phenotype in asthmatics which is responsible for disease-related activities, such as recruiting dendritic cells and initiation of the adaptive immune system (10). However, examining the 'fragility', a state which would seem to imply susceptibility to cell death, has not significantly advanced an understanding of why AECs of asthmatics exhibit apoptotic changes (170). This is evidenced by a scarcity of research regarding the role of the Inhibitor of apoptosis (IAP) proteins in asthma. Indeed, there is a limited amount of literature describing AEC apoptosis with regards to any of the endogenous regulators of cell death (170). Investigations of the IAPs have focused on their implications for cancer, with relatively little attention assigned to their role in those non-malignant diseases that also exhibit aberrations in mechanisms governing apoptosis. Given that the IAPs exhibit prosurvival functions in addition to caspase inhibition (169), and participate in inflammatory signalling (289), there is significant potential for the IAPs to participate in disease-related phenomenon associated with asthma. Further to this, genetic studies of the IAPs are surprisingly rare in asthma, especially as asthma exhibits a strong genetic component (404), and several reports have described epithelial destruction in the airways of asthmatics (75). Hence, although there are modes of caspase-independent cell death, apoptosis is primarily driven by the caspase cascade, which points towards a potential dysfunction in IAP-dependent caspase regulation.

Consequently, work presented in here links the IAPs to research focussing on the inflammation associated with asthma, and apoptosis of AECs. The initial, major hypotheses driving this thesis were that, i) the IAPs regulate caspase activation and apoptosis in normal AEC and ii) the IAPs exhibit intrinsic defects or genetic variations which promote apoptosis of AEC and contribute to the pathogenesis of asthma. The major objectives were to characterise the IAPs in primary AECs from asthmatic and healthy

individuals, and determine whether they exhibit altered abundance, which correlate with measures of accelerated apoptosis at the AE. Further to this, experimental manipulation of the IAPs (in particular, eliminating them with RNA-interference technology) in primary AECs subject to asthma-related inflammation, will serve to provide an understanding as to whether the function of specific IAPs are essential in maintaining proapoptotic changes. In addition, asthma exhibits a substantial genetic component. Hence, genetic polymorphisms in the IAPs were examined to determine whether IAP genetic polymorphism contribute to asthma susceptibility or severity.

Summary of Major Findings

The major findings of this study are, i) surprisingly, primary AECs do not undergo apoptosis after prolonged exposure to the proinflammatory cytokines IFNγ and TNFα *ex vivo*; ii) rather, IFNγ elicits a proapoptotic state in AECs, evidenced by, partial processing of procaspase-3, the absence of Poly (ADP-ribose) polymerase (PARP) cleavage, an increased *Bax*:*Bcl2* transcript ratio, and the absence of morphological changes associated with apoptosis; iii) both XIAP and cIAP1 were constitutively expressed in AECs, and protein levels were unaffected by cytokine treatment. In contrast cIAP2, initially weakly expressed, was strongly inducible by cytokine treatment; iv) no differences were observed between AEC from asthmatics and controls in terms of either basal IAP gene expression levels or their response to cytokine treatment; v) siRNA-mediated depletion of cIAP2-transcripts allows AEC to progress into apoptosis after extended culture conditions which also resulted in a decrease in both cIAP1 and Bcl2; vi) genetic polymorphism in the genes encoding, XIAP cIAP1 and cIAP2 do not associate with susceptibility for asthma. However, *cIAP1* polymorphism may modulate disease severity within asthmatics.

In general, the results of this investigation confirmed an important role for IAPs in regulating caspase activation in normal AEC, but suggest a role for dysregulation of IAPs in the accelerated apoptosis of AEC associated with asthma. Accelerated apoptosis was not observed; rather the IAPs were central in preventing apoptotic progression. However, genetic findings suggest the IAPs may modulate disease severity. Collectively, these results suggest that IAPs may contribute to the maintenance of a proapoptotic state in response to inflammation in the airway epithelium. It is possible that a sustained

proapoptotic state, without timely progression to apoptosis and non-inflammatory clearance, may contribute to observed AEC fragility in asthmatic airways.

Discussion of Findings

1. Surprisingly, primary AECs do not undergo apoptosis after prolonged exposure to the proinflammatory cytokines IFNγ and TNFα *ex vivo*.

Many investigations which have examined the effects of IFNγ or TNFα (and their combination) report apoptosis in a variety of cell types (Table 1.1 and 1.2 Chapter 1). It was a surprise when primary AECs stimulated with IFNγ and TNFα *ex vivo* did not enter into apoptosis, rather they persisted in a proapoptotic state (discussed in Chapter 3). One explanation for why IFNγ and TNFα did not produce the apoptosis observed by others (e.g. (97)), may centre on differences in cell culture models (discussed in (170)). Wen *et al.* demonstrated 20-35% of A549 lung epithelial cells underwent apoptosis, determined using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) and Annexin V, when exposed to IFNγ (214). The A459 cell line is derived from an alveolar tumour cell, but are often used to investigate phenomenon unrelated to malignancy (170). Further, secondary cell lines contain anomalies such as significant chromosomal artefacts imparted by transformation methods (e.g. SV40), which may modulate mechanisms governing cell survival (405, 406).

The closest approximation of primary AECs is the commercially available Normal human bronchial epithelial (NHBE) cell line. NHBE cells treated with IFNγ and TNFα support an anoikis-related death outcome (cell death through detachment). Trautmann *et al.* report NHBE cells exposed to 10 ng/ml IFNγ and TNFα exhibit high rates of anoikis (over 75%) after a 3 day culture period (97). However, the NHBE cells were seeded at a relatively low density, and unlike primary cells, NHBE cells propagate efficiently on plastic surfaces. Primary AECs are more resistant to apoptosis at higher densities (i.e. analogous to AECs in the airways), and when grown on surfaces which approximate the *in vivo* situation (209, 407, 408). To test this, here NHBE cells and primary AECs were propagated on a collagen substratum until cells were at 90-100% density. Both adopted a fragile appearance after application of IFNγ and TNFα (10 ng/ml), but for the most part

remained adherent after three days of cytokine exposure (Supplementary Figure 1). These results were supported by Shi *et al.* who demonstrated low density NHBE cells exhibited a five-fold decrease in viability when treated IFNγ (209). Further to this, Shi *et al.* tested NHBE cells in an air-liquid interface (ALI) culture system and observed approximately 25% of cells entered into apoptosis after treatment with IFNγ, determined by nuclear condensation and TUNEL staining. Hence, the choice of culture model may influence cellular responses to apoptotic cytokines, and approximating the situation *in vivo* may be an important consideration when examining AECs apoptosis.

There is also evidence that IFNγ and TNFα can cause cell death *in vivo*. AEC apoptosis due to IFNγ and TNFα has been reported in mouse models of airway inflammation, evidenced by DNA degradation, TUNEL staining, and Annexin-V fluorescence (196, 211, 212). Convincing evidence demonstrating the AE apoptosis in asthmatics came from Zhou *et al.* who examined the airways of asthmatics and controls post-mortem (161). They demonstrate bronchial biopsies from asthmatics exhibited an increase in caspase-3 and TUNEL positive cells, and pronounced epithelial loss. However, there are some concerns with examining apoptotic changes post-mortem, particularly as the condition was lethal for some asthmatic individuals. As the magnitude and extent of AEC likely varies with disease severity, these results may represent an extreme situation of AEC apoptosis. In addition, there have been some concerns voiced that extensive apoptosis and widespread AEC shedding seen in airway biopsies from asthmatics, may in part reflect artefacts brought about by sample handling and analysis techniques (170, 409).

Further to this, most *in vitro* and *in vivo* investigations of AEC apoptosis in asthmatic airways examine markers of apoptosis down-stream of the caspases, often using cytological methods such as TUNEL, Annexin-V, and caspase-3 staining (e.g. (161, 167, 410)). Although, these methods provide visual evidence of cell death, staining techniques such as TUNEL can label necrotic cells (411), and staining for elevated caspase-3 abundance does not provide evidence of caspase activation. Hence, although apoptotic changes in the AE of asthmatics are a common event, the extent of the apoptosis may be over-estimated. Further to this, important mechanisms governing the apoptosis, such as caspase activity and the influence of endogenous inhibitors of apoptosis have not been rigorously examined. For example, here it was shown the IAPs play an important role in

protecting AECs from the inflammation associated with asthma. Taken together, these results suggest there may be a spectrum of apoptosis-related changes seen in the airways of asthmatics, which is influenced by analysis techniques, genetic factors (412), and exogenous agents such as asthma medications (413).

2. Rather, IFNγ elicits a proapoptotic state in AECs, evidenced by, partial processing of procaspase-3, the absence of Poly (ADP-ribose) polymerase (PARP) cleavage, an increased *Bax*:*Bcl2* transcript ratio, and the absence of morphological changes associated with apoptosis

Here, primary AECs treated with IFNγ exhibited proapoptotic changes (TNFα had minimal effect), and did not progression into apoptosis. The treatment time and doses used here were within the boundaries of related investigations (e.g. (97)), although an accurate estimate for serum-IFNγ in asthmatics still needs to be determined (414, 415). The proapoptotic phenotype was associated with a time and dose dependant increase in the *Bax*:*Bcl2* transcript ratio, partial processing of procaspase-3, and the presence of unprocessed caspase-3 substrates such PARP (Chapter 3, Figure 1). There was also no evidence of necrotic changes as a result of the proinflammatory conditions (Chapter 3, Figure 1B). Interestingly, there was an increase in the fluorescence of active caspase-3 and -7 in treated AECs, which exhibited a punctate, cytosolic distribution (Chapter 3, Figure 1D). As western analysis indicated there was no apoptosis (i.e. no PARP cleavage), this observation may represent fluorophore-bound caspases sequestered into multiprotein complexes.

3. Both XIAP and cIAP1 were constitutively expressed in AEC, and protein levels were unaffected by cytokine treatment. In contrast cIAP2, initially weakly expressed, was strongly inducible with cytokine treatment.

The time and dose-dependent induction of cIAP2 by IFNγ, was associated with the proapoptotic phenotype in AEC derived from both asthmatics and control participants. The upregulation of transcript abundance of cIAP2 matched the increase in the *Bax*:*Bcl2*

transcript ratio (Chapter 3, Figure 2), and induction of cIAP2 protein correlated with the partial processing of procaspases-3 (e.g. Chapter 3, Figure 3A).

Indeed, observations from expression studies suggest primary AECs are well equipped to negatively regulate apoptosis. Basal level expression of XIAP, cIAP1 and Bcl2 protein (and transcript) is relatively high, suggesting a sentinel function (Chapter 3, Figures 2 and 3), and cIAP2 is highly inducible. The abundance of Smac mRNA did not alter with cytokine treatment, and although the abundance of XAF1 transcripts increased, a corresponding increase in XAF1 protein was not detected. This may have been due to powerful posttranscriptional regulation of XAF1, to maintain a homeostatic balance of XAF1 protein (416).

Hence, cIAP2-induction, at least in part, may play a role in preventing apoptosis of AECs exposed to IFNγ. In the handful of investigations which examined cIAP2 regulation in cells exposed to IFNγ, its expression was generally not altered, with the exception of an increase in cIAP2 in neutrophils (377) (Table 1.6). There was no change observed for cIAP2 abundance in the commonly used A549 AEC line, which exhibited cell death after exposure to IFNγ (e.g. (379)). Another commonly used secondary AEC, the 16HBE14o- cell line, were found here to express relatively high basal of cIAP2 (Supplementary Figure 2). One exception was NHBE cells, which were found to approximate the IAP expression profile seen in primary AECs (Supplementary Figure 2). These results support observations made by others that bronchial and nasal AECs respond similarly to cytokine exposure (417).

Interestingly, AECs were responsive to the influence of IFNγ (evidenced by the induction of cIAP2, and proapoptotic changes), whereas TNFα produced modest effects. The induction of cIAP2 by IFNγ, which mediates a prosurvival aspect of this cytokine, can be explained by the presence of an Interferon regulator factor-1 (IRF-1) binding element with in the *cIAP2* gene promoter (378). In addition, cIAP2 expression has been shown to respond to the activity of the IFNγ-regulated transcription factor, Signal transducer and activator of transcription 3 (STAT3) (377). However, the *cIAP2* gene promoter also contains two NF-κB elements (418), which are responsive to NF-κB-mediate gene expression (419). Hence, it was surprising that IFNγ produced a stronger response for cIAP2. One explanation may relate to IFNγ signals that crosstalk to the pathways which

activate NF-κB (e.g. (420-422)). This presents a scenario whereby IFNγ can influence the transcription of *cIAP2* through IRF-1, STATs, and two NF-κB response elements in the *BIRC3* promoter.

Results here suggest the cIAPs were also supported by the activity of XIAP to maintain the apoptotic threshold. The cIAPs have been reported to perform prosurvival activities using several mechanisms (169, 423), and can maintain the apoptotic threshold independent of XIAP (276). However, XIAP is a potent inhibitor of caspase-enzymatic activity (223), and whether the cIAPs (or any other IAP, with the exception XIAP) binds the proteolytic domain of caspases is contentious (223, 266, 271). There are three observations which suggested XIAP was also significant in preventing apoptosis. Firstly, in untrasfected cells (i.e. no siRNA treatment), IFNγ elicited partial procaspase-3 activation in AECs, evidence by the production of the caspase-3 p20 fragment (i.e. the proapoptotic state). Although the cIAPs are known to inhibit executioner caspases (e.g. (424)), XIAP is a more effective inhibitor of executioner caspases (271), while cIAPs are associated with inhibition of caspase-8 (e.g. (269, 270)) and other multiprotein death-inducing signal complexes. Hence, as partial procaspases-3 processing was observed, suggests the cIAPs were unable to completely inhibit all caspase-8 activation, allowing procaspase-3 to be partially processed. As PARP cleavage was not observed suggests complete activation of caspase-3 may have been prevented by XIAP.

Second, the relative abundance of caspase-3 subunits generated as a result of siRNA-mediated cIAP2 inhibition were unevenly proportioned, exhibiting an enlarged p20 (19 kDa) and reduced p17 subunit (17 kDa) (Chapter 3, Figure 5A, and Figure 1.6.B). Knockout of cIAP2 and downregulation of cIAP1 may elevate the rate of caspase-8-mediated processing of procaspases-3, increasing the abundance of the p20 (and p12) subunit. Although XIAP is abundant in AEC examined here, the increase in p20 (due to cIAP depletion, and increased caspase-8 activity) may have exceeded XIAP's capacity to inhibit autocatalysis of all p20 subunit. The inhibition of caspase-3 autocatalysis (removal of the small prodomain from the p20 subunit) by XIAP has been observed on at least two occasions (e.g. (425, 426)), but occurs through an undescribed mechanism. Hence, XIAP may affect a build-up of the p20 subunit by preventing a great majority of its autocatalysis, which in turn, strongly reduced the presence of the smaller p17 subunit. Finally, partial

cleavage of PARP was a consistent observation in AECs stimulated with the proinflammatory cytokines, when cIAP2 was depleted with siRNA. Consequently, the activity of XIAP downstream of cIAP2, likely prevented efficient PARP cleavage by inhibiting the proteolytic activity of some of the activated caspase-3 (229, 425).

The reduction in Bcl2 may have also contributed to the apoptosis seen after the downregulation of cIAP1 and knockdown of cIAP2 in AECs exposed to IFNγ. As IFNγ is known to cause mitochondrial damage and activation (e.g. (427)), the decrease in Bcl2 abundance may have initiated the intrinsic apoptotic pathway. Bcl2 inhibits the release of cytochrome *c* from the mitochondria, thereby limiting the formation of the apoptosome, and activation of caspase-9. Consequently, a reduction in Bcl2 may have contributed to the activation of caspase-3 by caspase-9 through the intrinsic (mitochondrial) apoptosis pathway. As XIAP is able to inhibit caspase-9 activity, this apoptosis pathway may have been limited. In addition, cIAP1 is also known to inhibit caspase-9 at the apoptosome via steric interference (252). As a result, the downregulation of cIAP1 by IFNγ may have contributed to elevating the activity of caspase-9.

4. No differences were observed between AEC from asthmatics and controls in terms of either basal IAP gene expression levels or their response to cytokine treatment.

Surprisingly, AECs from asthmatics and control participants exhibited similar induction of the *Bax*:*Bcl2* ratio (Chapter 3, Figure 2), and the absence of morphological changes associated with apoptosis, (as shown in Supplementary Figure 1). This suggests there were no intrinsic differences present in the AECs of asthmatics and controls which lower their apoptotic threshold, at least within the confines of this culture model.

AECs from asthmatic and control participants also exhibited similar regulation of IAPs (and their antagonists), in response to exposure to inflammatory cytokines (Chapter 3, Figure 2). Similar IAP expression profiles observed for asthmatics and control participants supported previous observations that asthma status does not heighten the susceptibility of AECs to cytokine-related apoptosis.

Further to this, transcript abundance of IAPs in primary nasal AECs biopsies from a paediatric cohort of asthmatic (n=17), and non-asthmatic (n=43) participants, did not

modulate with disease status (Supplementary Figure 3). Together, these results suggest asthmatics do not exhibit intrinsic defects in the mechanisms which regulate the expression of the IAPs (or other markers examined here), and that cIAP2 may be influential in maintaining AECs exposed inflammatory cytokines in a proapoptotic state.

Therefore, there does not appear to be intrinsic anomalies in IAP basal levels, nor the response to inflammatory cytokines in AEC from asthmatics.

5. siRNA-mediated depletion of cIAP2 sensitised AEC to apoptosis with concomitant downregulation of cIAP1 and Bcl2.

Given that the levels of cIAP2's closest paralogues cIAP1 and XIAP were elevated, suggests a specific role for cIAP2 in the protection of AEC from apoptosis elicited by IFNγ. A unique role for cIAP2 is supported by observations for primary AECs exposed to doxorubicin, which is a potent activator of caspases (428). In this situation, doxorubicin (1 μM) produced caspase-mediated apoptosis which correlated with the blockade of cytokine-mediated cIAP2-induction (Chapter 1, Figure 4). This is further evidenced by IFNγ and TNFα co-treatment not sensitising AECs to doxorubicin at concentrations below 1 μM, where cIAP2 protein levels were not affected by doxorubicin. Hence, low basal levels of cIAP2 may be a common requisite to enable AEC apoptosis. In addition, these results shows cIAP2 is not induced simply as a result of generalised proapoptotic stimuli. Consequently, these results raised the question of whether depletion of cIAP2 in AECs exposed to IFNγ allows these cells to progress from a proapoptotic state into apoptosis.

A scenario in which the upregulation of cIAP2 by IFNγ potentiates a proapoptotic state, suggests selectively depleting cIAP2 may permit AECs to progress into apoptosis. To assess this, cIAP2 transcripts were depleted with siRNA in AECs exposed to proinflammatory cytokines. However, knockdown of cIAP2 was not sufficient to induce apoptosis until a concomitant reduction in cIAP1 and Bcl2 protein was induced after extended cytokine exposure (Chapter 3, Figure 5). Indeed, a reduction in cIAP1 and Bcl2 protein was also observed when AECs were exposed to doxorubicin (Chapter 3, Figure 3A). Hence, cIAP2 appears to sustain the survival of AECs exposed to proinflammatory cytokines, as part of a co-operative of survival factors that may compensate for the loss of

single member. Indeed, prosurvival proteins often co-operate to increase the cellular apoptotic threshold (e.g. (429-431)), and the cIAPs behave in this manner to regulate large protein platforms such as the inflammasome (343), necrosome (247), ripoptosome (279, 432) and DISCs (418). Indeed, higher concentrations of IFNγ (10-50 ng/ml) elevated cIAP2 protein to levels matching that of cIAP1 in primary AECs (e.g. Chapter 3, Figure 3A). This may suggest the calling for a similar stoichiometric level of IAPs to participate in co-operative functions.

6. Genetic polymorphism in the genes encoding, XIAP cIAP1 and cIAP2 do not associate with susceptibility for asthma. However, cIAP1 polymorphism may modulate disease severity within asthmatics.

Asthma is a heritable, genetic disorder, and further increases in the prevalence of the disease are anticipated (433, 434). Genetic polymorphisms can modulate asthma-susceptibility, and potentiate phenotypes associated with the disease (35). Although *ex vivo* analyses suggest that the IAPs were not defective, this does not preclude potential genetic associations between IAP polymorphism and asthma. In addition to their role in abating apoptosis, each of the IAPs examined here have links with inflammation, and possess further functions which support a diverse range of cellular activities (169, 423). Interestingly, there are few reports attributing XIAP (encoded by *BIRC4*), cIAP1 (*BIRC2*), and cIAP2 (*BIRC3*) polymorphisms and susceptibility with disease. Therefore polymorphisms in the genes encoding several IAPs were examined for an association with asthma.

There was no evidence that single nucleotide polymorphisms (SNP) or copy number variation (CNV) in *BIRC4* associated with asthma (Discussed in Chapter 4, Part A (435)). This came as a surprise as the X-chromosome exhibits a disproportionately high frequency of genetic polymorphisms which produce biological consequence (293). Indeed, polymorphisms in XIAP are frequently associated with immunological consequences (described in (435)). One study reported, SNPs in the gene encoding XIAP ranked in the top 5% of 1,380 genes which brought about alterations in gene expression (297). In addition, several SNP in the gene encoding the IAP Survivin significantly associated with susceptibility for asthma (301). As the *BIRC4* gene is located in a region of known

structural variability (436), it was examined for structural polymorphisms related to asthma. Again however, there was no change in *BIRC4* copy number for any of the participants (i.e. two copies for women, and one copy for men) Together, these observations may reflect evolutionary processes which select against instability in *BIRC4* in humans (259, 260).

Similarly, *BIRC2* and *BIRC3* SNPs did not associate with asthma susceptibility (Chapter 4, Part B). However, the minor alleles of two *BIRC2* SNPs (rs4385869 and rs10895290) were significantly decreased in asthmatics requiring steroid therapy (Chapter 4, Part B, Table 3). Interestingly, one haplotype (an assembly of SNPs which are unambiguously inherited together) containing both rs4385869 and rs10895290, was protective of steroid use in asthma cases (OR 0.43, 95% CI 0.19, 0.95, p = 0.039). These observations suggest *BIRC2* polymorphisms may have a modulatory effect with regards to asthma severity. Another significant characteristic of these SNPs is they define the margins ("bookend") of an interrupted, long interspersed nuclear element (LINE) located in intron 3. Retroelement insertions can damage the genome (437), interfere with gene expression (438), and are associated with at a number of genetic diseases (439). Interestingly, LINEs possess pathological characteristics independent of the genome. For example, LINEs have been hypothesised to initiate immune diseases early in life by serving as an antigen which primes the onset of autoimmunity (440). As the cause of asthma is largely unknown, the influence of LINEs may help explain the pathogenesis of asthma in individuals who do not have a familial history of the disease. Consequently, the *BIRC2* SNPs may tag variation in this retroelements which associate with altered gene expression. Hence, it can be speculated SNPs rs4385869 and rs10895290 may reduce unscheduled airway epithelial cell death, depress the apoptotic threshold of inflammatory cells, or abate the proinflammatory potential of the cIAPs. Further examination of the expression of *BIRC2* in relation to these SNPs may provide insights into the functional consequence of the LINE.

One SNP in the *BIRC3* 3`-untranslated region (3`-UTR), rs3460, warranted attention. BIRC3 SNP rs3460 showed a significant association with asthma, which was lost after statistical adjustment for atopy (Chapter 4, Part B, Table 2). However, 3`-UTR's of mRNA are replete in sequence elements of significance (441), and can modulate disease-related processes (e.g.(442, 443)). In addition, the *BIRC3* 3`-UTR has attracted some attention for

its involvement in potentiating *cIAP2* transcription, and as a target for novels therapeutic applications for the management of cancer (444, 445). Consequently, bioinformatic prediction analyses were performed to identify a potential effect for this *BIRC3* variation. *BIRC3* SNP rs3460 was not predicted to generate alternative acceptor or donor splice-sites, and was distant (43 bp) from the closest predicted adenylate/uridylate-rich elements (AU-rich element), and those mapped by Kim *et al.* for cIAP2 (444). Interestingly however, microRNA binding analysis for the region encompassing rs3460, revealed the presence of the G>C substitution (generated by this polymorphism), may disrupt the binding of the microRNA 'hsa-miR-616' seed sequence (Supplementary Figure 4). MicroRNA's are endogenous inhibitors of RNA expression, and MicroRNA "seed sequences" are critical for effective binding to target transcripts (446). In addition, microRNAs are a potential therapeutic target for the management of asthma (447). Hence, there is the possibility the presence of rs3460 in cIAP2 mRNA may destabilise the binding of hsa-miR-616, leading to an elevated abundance of cIAP2.

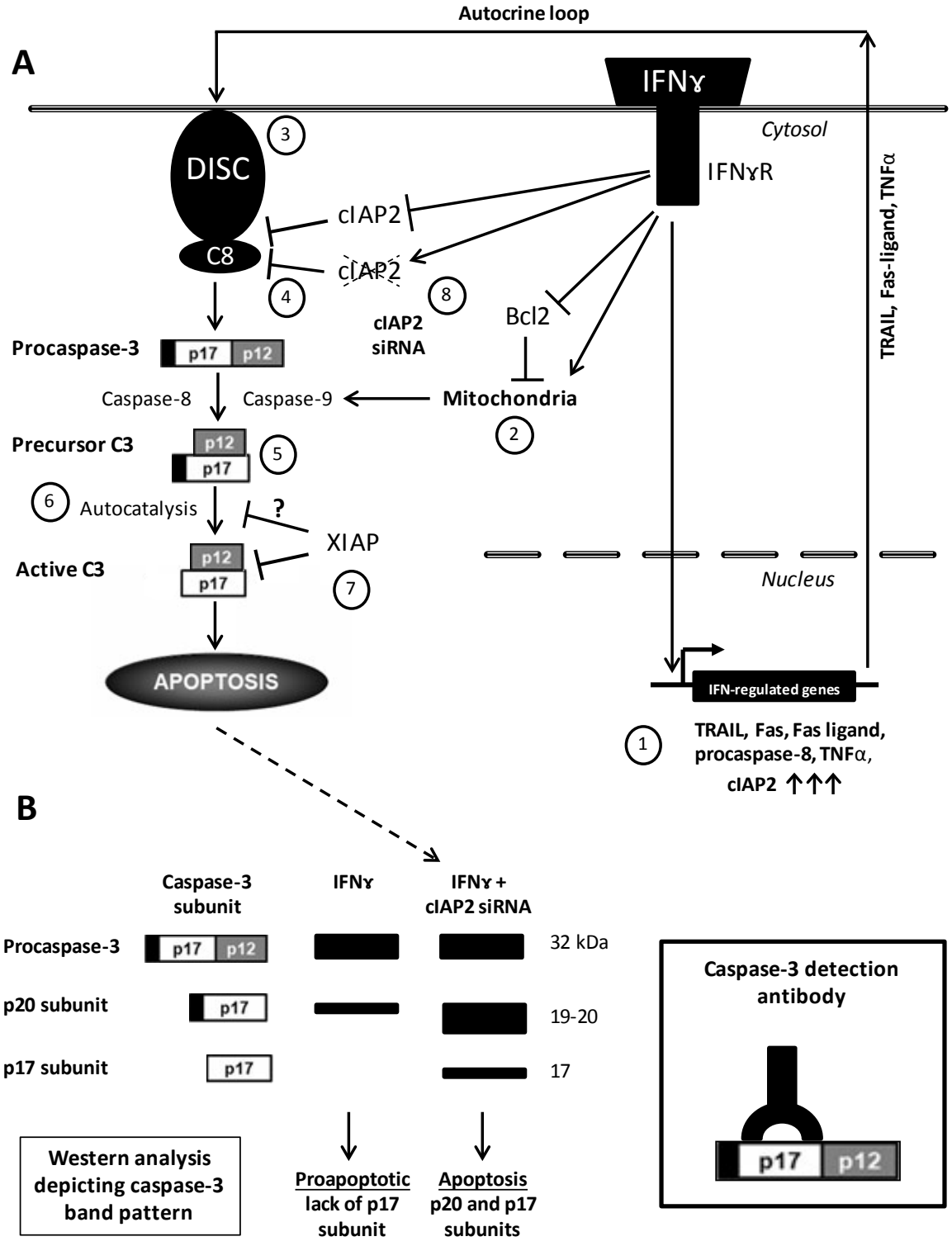
Biological Significance of Findings Presented in this Thesis

A model depicting IFNγ-induced apoptosis in AEC transfected with siRNA is presented in Figure 5.1. A number of the gene products generated by IFNγ signalling are able to potentiate the formation of the DISC, by binding death receptors in an autocrine loop (269). Three include TNF-related apoptosis-inducing ligand (TRAIL), TNFα, and Fas-ligand (200, 201, 448). Another common proapoptotic activity ascribed to IFNγ signalling is the induction of procaspase-8 expression (e.g. (449)). As a result, IFNγ-mediated formation of the DISC and upregulation of procaspase-8 provides conditions suited to the activation of the extrinsic apoptosis pathway. Hence, siRNA-mediate depletion of cIAP2 and a concomitant downregulation of cIAP1 may have released caspase-8 inhibition and potentiated apoptosis of AECs exposed to IFNγ. In addition, depletion of Bcl2 may have increased the influence of apoptotic stimuli generated by caspase-9. Hence, it is possible that cIAP depletion reduced the activation threshold of procaspase-8, thereby elevating the production of activated caspase-3. Subsequently, the high abundance of activated caspase-3 may have overwhelmed the protective effect provided by XIAP, leading to apoptosis.

Although there is no evidence directly linking IAPs with activity of caspases involved in the inflammatory response, indirectly alterations in IAPs may affect the production and secretion of proinflammatory cytokines produced by the inflammasome and the activation of NFkB signalling. For example, the production and secretion of mature forms of IL-18 and IL-1B are dependent on caspase-1 which is activated after formation of the inflammasome. The significance of alterd IAPs and inflammasome function could be investigated in *in vitro* models of inflammasome activation, which would include activation by microbial products, in AEC depleted of selected IAPs. Monitoring the production of mature IL-1B and IL-18, and the activity of caspase-1, would provide a means to determine if the absence of IAPs has a functional consequence for inflammasome activity.

Figure 5.1: The cIAPs block IFNγ-mediated apoptosis of AECs

Caspases are partially activated in primary AEC treated with IFNγ, producing a proapoptotic state. Apoptosis occurs in AECs exposed to IFNγ after siRNA-mediated depletion of cIAP2, and concomitant downregulation of cIAP1 and Bcl2. A: 1) Activation of the IFNγ-receptor leads to transcription of proapoptotic genes such as procaspase-8 (C8), and anti-apoptotic.cIAP2. 2) IFNγ can depolarise the mitochondria and downregulated the expression of Bcl2. This can lead to the activation of caspase-9, which processes caspasae-3. 3) Ligation of death receptors, leads to the formation of the death inducing signal complex (DISC) which recruits procaspase-8 (C8). 4) Induction of cIAP2 by IFNγ can inhibit the activity of caspase-8, thereby reducing the first cleavage of procaspases-3. 5) Some caspase-8 evades cIAP inhibition and allows the first cleavage of procaspases-3 to form the p12 and p20 subunits (p20 is composed of the prodomain (black) and p17). 6) The caspase-3 prodomain is removed from the p20 subunit via autocatalysis, leading to the formation of the p17 subunit, which is needed for the formation of caspase-3 p12/p17 heterodimers. 7) XIAP is able to inhibit the autocatalysis (with an undescribed mechanism), in addition to inhibiting the active form of caspase-3. Apoptosis was not observed; rather cells were maintained in a proapoptotic state, evidence by the absence of the p17 caspase-3 subunit in western analysis (depicted in section B). 8) Depletion of cIAP2 with siRNA and concomitant downregulation of cIAP1 (and Bcl2) releases the inhibition of caspase-8, leading to elevated production of the caspases-3 p20 and p12 subunits. This enables the formation of precursor caspase-3 at levels sufficient to evade complete inhibition by XIAP, producing an apoptotic outcome. B: A schematic representation of a typical caspase-3 western analysis after AECs was exposed to cytokines, with or without siRNA-mediated cIAP2 depletion. Cytokine-stimulated AECs exhibit a proapoptotic state, evidenced by partial procaspase-3 processing. The presence of partially processed procaspase-3 suggests cIAPs are not sufficient to completely block IFNγ-mediated caspase-8 activation, hence some of the initial cleavage of procaspases-3 is observed (the p20 subunit). Depletion of cIAP2 with siRNA and downregulation of cIAP1 by IFNγ may have decreased the activation threshold of caspase-8. Consequently, caspase-8 activated a greater proportion of caspase-3, which may have overwhelm XIAP's capacity to prevent caspase-3-mediated apoptosis. A build-up of the p20 caspase-3 subunit (compared to the p17 subunit) was observed after cIAP2 knockdown. The generation of the large p20 subunit was likely caused by XIAP blocking the autocatalysis of the prodomain. This would have the effect of reducing the abundance of the p17 subunit, and increasing the amount of p20 (prodomain attached). Insert: the caspase-3 western blot antibody used in this investigation binds procaspases-3, and the p20 and p17 subunits.


A
Autocrine loop
IFNγ
Cytosol
IFNγR
DISC
C8
3
cIAP2
cIAP2
4
8
cIAP2
siRNA
Bcl2
TRAIL, Fas-ligand, TNFα
Procaspase-3
p17
p12
Caspase-8
Caspase-9
Mitochondria
2
Precursor C3
p12
p17
5
6
Autocatalysis
?
XIAP
Active C3
7
Nucleus
APOPTOSIS
IFN-regulated genes
1
TRAIL, Fas, Fas ligand,
procaspase-8, TNFα,
cIAP2 ↑↑↑
B
Caspase-3
subunit
IFNγ
IFNγ +
cIAP2 siRNA
Procaspase-3
p17
p12
32 kDa
p20 subunit
p17
19-20
p17 subunit
p17
17
Caspase-3 detection
antibody
p17
p12
Western analysis
depicting caspase-3
band pattern
Proapoptotic
lack of p17
subunit
Apoptosis
p20 and p17
subunits

To summarise, both XIAP and cIAP1 (constitutively) and cIAP2 (inducible by cytokines) contribute to resistance to apoptosis in primary AEC. Supporting this, SNPs in the gene encoding cIAP1 are protective of steroid use in asthmatics. Hence, observations presented in the framework of this study suggest a protective role for the IAPs in the context of asthma. The IAPs may potentiate a disease-related function in the AE of asthmatics by sustaining a proapoptotic phenotype in AECs, particularly in the context of T_H1-driven (IFNγ) inflammation. There are three observations reported for AECs that may support this hypothesis. First, results here are consistent with reports that AECs from healthy airways are uniquely resistant to apoptosis (11, 450). Secondly, cells persisting in a proapoptotic state may contribute influential disease-associated factors, while accelerated apoptosis may be a comparatively favourable outcome (132, 451). Finally, many disease-related activities associated with the AE of asthmatics stem from a concept that AECs exhibit a fragile or, "chronically wounded" phenotype. This is characterised by a prolonged state of AEC activation, decrease intercellular integrity, and aberration in repair processes (10, 75). It is clear cytotoxic agents are produced by inflammatory cells which promote AEC death. Disease-related factors presented by the AE may require AEC to persist long enough for these phenotypes to be expressed. It seems logical the IAPs protect cells from unscheduled apoptosis, but in doing they support the disease-related activities of fragile AECs, which can produce TSLP and other mediators of inflammation. This raises the question of whether targeting the IAPs in AEC may have an ameliorative effect for airway inflammation in asthmatics.

Medical Significance

Modern therapeutics largely ignore disease-related phenomenon presented by the AE and target the inflammation and bronchoconstriction. As there are a number of refractory and steroid dependent asthmatics, suggests other disease-related sites, such as the epithelium, maybe important (12, 120, 452). Common asthma therapeutics such as dexamethasone, have been shown to increase the propensity for AECs of asthmatic and healthy individuals to undergo apoptosis (413, 453-455). Interestingly, asthma medications commonly used in combination with steroids, such as β-agonists, may influence epithelial repair and survival (456, 457). These therapies are not designed to manage epithelial survival and therefore

their effects are non-specific. There have been calls for novel therapeutics which target the epithelial fragility, to ameliorate the “chronic wound” scenario observed in the airways of asthmatics (12).

As fragility would seem to imply a propensity for death, raises the question of whether IAPs are responsible for contributing the ‘proapoptotic aspect’ of this phenomenon. If the proapoptotic state is associated with the fragile phenotype described for the AE in asthmatics, targeting issues associated with the apoptosis may have wider benefits to other disease-related phenomenon. Indeed, recently there has been great attention paid to the disease-related aspect of a ‘chronically wounded’ AE in asthmatics. However, research addressing AEC fragility in asthma is yet to provide a clearer understanding of the apoptosis in this scenario and the molecular basis of the fragile phenotype remains unclear (65).

Consequently, inquiry into the relationship between AEC apoptosis and fragility may provide a better understanding of how the airway epithelium can direct the clinical management of asthma. If the proapoptotic state initiates, precedes, or is directly linked with the fragility presented by the AE, addressing the apoptosis promises to help ameliorate disease-related factors such as the inflammation, airway remodelling, and disease progression (170, 454). As the IAPs are shown here to sequester AECs in a state where the mechanisms of apoptosis and life operate simultaneously, they may represent a target to address the source of multiple disease-related activities presented by the epithelium.

Interestingly, the *cIAP2* promoter contains a putative glucocorticoid response element (GRE), able to direct the upregulation of cIAP2 in A549 cells treated with dexamethasone, which conferred resistance to TNFα and IFNγ (214, 354). However, there have been no follow-up investigations to determine whether upregulation of cIAP2 (or any other IAP) has beneficial effects for the epithelium. Here, AEC from health participants were assessed for apoptotic changes and IAP expression in this response to asthma medications. Primary AECs treated with fluticasone caused significant apoptosis at concentrations associated with increased cIAP2 expression (Supplementary Figure 5 and 6), while the β-agonist Salbutamol had no effect on IAP abundance or cell viability (data not shown). Hence, it is possible current asthma therapeutics may be improved by promoting GRE-driven cIAP2

production (214). Hence, a single compound may be able to address the inflammation, and disease-related characteristic of the epithelium asthmatic.

However, a significant question is whether inhibiting IAPs to allow AEC which exhibit apoptotic changes to die, is a more favourable situation than increasing their activity to promote cell survival. On the face of it, restoring the AEC of asthmatics to health (i.e. increasing their survival by increasing IAPs) seems the best option. However, it is uncertain whether increasing the function of the IAPs would have this effect. Two important factors suggesting that depleting the IAPs is a safer option are that, cIAPs and XIAP are generally considered to participate in proinflammatory signalling complexes (341). In addition, therapeutics which decreases the activity of anti-apoptosis proteins raises fewer concerns for effects relating to cancer. Although resistance to apoptosis in only one hallmark of cancer (156), increased expression and function of the IAPs is a trait commonly ascribed with oncogenesis (e.g. (220)). Hence, the current understanding of the IAPs and the potentially deleterious effects of AECs persistently in an apoptotic state, seems to point to clearing AEC by downregulation IAP expression or function (132, 170). Indeed, results here suggest the IAPs are potent prosurvival factors in AECs subject to the inflammation associated with asthma. Hence, increasing the activity of the IAPs may further potentiate the proapoptotic state, and produce a more deleterious outcome for than allowing AECs to enter into apoptosis (451).

As outlined in Chapter 1 (section 1.5.12) downregulation of the IAPs may be achieved in a number of ways. Work examining the role of the IAPs in cancer represents a substantial source of information to target the IAPs for degradation. For example, increasing the activity of endogenous IAP antagonists such as Smac and XAF1, or applying antagonists exogenously, has been achieved through the delivery of cell permeable Smac mimetics (458). Indeed, all therapeutics targeting IAPs applied in the context of cancer research is aimed at removing IAPs, mainly by applying Smac mimetics and anti-sense oligonucleotides (220).

However, IAP-antagonists target several IAPs, and may produce non-specific effects, or significantly lower the apoptotic threshold of healthy cells. Here, cIAP2 was found to be inducible, and provided significant protection from apoptosis in AEC exposed to IFNγ. Indeed, cIAP2 has been described as a master switch to apoptosis in cells treated with

inflammatory cytokines (378). Consequently, the upregulation of cIAP2 in proapoptotic AEC provides an inducible target. On the other hand, cells free of proapoptotic changes ('healthy cells') would be expected to express low basal level-cIAP2, and therefore do not provide a less effective target. Hence, targeting cIAP2 using anti-sense oligonucleotides may provide a level of specificity for cells undergoing apoptotic changes at the AE. Further to this, cIAP2 (not cIAP1 or Mcl-2) is known to potentiate the survival of neutrophils through the activity of IFNγ (377). Hypothetically, two sources of disease-potentiating cells may be cleared, and may significantly ameliorate disease-related insults to the airways. However, results here show that for AECs to enter into apoptosis, at least in the context of the inflammation associated with asthma, both cIAP1 and cIAP2 need to be depleted. One way to achieve this is may be to use duel cIAP inhibitors, such as CS3 which is under development by Genetech (393). Presuming human trials were successful, this cell permeable drug could be aerosolised and delivered to the airways using inhaler technology, which is a familiar to asthma suffers.

Limitations of this Thesis

Investigations in complex areas such as asthma, apoptosis, and IAPs, are inherently limited in scope within these large areas of research. IAPs are referred to as enigmatic proteins because they possess a number of mechanisms to regulate apoptosis, and they participate in a diverse range of signalling pathways (231, 240). Consequently, the research field occupied by IAPs has increased considerably over the last five years. It was only recently that XIAP, and cIAPs were widely accepted to be influential regulators of TLR signalling and other mechanisms driving the inflammatory response (reviewed in (289)). In addition, the IAPs are not the only endogenous factors that are able to regulate apoptosis. Other endogenous inhibitors of apoptosis include Cellular FLICE-like inhibitory factors (459), Bcl-xL (460), Mcl-1 (461), and zinc (169), can also increase the apoptotic threshold in disorders of the epithelium. Here, XIAP and cIAPs were chosen as, among other reasons, they are potent inhibitors of caspases, and are abundant in AE of humans (462).

Further to this, asthma is a complex disease composed of a number of overlapping sub-phenotypes (6, 16). This is evidenced by (for example) the various forms of inflammation described in the airways of asthmatics (16), and the central importance of the AE in

pathogenesis of the disease (463). Here, IFNγ and TNFα were chosen as they are common to the inflammation seen in asthmatics, and can initiate the processes governing apoptosis. In line with this, the AE was examined as AECs are known to undergo disease-related apoptotic changes. However, there are more disease-related factors which potentiate apoptotic changes in AEC of asthmatics including, IL-1β (464), Fas ligand, and TGF-β (170), and the overlapping influence of related respiratory diseases (465, 466). Indeed, as pathologies of the airway are unified through the AE and inflammation (467), an effort was made to participate in research areas such as COPD and chronic rhinosinusitis ((468-470); Appendix 5-7). Hence, the work presented here may be consolidated by exploring further research directions, such as the potential involvement of the IAPs in airway inflammation, and the contribution of other factors which govern cell survival.

Given the reported fragile epithelium of asthmatics, it was hypothesised AEC from asthmatics exposed to proinflammatory cytokines would become apoptotic as a result of intrinsic defects in IAP expression. However, neither AECs derived from asthmatics or control participants became apoptotic, and they shared similar IAP expression profiles. Although, *ex vivo* models used in this investigation mitigated issues with secondary cell lines, submerged culture models may mask disease-related phenotypes. Growing primary AECs at an air-liquid interface (ALI) is used to promote the differentiation of ciliated, columnar cells, and mucus secreting goblet cells (e.g. Figure 2.1). In addition, ALI cultures allow the formation of tight junctions (TJ) at the apicolateral margins of adjacent AECs, which is a defining characteristic of a healthy epithelium *in vivo*. ALI culture models of epithelial cells from asthmatics have also been shown to emphasise their disease-related characteristics, such as the downregulation of TJ proteins, which is a central trait of the fragile AEC phenotype (e.g. (396, 471)). This said, there are also significant shortcomings with ALI culture models, such as they are expensive, require several weeks to propagate, and cells are not amenable to DNA transfection in this system. However, persisting with an ALI culture system may have provided a means to replicate the asthma-related phenotypes presented by primary AECs, which potentiate their susceptibility to apoptosis.

Nasal AECs were used during *ex vivo* investigations as, unlike bronchial cells, they can be accessed with minimal discomfort, and nasal brushings do not require participants to undergoing general anaesthesia. Nasal epithelia cells have been shown to serve as reliable

surrogates for bronchial AECs for the study of airway inflammation (417). In addition, NHBE cells and primary nasal AECs produced similar IAP expression profiles after exposure to proinflammatory cytokines (Supplementary Figure 1). However, the use of primary nasal AEC biopsies during expression analyses was problematic due to inflammatory cell contamination observed in samples from asthmatics. Hence, although bronchial biopsies are difficult to obtain, this may have been a worthwhile pursuit to improve expression studies. In addition, given the opportunity to revisit this investigation, every effort would be made to examine bronchial epithelia during an *ex vivo* ALI culture model.

Although *ex vivo* investigations offer important insights into asthma, progression into *in vivo* models can be an essential step. As mentioned, asthma is a complex disease. It is debatable whether mouse models of airway inflammation can replicate the pathophysiology of asthma (472). However, they may simulate the situation in humans more effectively than *ex vivo* models. Given time, a mouse model of asthma may have been used to revisit the question; do the IAPs become dysregulated due to airway inflammation which leads to AEC apoptosis, or do they associate with the fragile epithelial phenotype? Mouse models of disease provide the added benefit that several genes can be deleted from the mouse genome. Results here suggest multiple IAPs may be important to protect from apoptosis as a result of proinflammatory conditions. Knockout of several genes is not recommended with siRNA in vitro, due to (for example) the risk of depleting off-target transcripts. However, mice can be generated with multiple gene deletions, as gene expression is prevented at the level of the genome, rather than the transcriptome. Here, inhibiting multiple IAPs was attempted in AEC cultures using cIAP2 siRNA, in conjunction with Embelin, a cell permeable compound known to suppress the production and function of XIAP (473-476). However a decrease in XIAP abundance was not observed and increasing concentrations of Embelin caused AEC apoptosis (Supplementary Figure 7). Hence, given the opportunity, applying the aims used here to a mouse model of asthma, may provide further insight of a role for .

The power of the genetics study could have been improved with increased participant numbers. A strength of the genetic arm of this thesis was that participants were derived from the same geographic location (North-Western suburbs of Adelaide), and share similar

ethnic backgrounds. As asthma exhibits complex gene-environment interactions (reviewed in (477)), measures for lung function and allergy which were used to identify asthmatic and atopic cases, can be affected by factors such as geography and ethnicity. Consequently, participant numbers were limited based on these parameters, from the larger pool of available individuals. In addition, participants were excluded to match gender and age between each case. Although there is no reason to doubt the results presented here, this investigation was not adequately powered to identify very small associations between IAP polymorphisms and asthma. Given the chance to perform these studies again, all available genomic DNA from asthmatic participants could be analysed to impove sample size, and results adjusted to rule out the influence of demographic imbalances in asthmatic and control cases.

In addition, IAP genetic analyses may have been expanded to include additional SNPs identified for XIAP, and the cIAPs. Here, SNP were chosen which are frequently represented in Caucasian individuals (minor alleles frequency greater than 5%), and which exhibit strong linkage disequilibrium across the genomic region containing the respective IAP genes. Although it is necessary to examine SNPs which are frequently represented to produce unambiguous results, the location of SNPs in relation to gene structure is also important. For example, polymorphisms in promoter regions, and non-synonymous SNPs which alter amino acid sequences, can have a direct biological effect on protein expression, structure and function. Hence, although results presented here were informative within the boundaries of this investigation, in the future, alternative SNPs in the IAP genes may be examined to expand the scope of this inquiry.

The CNV genotyping performed exceptionally well, with gene copy number assigned with high confidence (Chapter 4, Figure 3). Interestingly, without exception *BIRC4* copy number was strictly maintained in each individual. However, the primers used to amplify the *BIRC4* genomic sequence were directed to exon 2. Consequently, *BIRC4* regions up and downstream of the 200 bp amplicon were not assessed. This means any truncated amplifications could not be identified. Given the chance to perform this analysis again, a greater number of priming sites would be chosen to span the *BIRC4* genomic sequence.

There were also a number of obstacles which were overcome during the production of this thesis. As discuss previously, the expression of the IAPs varies between commonly

used AEC cell lines, and may have produced misleading results during this investigation. To overcome this, primary airway epithelial cells were selected for used in *ex vivo* models. Although propagating primary cells was technically challenging, and require relatively long growth periods, they lent to the production of informative results. In addition, unlike secondary cell lines, primary cells from asthmatics can contain disease-related genetic aberrations, which promote the production of phenotypes associated with asthma in an *ex vivo* setting (75). The use of primary cells did pose a problem while optimising transfection experiments however. As a protocol for internalising siRNA into primary AEC cells was not available, significant effort was spent to develop a protocol which allowed IAP depletion, without deleteriously affecting cell viability. This was particularly important here because, as IAPs promote cell survival, their deletion has the effect of lowering the apoptotic threshold. Hence, as the transfection process can have cytotoxic effects (e.g. (478)), protocols were tuned to permit maximum IAP knockdown without potentiating apoptosis in resting cells.

Another obstacle was the availability of quality molecular tools to investigate the IAPs, and other markers examined here. For example, primers for qRT-PCR were not available early in this investigation, and even now commercial cDNA primers promote the amplification of off-target templates sequences such as genomic DNA. This was overcome by developing skills to design, test, and validate qRT-PCR primers (described in Chapter 2, sections 2.6.1 and 2.6.2). The use of western analysis to examine protein expression was also highly informative. However, it was difficult to predict whether various primary antibodies would operate effectively in western transfer system. Several antibodies did not bind target protein under particular transfer conditions, and required extensive trouble shooting before acceptable blots were obtained. In one case, the phospho-XIAP antibody (developed by AbCam, reference number ab59289) did not produce protein blots at all, and a review of the literature revealed this anti-body was not used to examine XIAP phosphorylation by other investigators, for obvious reasons. Indeed, it was important to follow up qRT-PCR results with reliable protein analyses in the context of investigation. For example, substantial increases in XAF1 transcript observed in AECs treated with proinflammatory cytokines did not lead to an increase in protein abundance. This was likely due to strict post-transcriptional regulation (Chapter 3, Figure 5B and 5C). Although

significant time was spent to work-up the technology used here, these efforts added significant value to the outcomes of this investigation.

Questions Which Arise From this Thesis

One question which arose from this thesis is whether the of maintenance AECs in a proapoptotic state is a significant disease-related phenomenon in the airways of asthmatics. There is evidence suggesting that widespread epithelial shedding, apoptosis, and inefficient efferocytosis promotes (for example) bronchial hypersensitivity and airway remodelling (161, 410, 479). Conversely, allowing AECs to undergo apoptosis is also understood to provide an ameliorative effect through, for example, anti-inflammatory influences of apoptotic cells (e.g. (480)). Supporting this, insults which initiate apoptotic changes can also potentiate AEC activation and disease-related changes, such as the secretion of proinflammatory cytokines (9). Indeed, it is possible that a number of cytotoxic stimuli common in the airways of asthmatics can elicit proapoptotic changes and activation of AEC. Consequently, factors which prevent rapid AEC apoptosis, such as the IAPs, may potentiate their disease-related activities. Importantly, there is evidence that activated AEC in the lungs of asthmatics promote cell survival. For example, TLRs expressed on activated AEC initiate signalling events which prevent AEC apoptosis, and drive the inflammation (481). Indeed, if the epithelium actively participates in asthma pathogenesis, it would seem that one requirement would be that disease-related activities occur while the cells are viable, at least for a period of time.

Consequently, identifying a relationship between proapoptotic and ‘fragile’ AECs may be a key factor to determine whether these are interdependent phenomenon. An informative first step may involve exposing ALI cultures of AECs from asthmatic (and control) participants, to sub-lethal doses of apoptosis inducing agents over an extended period. This may help to determine whether signs of epithelial fragility (e.g. TJ disruption, TSLP production, expression of pattern recognition receptors) are produced under proapoptotic conditions. Some aims could be; 1) to ascertain whether the induction of proapoptotic conditions (e.g. elevation if cIAP2 and caspase activity) occurs before, during, or after signs of AEC fragility are detected, 2) determine the relative influence of distinct proinflammatory cytokines for the induction of either phenotype, 3) assess the

relationship between these outcome measures, and the expression of endogenous regulators of apoptosis. If the fragile-epithelium and proapoptotic phenotypes occur together, the next challenge would be to elucidate the signalling pathways allowing them to arise simultaneously, and whether they are potentiated by a common mechanism. In line with the aims of the investigation undertaken here, the IAPs would be assessed for their prosurvival (and proinflammatory) influences. Important discoveries may be consolidated in a mouse model of airway inflammation, and by examining appropriate markers of epithelial apoptosis and fragility, in bronchial biopsy from asthmatics.

Another significant question is why primary AECs and NHBE cells exposed to IFNγ exhibit a vast induction of cIAP2, while its basal levels were barely detectable. To our knowledge this was the first time an up-regulation has been reported for cIAP2 of this magnitude (e.g. 40-fold increase at the protein level, $P > 0.001$). Further, only in neutrophils has IFNγ been shown to modestly increase cIAP2 transcript abundance (protein was not tested; (377)). This large increase implies a specific function for cIAP2 in AECs stimulated with a T_H1 cytokines, perhaps in addition to its survival role at the DISC. As mentioned previously, cIAP2 protein increased to levels matching XIAP and cIAP1, which may suggest a co-operative function for the IAPs. Interestingly, one target of cIAP2 is the necrosome, where it has been shown to participate with cIAP1 to prevent necroptosis in macrophages (247). Hence, as the necrosome can be assembled in epithelial cells (482), cIAP2 be involved in the regulation of this death inducing complex. One way to examine this may be to stimulate AECs with IFNγ and perform immunocytochemistry to determine if cIAP2 aggregates with proteins involved in the assembly of the necrosome. In addition, cIAP2 associated with other multiprotein complexes, such as the, ripoptosome, and DISC, may also be examined. Further to this, a role for cIAP2 in potentiating the inflammation observed in the airways of asthmatics could be determined by examining its incorporation into the inflammasome..

Another interesting observation which warrants further investigation was the co-localisation of cIAP2 and XAF1 to the nucleus (Chapter 3, Figure 3C). XAF1 may interact with cIAP2 and sequester XAF1-cIAP2 complexes to the nucleus, after treatment with TNFα and IFNγ. This may represent an unidentified mode of cIAP2 inhibition, whereby XAF1-cIAP2 complexes are sequestered to the nucleus. Nuclear localisation of XAF1-

XIAP complexes are known to suppress XIAP's caspase inhibition activity (483). Consequently, a similar mechanism may occur for cIAP2, particularly as cIAP2 has been reported by other groups to localise to the nucleus (484). The association of cIAP2 with XAF1 could be confirmed by separating the nuclear and cytosolic portions of treated cells and performing immunoprecipitation studies to determine whether XAF1-cIAP2 complexes are elevated in the nucleus. If this hypothesis was valid , it has implications for using XAF1 to target cIAP2, to ameliorate the proapoptotic phenotype exhibited by AEC treated with cytokines.

Although results from genetic studies suggest XIAP and the cIAPs are not asthma susceptibility genes, follow up studies in other cohorts may be used to confirm these observations. Studies in different ethnic backgrounds will be important (485). However cIAP1 polymorphism examined here suggest they may modulate asthma severity, as it was observed two non-coding cIAP1 SNPs (rs4385869 and rs10895290), w hich were in strong linkage disequilibrium, were associated with steroid use within asthmatics. As mentioned previously, future investigations may be performed to confirm this association in a larger sample population which incorporates a higher frequency of corticosteroid-dependent asthmatics. To demonstrate the functional significance of the cIAP1 polymorphisms in asthmatics vs. control participants, further studies are required to characterise whether these SNPs are associated with differential cIAP1 gene expression.

Concluding Remarks and Future Directions

This thesis demonstrated that AECs are well protected from apoptotic stimuli elicited by the proinflammatory cytokines IFNγ and TNFα. While IAPs may be key regulators of apoptosis in the asthmatic airway epithelium, there was no evidence of differences in relation to IAPs expression, function or genetics, between asthmatics and controls.

Over the last ten years, convincing evidence has implicated the AE as a site which is involved in the pathogenesis of asthma. A trait common to most asthmatics is epithelial fragility and destruction. Consequently, disease-related properties of the IAPs in AECs may manifest through their normal prosurvival activities, i.e. sustaining cell survival in the context of chronic inflammatory stimuli.

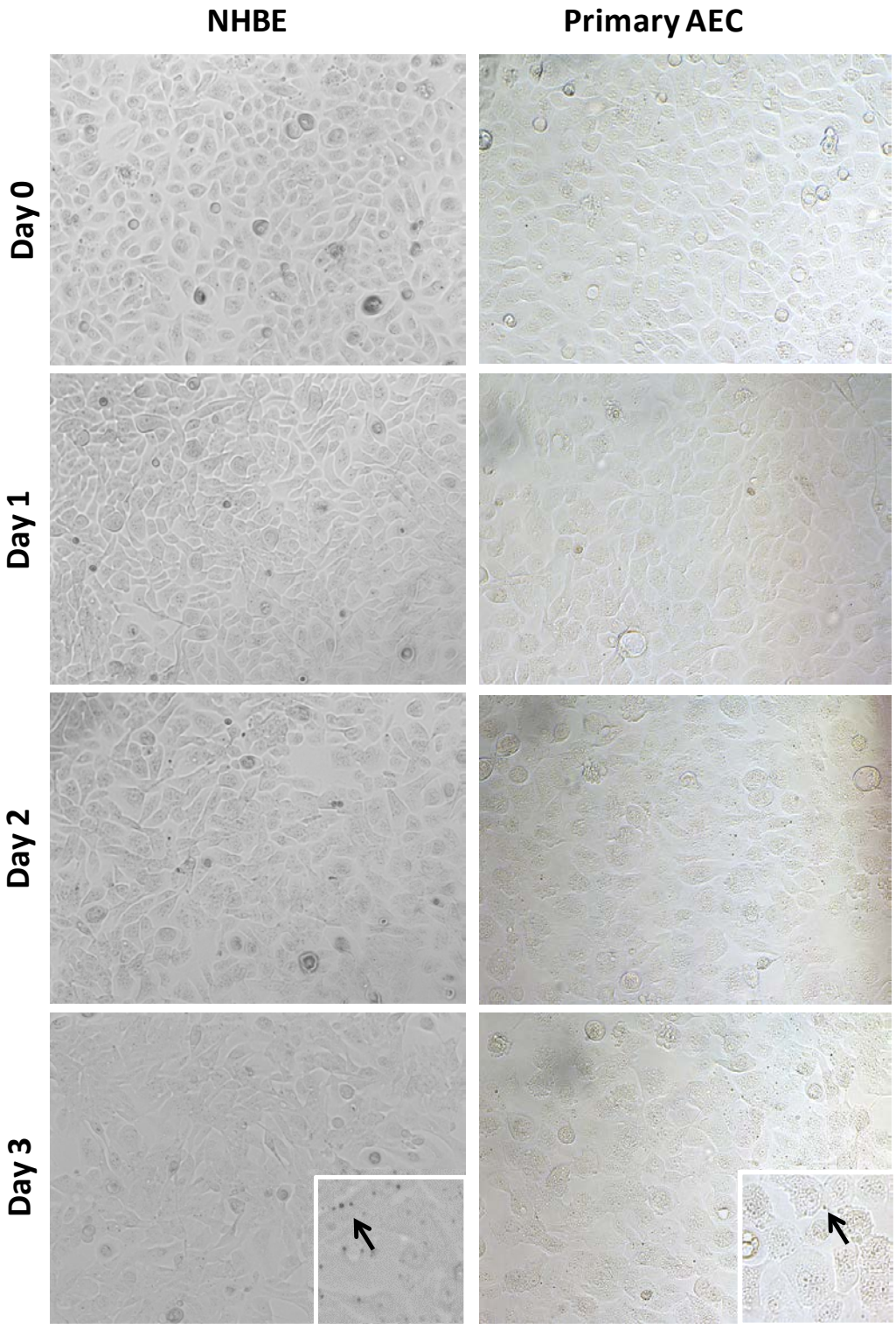
Although, observations reported in this investigation suggest AECs are hardy, the situation *in vivo* is more complex, and additional stimuli may provoke apoptosis in the epithelium of asthmatics by reducing the activity of the IAPs. To address this, the anti-apoptotic function of IAPs could be assessed in an *in vivo* model of airway inflammation. Further, the role of IAPs as regulators of the inflammation may also be examined. in a mouse model of airway inflammation.

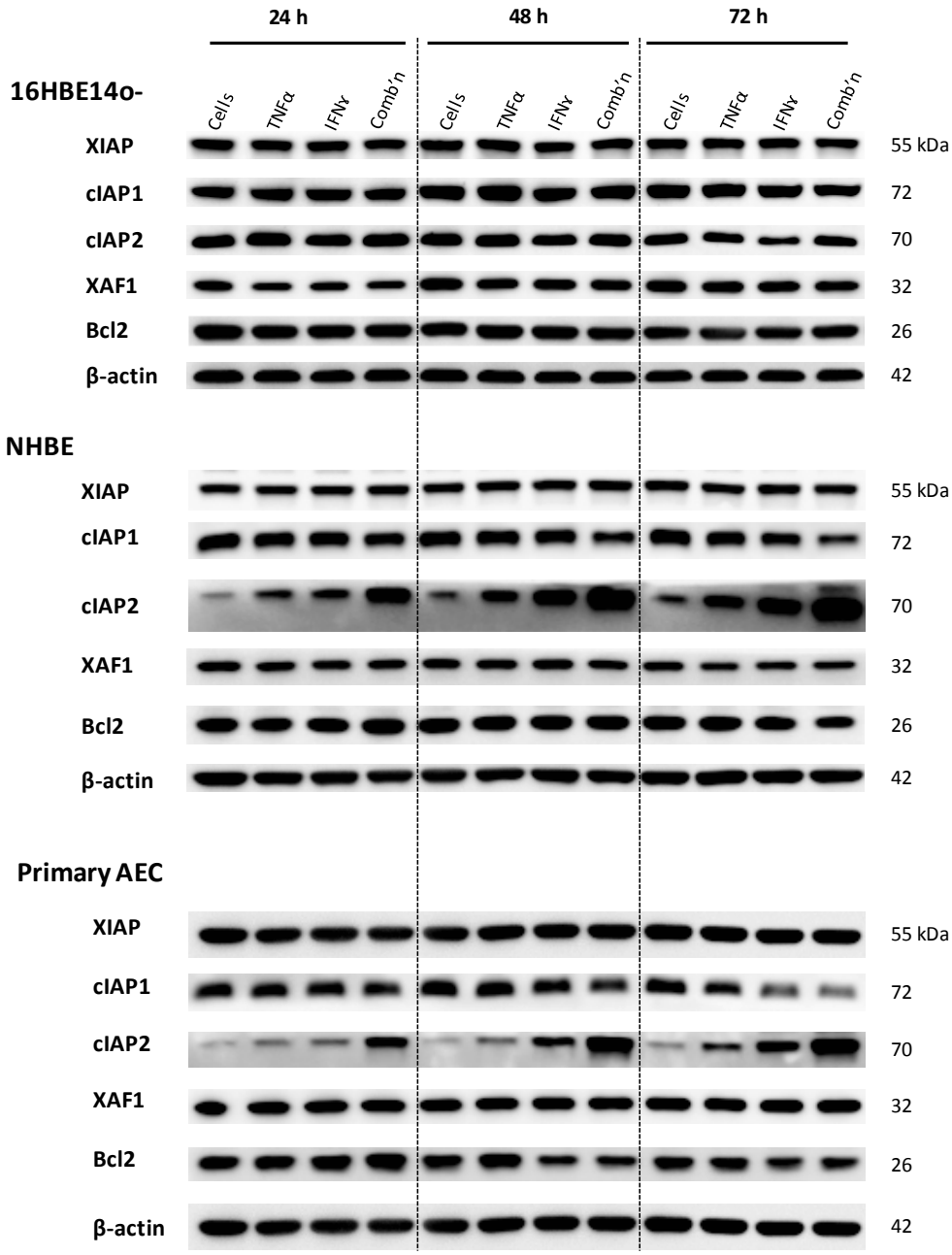
One question is whether IAPs maintain AEC in a proapoptotic state contributes to the pathogenesis of asthma. Future directions of this research should focus on delineation of the molecular and cellular properties which govern apoptotic changes, apoptosis resistance and epithelial fragility in asthma. Ultimately, this research may lead to the identification of new therapeutic targets for asthma.

Supplementary Information

Supp. Figure 1: Morphology of AECs exposed to TNFα and IFNγ

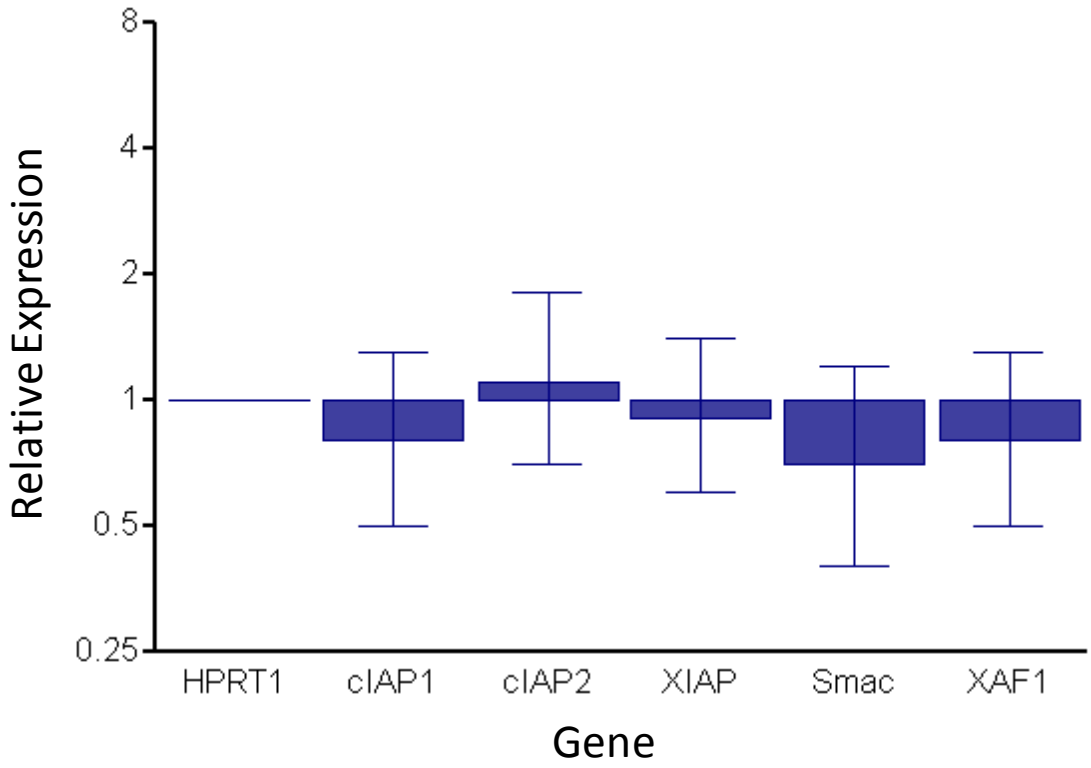
Normal human bronchial epithelial (NHBE) cells and primary airway epithelial cells (AEC) were grown in the presence of IFNγ and TNFα (10 ng/ml), for three days, to replicate the conditions used by Trautmann *et al.* (97). Two changes were made to the system used by Trautmann *et al*; culture flasks were coated with collagen, and cells were seeded to allow neighbouring cells to approximate with each other. At day 0, cell density is approximately 90-100%, and cells show signs of contact, adopting a polygonal morphology, with defined margins. After 24 hours exposure, cells show signs of losing their polygonal morphology. At day 2, the effects of the proinflammatory cytokines become more evident, with cells becoming enlarged, and they exhibit a reduction in cell-to-cell adherence. After 72 hours cell margins are less defined and cytoplasmic regions become translucent. Although AECs often exhibit punctate regions of dark speck, they are larger and more pronounced after three days exposure to the cytokines (indicated by an arrow in magnified insert). Hence, cells here adopt a fragile appearance, but there are relatively few signs of detachment, or morphological changes associated with apoptosis or necrosis. Images are representative of a number of cell cultures performed during the course of this investigation, using AECs derived from both asthmatic and control participants.


NHBE
Primary AEC
Day 0
Day 1
Day 2
Day 3



Supp. Figure 2: IAP expression in AEC exposed to TNFα and IFNγ

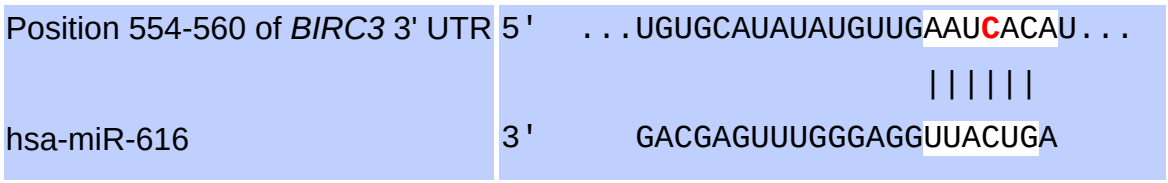
Cells were exposed to either or both of IFNγ and TNFα (50 ng/ml) for up to 72 hours, and assessed for expression of the IAPs, XAF1 and Bcl2 protein. Unlike NHBE cells and primary AECs, the 16HNE14o- cell line expresses high basal levels of cIAP2, and do not potentiate a decrease in cIAP1 and Bcl2 after extended culture in the presence of proinflammatory cytokines. In contrast, NHBE cells present a similar expression profile to those generated for primary AECs. Results are representative of three experiments.



Supp. Figure 3: IAP expression in nasal AEC biopsies from asthmatics vs. controls

Nasal brushing biopsies from asthmatic (n = 17; 53% with atopy) and control (n = 43; 44% with atopy) children attending the Princess Margaret Hospital for Children, were analysed using qRT-PCR. There was no significant difference between asthmatic and control subjects with regard to the expression of the IAPs, or their antagonists. Y-axis values represent log fold-change in relative expression, normalised to Hypoxanthine phosphoribosyltransferase-1 (HPRT1). Error bars represent 95% confidence intervals.

RNA samples used to generate cDNA were a kind gift from Dr Anthony Kicic.

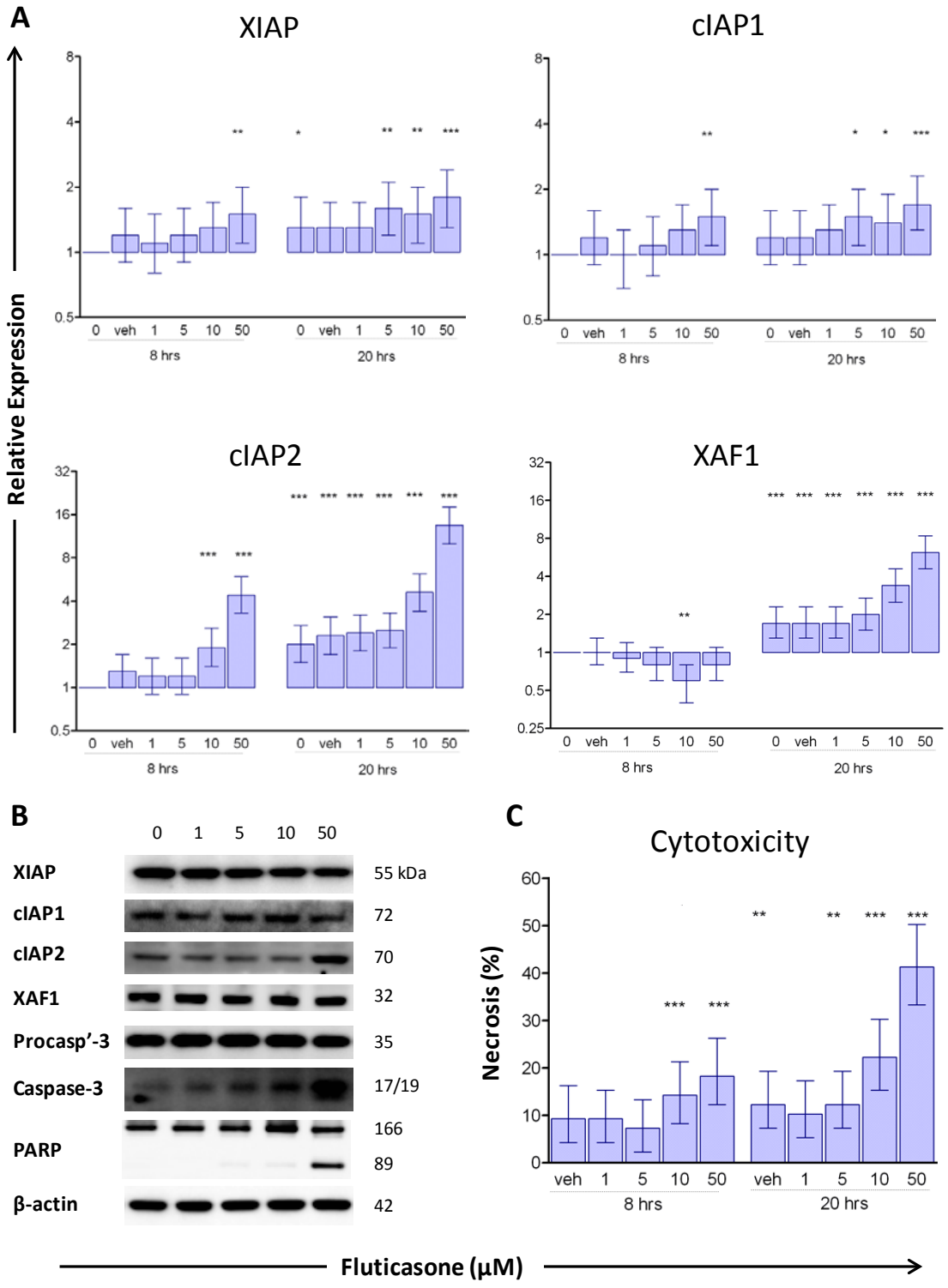


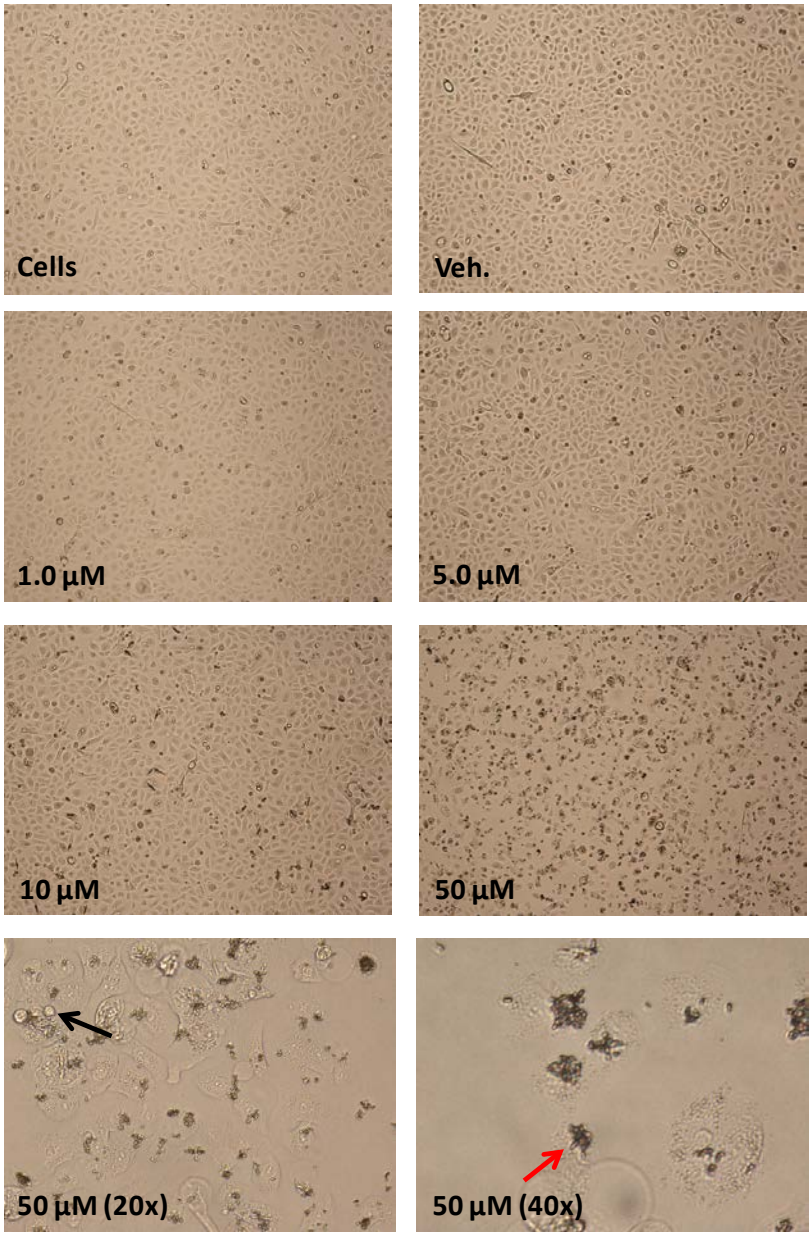
Supp. Figure 4: The *BIRC3* SNP rs3460 within the microRNA hsa-miR-616

The presence of the *BIRC3* SNP rs3460 was assessed to determine whether the base change G>C affects microRNAs predicted to bind *cIAP2* transcripts. The presence of the minor allele of rs3460 (presented in red text) may destabilise the binding of the has-miR-616 seed sequence (white). This bioinformatics analysis was performed using TargetScanHuman Prediction of microRNA targets (http://www.targetscan.org/vert_61/).

Supp. Figure 5: Expression analysis of AECs treated with Fluticasone propionate

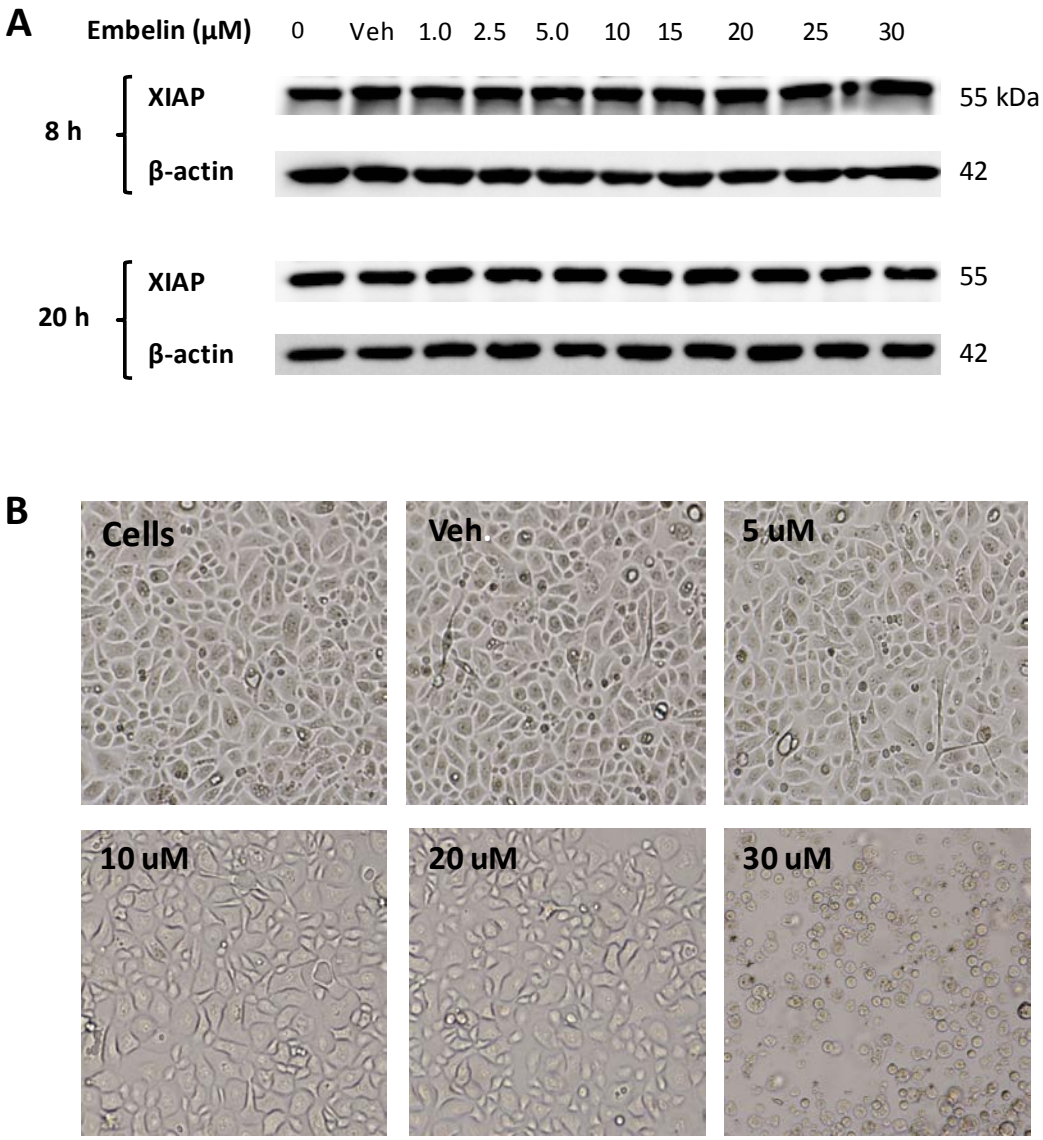
Primary airway epithelial cells (AEC) from healthy participants were assessed for alterations in IAP expression, apoptosis, and necrosis, following treatment with fluticasone propionate. High levels of fluticasone lead to induction of cIAP2, cleavage of caspase-3, and significant necrosis. In this situation, the induction of cIAP2 was insufficient to protect from apoptosis as a result of high levels of fluticasone treatment. In addition, the induction of *XAF1* transcripts was not reflected by an increase in XAF1 protein. **A:** Real-time PCR analysis of primary cells from healthy participants (n=5) were treated with increasing concentrations of fluticasone and assessed for alterations in *IAP*, and *XAF1* transcript abundance. Dimethyl sulfoxide was used as the vehicle (veh) for Fluticasone. Gene expression data was baselined to the 8 hour untreated sample, and normalised to Hypoxanthine phosphoribosyltransferase-1 and TATA-Box binding protein reference genes. "*" denotes $P < 0.05$, "**" $P < 0.01$, and "***" $P < 0.001$. Error bars represent 95% confidence intervals. **B:** Primary AECs from a single healthy participant were treated with Fluticasone for 20 hours and assessed for IAP, and XAF1 protein abundance, and markers of apoptosis. **C:** Cultures assessed for relative transcript abundance were also examined for cytotoxicity, by measuring lactate dehydrogenase release into culture media. "**" denotes $P < 0.01$, and "***" $P < 0.001$. Error bars represent 95% confidence intervals.


A
XIAP
cIAP1
cIAP2
XAF1
Relative Expression
0 veh 1 5 10 50
8 hrs
20 hrs
B
0 1 5 10 50
XIAP
cIAP1
cIAP2
XAF1
Procasp'-3
Caspase-3
PARP
β-actin
55 kDa
72
70
32
35
17/19
166
89
42
C
Cytotoxicity
Necrosis (%)
Fluticasone (μM)



Supp. Figure 6: Morphology of AECs treated with Fluticasone propionate

Primary AECs from a single participant were examined using bright field microscopy to examine morphological changes associated with treatment with the corticosteroid Fluticasone for 20 hours. Cells exhibit membrane blebbing (black arrow) indicative of disintegration of the cytoskeleton, and nuclear condensation (red arrow). Both of these events are potentiated by activated executioner caspases, and occurred after 20 hours of 50 µM Fluticasone treatment.



Supp. Figure 7: XIAP is not modulated in AEC exposure to Embelin

The XIAP inhibitor Embelin was applied to primary epithelial cells in an attempt to decrease XIAP protein abundance. Increasing concentrations of Embelin did not potentiate a decrease in XIAP protein abundance (A), and consistently caused cell death at concentrations approaching 30 μM (B). The vehicle (Veh) used to resuspend Embelin was dimethyl sulfoxide. Results are representative of two experiments, and were similar for experiments using the 16HBE14o- AEC line.

Appendices

Appendix 1: Reagents, Kits and Manufacturers

Cell Culture

Cell Culture Reagents

Amphotericin-B	Gibco Inc, Billings, MT, USA
BEGM® Bullet Kit	Lonza Group Pty Ltd, Walkersville, MD, USA
Cryostor® CS5	Stemcell Technologies Inc., Vancouver, BC, Canada
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Corp., St Louis, MO, USA
Dulbecco's phosphate buffered saline powder	Gibco Inc., Billings, MT, USA
Gentimicin solution	Gibco, Billings, MT, USA
Hank's balanced saline solution	Gibco Inc., Billings, MT, USA
Heat inactivated foetal calf serum	PAA Laboratories Pty Ltd, Morningside, QLD, Australia
Human recombinant epidermal growth factor	Sigma-Aldrich Corp., St Louis, MO, USA
L-glutamine solution	Gibco Inc., Billings, MT, USA
Minimum essential medium	Gibco Inc., Billings, MT, USA
Monoclonal Mouse Anti-Human CD68	DakoCytomation Inc., Carpentaria, CA, USA
Pencillin/Streptomycin solution	Gibco Inc., Billings, MT, USA
RPMI	Gibco Inc., Billings, MT, USA
Trypan blue stain	Gibco Inc., Billings, MT, USA
Trypsin-EDTA solution	Gibco Inc., Billings, MT, USA
Ultroser™ G powder	Pall Corp., Port Washington, NY, USA

Treatment Agents

Doxorubicin	Sigma-Aldrich Corp., St Louis, MO, USA
Fluticasone propionate	Sigma-Aldrich Corp., St Louis, MO, USA
Interferon-gamma	Sigma-Aldrich Corp., St Louis, MO, USA
Salbutamol	Sigma-Aldrich Corp., St Louis, MO, USA
Tumour necrosis factor-alpha	Sigma-Aldrich Corp., St Louis, MO, USA
PneumaCult® cilium differentiation supplements	StemCell Technologies Inc., Vancouver, BC, Canada

Immunocytochemistry

Reagents

Fluorescence mounting media	DakoCytomation Inc., Carpentaria, CA, USA
Neutral buffered formalin 10%	Sigma-Aldrich Corp., St Louis, MO, USA
Serum free blocker	DakoCytomation Inc., Carpentaria, CA, USA
Sodium dodecyl sulfate	Sigma-Aldrich Corp., St Louis, MO, USA
4', 6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich Corp., St Louis, MO, USA

Antibodies and IgG controls

Donkey anti-goat 488 conjugated IgG	Jackson ImmunoResearch Labs Inc., West Grove, PA, USA
Donkey anti-rabbit 594 conjugated IgG	Jackson ImmunoResearch Labs Inc., West Grove, PA, USA
Donkey anti-mouse 594 conjugated IgG	Jackson ImmunoResearch Labs Inc., West Grove, PA, USA

Goat IgG control antibody	Life Technologies Corp., Carlsbad, CA, USA
Human cIAP2 polyclonal goat IgG	R&D Systems Inc., Emeryville, CA, USA
Human XAF1 polyclonal rabbit IgG	AbCam Inc., Cambridge, MA, USA
Human ZO-1 monoclonal mouse IgG	Life Technologies Corp., Carlsbad, CA, USA
Rabbit IgG control antibody	Life Technologies Corp., Carlsbad, CA, USA

Western Analysis

Reagents

Antioxidant solution	Life Technologies Corp., Carlsbad, CA, USA
Cellulose blotting paper	Sigma-Aldrich Corp., St Louis, MO, USA
Dithiothreitol (DTT)	Sigma-Aldrich Corp., St Louis, MO, USA
Electrophoresis running buffer	Life Technologies Corp., Carlsbad, CA, USA
Full range protein molecular weight marker	GE Healthcare Life Sciences Corp., Sweden
Gradient (4-12%) Bis-Tris electrophoresis gels	Life Technologies Corp., Carlsbad, CA, USA
Halt® protease inhibitor cocktail	Thermo Fisher Scientific Inc., Rockford, IL, USA
Loading buffer	Life Technologies Corp., Carlsbad, CA, USA
Nitrocellulose membrane	GE Healthcare Life Sciences Corp., Sweden
Methanol (analytical grade)	Merck Pty Ltd, Darmstadt, Germany

M-Per® mammalian cell extraction reagent	Thermo Fisher Scientific Inc., Rockford, IL, USA
Phenylmethanesulfonyl fluoride	Sigma-Aldrich Corp., St Louis, MO, USA
Skim milk powder	Fronterra Fodd Services Ltd, Auckland, New Zealand
Transfer running buffer	Life Technologies Corp., Carlsbad, CA, USA

Kits

DC™ protein assay	Bio-Rad Laboratories Inc., Hercules, CA, USA
ECL™ Advanced protein detection reagents	GE Healthcare Life Sciences Corp., Sweden

Antibodies

Anti-rabbit IgG-HRP secondary antibody	R&D Systems Inc., Emeryville, CA, USA
Anti-mouse IgG-HRP secondary antibody	R&D Systems Inc., Emeryville, CA, USA

Primary antibody manufacturers are listed in Table 2.1

Real-Time Polymerase Chain Reaction

Reagents

Agarose I powder	Amresco Inc., Solon, OH, USA
Boric acid powder	Sigma-Aldrich Corp., St Louis, MO, USA
Deoxyribonucleotides	Promega Ltd, Madison, WI, USA
GelRed DNA stain solution	Biotium Inc., Hayward, CA, USA
Nuclease free water	Life Technologies Corp., Carlsbad, CA, USA
PCR grade ultra-pure water	Fisher Biotec Pty Ltd, Wembley, WA, Australia

pUC19 DNA ladder	GeneWorks Pty Ltd, Hindmarsh, SA, Australia
Random hexamers	QIAGEN Scientific Inc., Hilden, Germany
Syto-9 DNA stain	Life Technologies Corp., Carlsbad, CA, USA
6x DNA loading dye	Fermentas Inc. Hanover, MD, USA

Kits

Hotstar® Taq PCR kit	QIAGEN Scientific Inc., Hilden, Germany
M-MLV Reverse Transcriptase Kit	Life Technologies Corp., Carlsbad, CA, USA
QIAshredder® cell extraction clean up kit	QIAGEN Scientific Inc., Hilden, Germany
QIAquick® PCR product purification kit	QIAGEN Scientific Inc., Hilden, Germany
RNeasy® RNA purification kit	QIAGEN Scientific Inc., Hilden, Germany

Cell Viability, Apoptosis and Necrosis Assaying

Kits

Cytotoxicity detection kit (LDH assay)	Roche Pty Ltd, Mannheim, Germany
FAM-FLICA™ caspase-3/7 detection kit	ImmunoChemistry Technologies Ltd, Bloomington, MI, USA

siRNA Procedures

Reagents

FlexiTube® siRNAs	QIAGEN Scientific Inc., Hilden, Germany
Fluorescence siRNA control	QIAGEN Scientific Inc., Hilden, Germany

HiPerFect™ siRNA transfection reagent	QIAGEN Scientific Inc., Hilden, Germany
Scrambled siRNA control	QIAGEN Scientific Inc., Hilden, Germany

Copy Number Analysis

TaqMan® copy number reference assay RNaseP	Life Technologies Corp., Carlsbad, CA, USA
TaqMan® copy number assay to XIAP	Life Technologies Corp., Carlsbad, CA, USA
TaqMan® GT master mix	Life Technologies Corp., Carlsbad, CA, USA

General Reagents

Absolute ethanol 200 proof	Sigma-Aldrich Corp., St Louis, MO, USA
B-Mercaptoethanol	Sigma-Aldrich Corp., St Louis, MO, USA
Bovine serum albumin	Sigma-Aldrich Corp., St Louis, MO, USA
Chloroform	Merck Pty Ltd, Darmstadt, Germany
EDTA	Sigma-Aldrich Corp., St Louis, MO, USA
Neutral buffered formalin (10%)	Sigma-Aldrich Corp., St Louis, MO, USA
Sodium chloride	Sigma-Aldrich Corp., St Louis, MO, USA
Sodium citrate	Sigma-Aldrich Corp., St Louis, MO, USA

Triton-X 100	Sigma-Aldrich Corp., St Louis, MO, USA
Tris base (Tris HCl) powder	Sigma-Aldrich Corp., St Louis, MO, USA
Tween-20	Sigma-Aldrich Corp., St Louis, MO, USA

Appendix 2: Media, Buffers and Solutions

Cell Culture

Anti-CD68 solution

Anti-CD68 (32 mg/l stock) monoclonal antibody was diluted 1:500 in PBS

Bovine Serum Albumin (0.1%)

Components:

Bovine serum albumin

Hanks balanced salt solution

For 100 ml:

1. Add 100 mg of BSA to 100 ml of HBSS
2. Stir with mild heating until dissolved
3. Filter sterilise

Epidermal Growth Factor

Components:

Human recombinant epidermal growth factor (lyophilised)

0.1% BSA solution

Add 200 μg EGF to 8 ml 0.1% BSA solution for 25 μg/ml final concentration

Phosphate Buffered Saline

Add 9.55 g PBS powder to 1 L sterile ultra-pure water and autoclave

16HBE14o- (HBE) Growth Media

Components:

Minimum essential medium

Heat inactivated foetal calf serum

Penicillin/streptomycin (10,000 U/ml)

L-glutamine (100x)

For 500 ml of 16HBE14o- growth media:

1. Transfer 440 ml of Bulbecco's Modified Eagle Medium to an autoclaved bottle
2. Add 50 ml FCS for 10% v/v
3. Add 5 ml of penicillin/streptomycin solution for 100 U/ml
4. Add 5 ml of L-glutamine for 1x

Normal Human Bronchial Cell Growth Media

Components:

BEGM™ growth media

BEGM™ single quots (concentrations unknown; provided by manufacturer with media)

which include:

1. Bovine pituitary extract
2. Hydrocortisone
3. Human epidermal growth factor
4. Adreniline
5. Insulin
6. Triiodothyronine
7. Transferrin
8. Gentiamicin
9. Amphotericin-B
10. Retanoic acid

For 500 ml of NHBE growth media add all single quots to 500 ml of BEGM®

Primary Human Airway Epithelial (AEC) Collection Media

Components:

RPMI

Amphoteracin-B (250 μg/ml)

Penicillin/streptomycin (100,000 U/ml)

Gentamicin (50 mg/ml)

Foetal calf serum

For 100 ml of hAEC collection media:

1. Add 20 ml FCS to 79 ml RPMI
2. Add 100 μl of amphoteracin-B for 2.5 μg/ml final concentration
3. Add 1 ml of penicillin/streptomycin solution for 100 U/ml
4. Add 100 μl of gentimicin solution for 50 μg/ml final concentration

Primary Human Airway Epithelial Cell (AEC) Growth Media

Components:

BEGM® growth media

BEGM® single quots

Amphoteracin-B (250 μg/ml)

Penicillin/streptomycin (100,000 U/ml)

Gentamicin (50 mg/ml)

Ultroser™ G serum substitute powder

Human recombinant epidermal growth factor (25 μg/ml)

For 500 ml of hAEC growth media:

1. Combine BEGM growth media with BEGM® single quots
2. Resuspend Ultroser® G powder in 20 ml sterile water then add 10 ml to BEGM for 2% v/v
3. Add 500 ul of amphoteracin-B for 2.5 ug/ml final concentration
4. Add 5 ml of penicillin/streptomycin solution for 100 U/ml
5. Add 500 ul of gentimicin solution for 50 μg/ml final concentration
6. Add 500 μl of EGF for 0.025 μg/ml final concentration

Treatment Agents

Doxorubicin (Sigma, D1515): reconstituted in DMSO for a 1 mM stock solution

Fluticasone Propionate (Sigma number F9428): reconstituted in DMSO 50 mM stock solution

Interferon-gamma (Sigma number I3265): reconstituted to 100 μg/mL in PBS (pH 8.0), then further diluted in RPMI plus 10% FCS for a for 2 μg/ml stock solution

Salbutamol (Sigma number S8260): reconstituted in sterile PBS for a 50 mM stock solution

Tumour Necrosis Factor-alpha (Sigma number T6674): reconstituted in filter sterile PBS for a 2 μg/ml stock solution

Immunocytochemistry

Neutral Buffered Formalin (2.5%)

10% Neutral buffered formalin was diluted 1:4 in PBS for 2.5% NBF/PBS

Tris-Buffered Saline 0.05% Tween-20

Components

NaCl

Tris base (Tris HCl)

Tween-20

For 1 L:

1. Make up 1 M Tris HCl solution by adding 157.6 g Tris base to 700 ml of ultra-pure water and pH to 7.5 using 10 N HCl, then top to 1 L with ultra-pure water
2. Make up 5 M NaCl solution by adding 292.2 g to 700 ml ultra-pure water, then top to 1 L with ultra-pure water
3. Add 10 ml 1 M Tris HCl solution to 30 ml 5 M NaCl solution and top to 1 L
4. Add 500 µl of Tween-20 for 0.05% final concentration v/v

Sodium Dodecyl Suphate 0.1% Solution

Components

PBS solution

Sodium dodecyl sulphate

For 100 ml add 100 ul sodium dodecyl sulphate to 100 ml of PBS solution for 0.1% v/v

Serum free blocker 10% in PBS

For 50 ml add 5 ml serum free blocker solution to 45 ml of PBS

Western Analysis

Phenylmethanesul fonylfluoride (PMSF) 100 mM

For 1 ml add 17.4 mg of PMSF to absolute ethanol and mix by pipetting. Store immediately at -20°C after, and use within 10 min of thawing

Dithiothreitol (DTT) 500 mM

For 1 ml add 77 mg of DTT to ultra-pure water

Protein Extraction Buffer

Components:

M-Per® mammalian cell lysis buffer

Protease inhibitor cocktail (100x)

PMSF solution (100 mM)

To 1 ml of M-Per® mammalian cell lysis buffer add 10 µl of 100 mM PMSF, 10 µl of protease inhibitor cocktail and 20 µl of phosphatise inhibitor cocktail

Electrophoresis sample buffer

Components:

Loading buffer (4x)

DTT (500 mM)

Ultra-pure water

For 10 µl add 1 µl of 500 µM DTT to 2.5 µl of sample loading buffer. Add water and protein sample to 10 µl

Electrophoresis running buffer (1x)

Components:

MOPS SDS running buffer (20x)

Antioxidant solution

For 1 L at 1x concentration add 50 ml 20x MOPS SDS running buffer to 950 ml ultra-pure water and fill the outer reservoir of the electrophoresis tank. Fill the inner reservoir with 1x running buffer supplemented with 500 µl of anti-oxidant and use within 30 min.

Western transfer running buffer (1x)

Components:

Transfer buffer (20x)

Analytical grade methanol

Antioxidant solution

For 1 L at 1x concentration for a two membrane transfer add 50 ml of 20x transfer buffer to 749 ml of ultra-pure water and top to 1 L using 200 ml methanol, then add 1 ml of antioxidant solution. For 1 L at 1x concentration for a one membrane transfer add 50 ml of 20x transfer buffer to 849 ml of ultra-pure water and top to 1 L using 100 ml methanol, then add 1 ml of antioxidant solution. Use transfer buffer cold from 4°C.

Tris buffered saline Tween-20 (20x)

Components:

NaCl

Tris base (Tris HCl)

Tween-20

For 2 L of 20x TBST:

1. Add 1,700 ml ultra-pure water to a 2 L beaker on a stirrer
2. Add 121 g Tris base (Tris HCl) for 20 mM final concentration
3. Add 351.2 g NaCl for 1.5 mM final concentration
4. Mix and pH to 7.4with 10 N HCl and allow to equilibrate to room temperature
5. Add 40 ml Tween-20
6. Top to 2 L with ultra-pure water

For 1 L 1x TBST add 50 ml to 950 ml ultra-pure water. Tween-20 will be 0.1% v/v.

Skim milk blocking buffer (5% w/v)

Components:

Diploma skim milk powder

TBST 1x

For 10 ml add 0.5 g diploma skim milk to 10 ml TBST and stir until dissolved. Make fresh before use.

Bovine serum albumin blocking buffer (5% w/v)

Components:

Bovine serum albumin

TBST (1x)

For 10 ml add 0.5 g BSA to 10 ml TBST and stir until dissolved on a hot plate set to low heating. Make fresh before use.

Real time Polymerase Chain Reaction

Ethanol (70%)

For 10 ml add 7 ml of absolute ethanol 200 proof to 30 ml of nuclease free water

Sodium citrate 0.1 M in 10% ethanol

For 100 ml:

1. Add 10 ml of absolute ethanol to 80 ml of nuclease free water
2. Add .2.56 g of sodium citrate to and mix
3. Top to 100 ml with ultra-pure water
4. Autoclave

TE solution pH 8.0 (1x)

Components:

Tris base

Ethylenediaminetetraacetic acid (EDTA)

1. For10 ml of 1 M Tris base solution dissolve 242.28 mg of Tris base in ultra-pure water
2. For 2 ml of 0.5 M EDTA solution dissolve 372.2 mg EDTA in ultra-pure water
3. Add 10 ml of 1 M Tris base solution and 2 ml of 0.5 M EDTA solution to 988 ml ultra-pure water and adjust pH to 8.0 using HCl. The solution will be 10 mM Tris base with 1 mM EDTA
4. Autoclave

Syto®-9 DNA staining solution

Syto®-9 is supplied as a stock solution at 5 mM in DMSO. Dilute 4 µl Syto-9 in 196 µl PCR grade ultra-pure water for a 0.1 mM stock and add 1 µl into PCR reactions.

TBE buffer (0.5x)

Components:

Tris base

Boric acid

EDTA solution (0.5 M, pH 8.0)

For 1 L:

Dissolve 5.4 g Tris base and 2.75 g boric acid to 800 ml of ultra-pure water then add 2 ml 0.5 M EDTA solution (made as shown above). Make up to 1 L with ultra-pure water.

Appendix 3: Equipment List

Cell culture CO_2 incubator	Sanyo North American Corp., CA, USA
Collagen coated T75 culture flask	Thermo Fisher Scientific Inc., IL, USA
Collagen coated 6-well culture plate	Thermo Fisher Scientific Inc., PA, USA
Collagen coated 12-well culture plate	Becton Dickinson Pty Ltd, MA, USA
Culture slide, 8-chamber	Becton Dickinson Pty Ltd, MA, USA
Cytology brush	Medico, VIC, Australia
Electrophoresis (DNA) cell	BioRad Laboratories Inc., CA, USA
Eppendorf 5810r centrifuge	Eppendorf Inc., Hamburg, Germany
FLUOstar Optima microplate reader	BMG Lab Tech Pty Ltd, Ortenberg, Germany
Haemocytometer	Hirschmann EM Techcolor Ltd., Eberstadt, Germany
Heraeus Fresco 17 bench centrifuge	Thermo Fisher Scientific Inc., PA, USA
LAS4000 luminescent image analyser	Fujifilm Corp. Ltd, Tokyo, Japan
NanoDrop spectrophotometer	Nano Drop Technologies Inc., DE, USA
Nikon Eclipse 90i microscope	Nikon Inc., Tokyo, Japan
Orbital shaker	Barnstead/Thermolyne Corp., IA, USA
pH meter	Hanna Instruments Pty Ltd, RI, USA
Power pack	BioRad Laboratories Inc., CA, USA
PTC Peltier 200 thermal cycler	MJ Research Inc., Waltham, MA, USA
Rotor-Gene™ 6000 real-time PCR cycler	Corbett Life Science Pty Ltd, NSW, Australia
Shandon Cytospin 4 centrifuge	Thermo Fisher Scientific Inc., IL, USA
Superfrost/Plus microscope slide	HD Scientific Supplies Pty Ltd, NWS, Australia

Syngene gel documentation system	Syngene Ltd, Frederick, MD, USA
Transwells membrane insert, clear, 0.4 μm	Becton Dickinson Pty Ltd, MA, USA
XCell Surelock™ electrophoresis cell	Life Technologies Corp., CA, USA
XCell Surelock™ western module	Life Technologies Corp., CA, USA
Zeiss Axio Observer Z1 microscope	Carl Zeiss Pty Ltd., Oberkochen, Germany
5100 Cryo 1˚C freezing container	Thermo Fisher Scientific Inc., IL, USA
7300 Real-Time PCR System	Applied Biosystems Inc., CA, USA

Appendix 4: Copy of review publication

Roscioli E, Hamon R, Lester S, Murgia C, Grant J, Zalewski P. Zinc-rich inhibitor of apoptosis proteins (IAPs) as regulatory factors in the epithelium of normal and inflamed airways. Biometals 2013; 26(2):205-27.

Roscioli, E., Hamon, R., Lester, S., Murgia, C., Grant, J. & Zalewski, P. (2013) Zinc-rich inhibitor of apoptosis proteins (IAPs) as regulatory factors in the epithelium of normal and inflamed airways. *Biometals, v. 26(2), pp. 205-227*

Appendix 5: Copy of published manuscript

Lang C, Hansen M, Roscioli E, Jones J, Murgia C, Leigh Ackland M, *et al.* Dietary zinc mediates inflammation and protects against wasting and metabolic derangement caused by sustained cigarette smoke exposure in mice. Biometals 2011; 24(1):23-39.

Lang, C.J., Hansen, M., Roscioli, E., Jones, J., Murgia, C., Ackland, M.L., Zalewski, P., Anderson, G. & Ruffin, R. (2011) Dietary zinc mediates inflammation and protects against wasting and metabolic derangement caused by sustained cigarette smoke exposure in mice.
Biometals, v. 24(1), pp. 23-39

Appendix 6: Copy of published manuscript

Murgia C, Grosser D, Truong-Tran A, Roscioli E, Michalczyk A, Ackland M, *et al.* Apical localization of zinc transporter ZnT4 in human airway epithelial cells and its loss in a murine model of allergic airway inflammation. Nutrients 2011; 3(11):910-28.

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Article

Apical Localization of Zinc Transporter ZnT4 in Human Airway Epithelial Cells and Its Loss in a Murine Model of Allergic Airway Inflammation

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Abstract: The apical cytoplasm of airway epithelium (AE) contains abundant labile zinc (Zn) ions that are involved in the protection of AE from oxidants and inhaled noxious substances. A major question is how dietary Zn traffics to this compartment. In rat airways, *in vivo* selenite autometallographic (Se-AMG)-electron microscopy revealed labile Zn-selenium nanocrystals in structures resembling secretory vesicles in the apical cytoplasm. This observation was consistent with the starry-sky Zinquin fluorescence staining of labile Zn ions confined to the same region. The vesicular Zn transporter ZnT4 was likewise

prominent in both the apical and basal parts of the epithelium both in rodent and human AE, although the apical pools were more obvious. Expression of ZnT4 mRNA was unaffected by changes in the extracellular Zn concentration. However, levels increased 3-fold during growth of cells in air liquid interface cultures and decreased sharply in the presence of retinoic acid. When comparing nasal *versus* bronchial human AE cells, there were significant positive correlations between levels of ZnT4 from the same subject, suggesting that nasal brushings may allow monitoring of airway Zn transporter expression. Finally, there were marked losses of both basally-located ZnT4 protein and labile Zn in the bronchial epithelium of mice with allergic airway inflammation. This study is the first to describe co-localization of zinc vesicles with the specific zinc transporter ZnT4 in airway epithelium and loss of ZnT4 protein in inflamed airways. Direct evidence that ZnT4 regulates Zn levels in the epithelium still needs to be provided. We speculate that ZnT4 is an important regulator of zinc ion accumulation in secretory apical vesicles and that the loss of labile Zn and ZnT4 in airway inflammation contributes to AE vulnerability in diseases such as asthma.

Keywords: zinc; zinc transporter; ZnT4; airway epithelium; airway inflammation; asthma; Zinquin; Se-Autometallography (Se-AMG)

1. Introduction

Airway epithelium (AE) is a pseudo-stratified columnar epithelium that lines the conducting nasal and tracheo-bronchial airways of the respiratory system. Amongst many things, AE provides a physical barrier to inhaled noxious substances, produces smooth muscle relaxing factor and provides mucociliary clearance that helps to maintain airway sterility and integrity [1,2]. The predominant cell type in AE is the ciliated columnar cell, each containing a few hundred cilia which protrude into the airway lumen and beat in a coordinated manner to force mucin and trapped foreign substances up the respiratory tract. AE is altered and often damaged by chronic smoking and in chronic inflammatory airway diseases such as asthma [3,4].

One factor that may influence airway integrity is the essential micronutrient zinc (Zn), a metal belonging to the group IIb series. Zn is the second most abundant metal in the body although approximately two-fold less than iron. The metal is obtained largely from protein-rich foods. Zn is involved in many physiological and pathological processes being present in a multitude of metalloenzymes and an essential player in the three dimensional structure of many proteins; free or labile Zn ions play important roles as anti-oxidants and in the regulation of cell death pathways [5]. Of particular importance is the central role it plays in the immune system, in the central nervous system and in epithelial integrity [6].

Zinc deficiency was reported to complicate some chronic diseases, including asthma [7]. The NHANES II survey of 9074 adults in the general population in USA found a negative relationship between wheezing and the serum Zn to Cu ratio [8] while a case-control study in Scotland noted an increase in the presence of atopy, bronchial reactivity and the risk of allergic-type symptoms in adults

with the lowest intake of dietary Zn [9]. Several other studies (reviewed in [10]) have revealed that asthmatics are likely to have hypozincaemia and/or low hair Zn levels, suggestive of an underlying Zn deficiency or, at least, altered Zn metabolism. Particularly affected by Zn deficiency is the AE, which is normally quite resistant to oxy-radical and cytokine-induced apoptotic cell death but becomes susceptible to damage when depleted of Zn *in vitro* [11,12]. *In vivo*, mild nutritional Zn deprivation significantly worsened airway inflammation in the mouse allergic airway inflammation model and this was associated with high rates of apoptosis of epithelial cells in AE [13]. The major bulk of tissue Zn is tightly incorporated in metalloenzymes and Zn finger transcription factors. Other pools of loosely bound (labile) Zn are involved in growth regulation, apoptosis, cell signaling and secretion (reviewed in [14,15]). These labile pools have been visualized using Zn-specific fluorophores including Zinquin [16] or by conversion *in vivo* and *in vitro* to Zn-sulphur or Zn-selenium nanocrystals, which are then made visible in semi-thin Epon sections by autometallographic (AMG) silver enhancement [17–20].

In our previous studies, when Zinquin was added to cryosections of trachea or lung, strong fluorescence was seen at the luminal end of the airway epithelial cells, suggesting an abundance of labile Zn in the apical cytoplasm of the columnar cells. This was confirmed by adding Zinquin to isolated normal human AE cells (hAEC); strong fluorescence was observed in their apical cytoplasm, immediately below the ciliary apparatus and within the cilia, themselves [12,14,21]. Delivery of Zn ion to the apical AE cytoplasm may involve a vesicular pathway since the Zinquin studies showed a punctate fluorescence indicative of pools of labile Zn within some form of cytoplasmic vesicle (zincosome), in accordance with what has been shown in other cell types [22].

Intracellular zinc homeostasis is achieved by the activity of specific proteins involved in uptake, efflux and intracellular compartmentalization. In the past decade a number of mammalian zinc transporters have been identified and the corresponding genes cloned [7,23,24]. Based on their sequence homology and structural properties, they have been assigned to two distinct families: SLC39A (or ZIP, ZRT/IRT-related protein) and SLC30A (or ZnT). ZIPs are mainly involved in uptake of Zn across the plasma membrane into cytosol; while ZnTs are believed to facilitate efflux of Zn from cells and mobilization of the metal in intracellular organelles [24]. It is believed that Zn ions, derived from a circulating reservoir of Zn ion carrier proteins in the sub-epithelial capillaries, are transported into the AE by one of the plasma membrane Zn transporters. Gene expression analysis of Zn transporters have been carried out in several tissues showing that any given cell type expresses a set of these proteins [24]. With respect to airway Zn, knowledge has mostly derived from studies in animal models. A comprehensive gene expression analysis of Zn transporters in the lungs of Balb/c mice by real time qPCR revealed expression of several genes belonging to both ZIP and ZnT families with a prevalence of ZIP1, ZIP6, ZnT1, ZnT4 and ZnT6 [25]. Our previous study has shown the importance of Zn ions in the maintenance of airways homeostasis in the ovalbumin (OVA) induced acute, allergic airway inflammation model [13]. In this model that replicates some of the features of human asthma, these mice show marked reductions in the labile apical Zn pools in AE [13]. In this model the effect of inflammation on the expression of Zn transporters was up-regulation, (>2-fold) of ZIP1, ZIP6 and ZIP14 and down-regulation (>2-fold) in ZIP2, ZIP4, ZIP7, ZnT6 and ZnT4, with the latter showing the most pronounced decrease [25].

The vesicular Zn transporter ZnT4 [22,26,27] is thought to be responsible for packaging Zn in cytoplasmic vesicles and endosomes. The gene was initially identified as the molecular basis of the

mouse mutant *lethal milk* syndrome in which the ZnT4-*lm* transcript carries a premature stop codon at amino acid 297 and the incomplete protein is then rapidly degraded [28]. Dams carrying this mutation produced milk with reduced content of Zn, pointing to ZnT4 as important for Zn secretion [26,29]. ZnT4 belongs to a subfamily of ZnT transporters, which includes also ZnT2 and ZnT8. These proteins share higher degree of homology among themselves than with the remaining ZnT family members and they all appear to associate with specific secretory vesicles [30–32].

The substantial down regulation of ZnT4 mRNA expression in the mice with airway inflammation [25] suggests that this transporter may also be of relevance to changes in Zn metabolism in airway inflammatory diseases such as asthma. This study was limited, however, by the lack of knowledge as to the level of expression of ZnT4 in different lung cell types and whether this was reflected at the protein level. The major aim of the current study was to investigate the subcellular distribution of ZnT4 protein in human AE as well as in murine AE, before and after induction of allergic airway inflammation. In addition, we used Se-AMG to further explore intracellular Zn localization and distribution in AE.

2. Materials and Methods

2.1. Airway Epithelial Cells and Cultures

Normal human bronchial/tracheal epithelial cells (NHBE, passage 1) and Bronchial Cell Epithelial Growth Media (BEGM) were purchased from Lonza Australia, Mt Waverley. Cells were expanded (2 passages) and stored in aliquots in liquid nitrogen according to manufacturer's instructions. For NHBE cultures, cells were seeded onto filters to near-confluent levels on 0.4 mm PET transparent transwell filters (Becton-Dickinson, France) in BEGM medium. Filters were maintained submerged until day 7 and then the medium was removed from the apical side and continued as air liquid interface cultures. On day 9, some filters received all-trans retinoic acid (RA, 50 nM, Sigma Chemicals, St Louis, MO, USA) in the basal medium. RA was replenished daily.

Human nasal brushings were obtained using a cytology brush from consenting volunteers. Human bronchial brushings were collected from consenting patients undergoing bronchial biopsies at The Queen Elizabeth Hospital. Samples were taken from healthy looking tissues lining the bronchi through a vigorous brushing action and placed in BEGM medium. Cells were cytospun onto slides or used immediately as cell suspensions for further studies. In some subjects, both nasal and bronchial cells were collected for comparison. All procedures were approved by The Human Ethics Committee of The Queen Elizabeth Hospital.

2.2. Measurement of Trans-Epithelial Electrical Resistance (TEER)

TEER of NHBE cells in air liquid interface cultures was measured daily using a volt ohmmeter (EVOM, WPI, Sarasota, FL, USA) according to manufacturer's instructions. Before measurement, medium was added to apical side of wells and allowed to equilibrate for 10 min.

2.3. Quantitative Real Time RT-PCR (qRT-PCR)

After collection, airway epithelial cells (AEC) were immediately placed in RLT buffer and total RNA was isolated and digested with DNase, as per kit instructions (RNeasy mini kit, Qiagen, Doncaster, Victoria, Australia). cDNA was prepared from 2 μg of total RNA using a High capacity cDNA reverse transcription kit (Applied Biosystems, Mulgrave Victoria, Australia). PCR was performed using 50 μg of cDNA as template and pre-made TaqMan primer/probes (SLC39A3 Hs00536788_m1, SLC39A4 Hs00214912_m1, SLC39A6 Hs00202392_m1, SLC39A7 Hs00199596_m1, SLC39A8 Hs00223357_m1, SLC39A14 Hs00299262_m1, SLC30A4 Hs00203308_m1, SLC30A6 Hs00215827_m1. Primer/probes, TaqMan gene expression master mix and the 7300 Real Time PCR system were all from Applied Biosystems (Mulgrave Victoria, Australia). PCR conditions were standard; 50 °C for 2 min, 95 °C for 10 min, (95 °C for 15 s, 60 °C 1 min), 40 cycles. Hypoxanthine guanine phosphoribosyl transferase (HPRT) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) house-keeping genes were used as the internal standards. A sample without reverse transcriptase enzyme was included to check for DNA contamination. The cycle thresholds (C_t) were obtained for test and house-keeping genes, and their differences calculated (ΔC_t). Results were presented as $\log_2 RQ$ (relative quantity) as fold difference compared to housekeeping gene ($1^{-2\Delta C_t}$) as described by Applied Biosystems (User Bulletin #2ABI).

2.4. Zinquin Labeling

Zinquin, ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate (Dr. A.D. Ward, Department of Chemistry, University of Adelaide, South Australia) was dissolved in dimethylsulfoxide (DMSO) at 5 mM and stored at −20 °C in the dark. Cells were incubated for 30 min in 25 μM Zinquin at room temperature (RT). Zinquin was freshly diluted in 1× phosphate-buffered saline (PBS) immediately before addition. A Bio-Rad MRC-1000 UV Laser Scanning Confocal Microscope System, equipped with UV-Argon laser, was used in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode [21]. Fluorescence excitation was at 351/8 nm and emission at 460 long pass (LP). Images were collected using a 40× water immersion objective lens with NA 1.15. Each image was averaged over 6 scans by Kalman filtering.

2.5. Immunofluorescence and Antibodies

Air dried cryosections and cytospun cells were fixed in 100% chilled acetone for 10 min at RT while cytospun cells were fixed in 4% paraformaldehyde for 15 min at RT, before washing with PBS containing 4% BSA (3× for 5 min each), to minimize non-specific binding. Primary antibody used in this study was rabbit polyclonal anti-ZnT4 [22]. Rabbit anti-rat ZnT4 was used at 1:250 dilution overnight at RT in a humidified chamber. Secondary antibody was fluoroscein isothiocyanate-conjugated goat anti-rabbit IgG (Rocklands, Gilbertsville, PA, USA) at 1:250 dilution for 2 h at RT. A drop of Anti-Fade fluorescent mounting medium (DAKO Corporations, CA, USA) was added, the coverslip-mounted and immediately (Excitation 488/10 nm and emission at 522/35).

2.6. Dual Labeling of ZnT4 and Zinc

For dual labeling studies of ZnT4 and Zn on cytospun cells, a similar protocol was used for that of ZnT4 alone except that, after secondary antibody addition, cells were labeled for Zn by Zinquin. Slides were washed and Zinquin was added to a final concentration of 25 μM in PBS. Slides were washed again in PBS and mounted as above. Where dual staining was performed, fluorescence images were merged using Confocal Assistant (Version 4.02) software package.

2.7. Murine Airway Inflammation Model

All experiments were performed under the University of Adelaide Animal Ethics Committee, approval number M-54-2001 and in compliance with "Principles of Animal Care" publication number 86-23 of the National Institute of Health and the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes", 6th ed. The method is described in detail elsewhere [13]. Briefly, female Balb/c mice (age 4–6 weeks; pathogen free) were randomly divided into experimental groups and housed at 21 °C with a 14-h light/10-h dark cycle. Mice received 50 μg of chicken ovalbumin (OVA) per 1 mL of alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline, intra-peritoneal (i.p.) on days 0 and 14. Control (SAL) mice received alhydrogel in 0.9% saline alone. Mice sensitized to OVA were then aerochallenged with 10 mg/mL OVA in 0.9% saline from day 22 to day 32, for 30 min, three times a day, every 2nd or 3rd day, using a side-stream nebulizer, which produced particles of 1–3 μm (Fisher and Paykel, Sydney, Australia). SAL-treated groups were nebulised with 0.9% saline alone. Mice were sacrificed over 2 days, on the day following their last nebulisation. Tissues were collected and processed as described in [13].

Cryosections of ~5 μm width were cut from regions of the lung rich in airways. Images (both bright-field and fluorescence) were captured at several sites along the epithelium. Profile lines were drawn across the epithelium based on bright-field photographs and superimposed on the corresponding fluorescence images. This was necessary for the OVA-treated mice as it was often difficult to see the epithelium in the fluorescence sections. At least 5 lines were drawn for each image for 5–10 images at randomly-chosen spots along the AE for each mouse); a total of 10 SAL and 11 OVA mice were measured in this way. Because of varying epithelial thickness, mean fluorescence intensity was calculated for each tenth interval across the epithelium, beginning at the luminal surface and ending at the basal surface.

2.8. In Vitro *Se Autometallography in Pig Tracheal Epithelial Cells*

Pig trachea was obtained freshly from the local abattoir. Ciliated epithelial cells were dislodged by rapid shaking, washed and resuspended to 10^7/mL in 5 mL of 0.1M PBS (Sorenson's, pH 7.4) containing 0 or 10 mg/mL sodium selenide (Sigma). After 60 min at RT, 5 mL of 3% glutaraldehyde in PBS was added and cells fixed overnight at RT. The following day cells were washed 3× with PBS. Cells were smeared on glass slides, air-dried and stained with AMG developer [17,18] for 70 min at 26 °C in a dark box. Smears were post-fixed with 70% ethanol for 30 min and counterstained with 0.1% aqueous toluidine blue pH 4.0. Zn staining was assessed by light microscopy.

2.9. In Vivo *Se Autometallography in Rat Tracheal Epithelium*

The specificity of this technique for detecting Zn ions has previously been well-characterized in sections of brain and other tissues [18]. Zn-Se AMG has not previously been used for tracing of Zn ions in lung tissue and minor adaptations have therefore been introduced in order to obtain maximum outcome. Male Wistar rats were euthanized and 10 mL of a 10 mg/mL solution of sodium selenide was infused into the trachea and lungs for 3 min, followed by 3% glutaraldehyde in PBS for a further 3 min. Lungs and trachea were removed, cut into 4 pieces each and left in 3% glutaraldehyde in PBS overnight at RT. 100 μm sections were cut using a vibratome, dipped in 0.5% gelatin and developed with AMG while floating, according to standard methods as described elsewhere [17,18]. To stop the reaction, 5% sodium thiosulphate was added for 10 min. Sections were rinsed in distilled water, counterstained with 0.1% toluidine blue and examined by light microscopy. Interesting areas were cut into smaller blocks, fixed in 1% osmium tetroxide for 1 h and stained with uranyl acetate. Sections were alcohol-fixed, embedded in Epon and prepared for transmission electron microscopy.

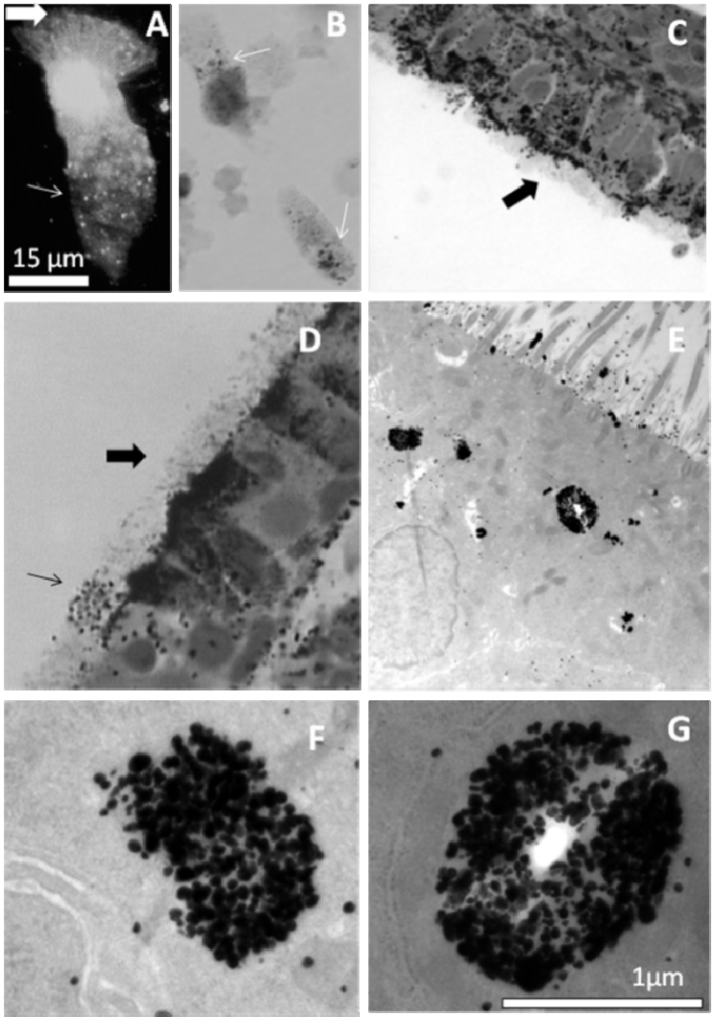
3. Results

3.1. Zinquin Fluorescence and Autometallography of Metal Ions in Apical Cytoplasm of AE

For labeling of Zn, brushed human ciliated columnar bronchial epithelial cells were treated in suspension with the Zn fluorophore Zinquin and examined by fluorescence microscopy. We have preferred to use Zinquin in these experiments rather than the more commonly used FluoZin3, as in our hands, Zinquin gives sharper images with these airway epithelial cells. Others have shown that Zinquin and FluoZin3 label different Zn-containing compartments in islet beta cells, granules and cytosol, respectively [33]. As previously shown [21], Zinquin stained AE cells in a vesicular manner throughout the cytoplasm but with a very high intensity in the ciliated, apical portion of the cell (thick arrow, typical shown in (Figure 1A). Close examination of the apical cytoplasm shows that the fluorescence was largely within vesicles as well as in the cilia. Some vesicles (thin arrow in Figure 1A) were also seen towards the basal end of the cell. Fluorescence was completely quenched by the specific, membrane-permeable Zn chelator TPEN (not shown).

A vesicular localization of Zn ions was confirmed by Se-AMG. Figure 1B shows Zn-Se nanocrystals (thin arrows), in the apical cytoplasm of pig tracheal epithelial cells after exposure of the isolated cells to sodium selenide *in vitro*. Next, we introduced sodium selenide into the trachea of anaesthetized rats, followed by fixative. Bright field images show intense labeling of metal ions in the apical cytoplasm (thick arrows in Figure 1C,D). Interestingly, occasional cells showed staining restricted to only the cilia (thin arrow in Figure 1D), giving the impression that the cell had secreted its Zn ions across the apical membrane. Electron micrographs confirmed the strong labeling in the apical cytoplasm to be located both in vesicles and in the cytoplasm and on the surface of the lower part of the cilia (Figure 1E–G).

Figure 1. Zinquin fluorescence and autometallography of metal ions in apical cytoplasm of airway epithelium (AE) (**A**) Labeling of labile Zn by Zinquin in a typical human ciliated bronchial epithelial cell. Note the intense fluorescence in the apical cytoplasm, fluorescence in cilia (thick arrow) and vesicular-like fluorescence extending into the basal region of the cell (thin arrow); (**B**) *In vitro* silver enhanced ZnSe nanocrystals (thin arrows) in the apical cytoplasm of pig tracheal epithelial cells; (**C**–**G**) *In vivo* Se-AMG-stained rat tracheal epithelium. Note the labeling of Zn ions in the cytoplasm below the cilia (thick arrow). In one of the AE cells (Panel D, thin arrow), Zn ions are largely absent from the cytoplasm, but seem to have moved out of the cell and onto the surfaces of the cilia. In panels E–G, typical transmission electron micrographs show clusters of zinc-selenium nanocrystals in the apical cytoplasm and around the cilia. Some clusters (e.g., panel G), show a structure that appears to be enclosed by a membrane and to contain a hollow core surrounded by metal ion.

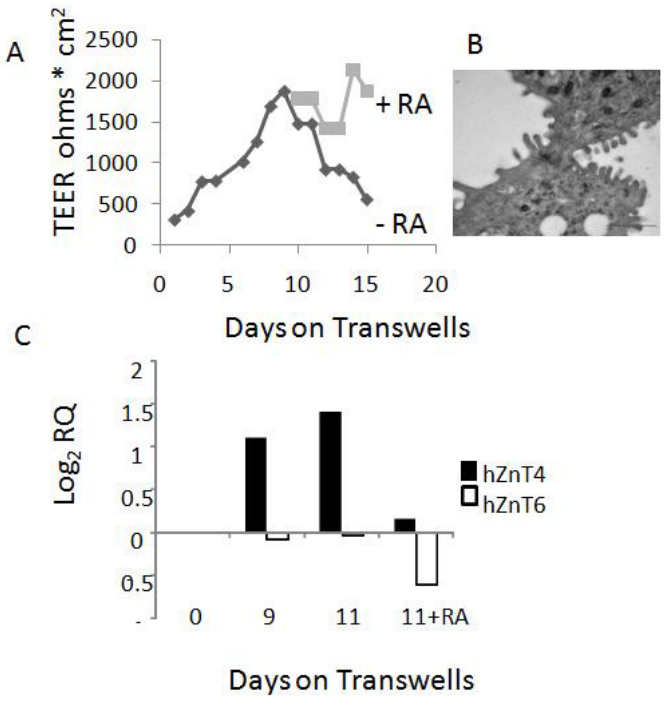


3.2. Expression of Vesicular Zn Transporter ZnT4 by qPCR in Human AEC

Our previous studies in whole mouse lung showed that ZnT4 is expressed at the mRNA level, along with a number of other ZIP and ZnT Zn transporters [25]. To confirm that ZnT4 is also expressed at the mRNA level in human AEC, we used real time qPCR initially on NHBE cells that had been seeded

on filters in submerged cultures and after a few days converted to air-liquid interface (ALI) cultures to mimic airways environment [34]. ALI culture caused a marked increase of trans-epithelial electrical resistance (TEER), peaking at day 9 (Figure 2A); this was likely to be due to formation of tight junctions, an important feature of functional AE. Cultures beyond day 9 resulted in a dramatic decline in TEER but this decline could be reversed by addition of retinoic acid (RA) to the cultures (Figure 2A). Electron microscopy studies confirmed that these culture conditions promoted the formation of morphologically recognizable tight junctions (example shown in Figure 2B). Levels of ZnT4 mRNA increased almost 3-fold during ALI culture of NHBE cells on air-liquid interface, peaking at days 9–12 (Figure 2C). Addition of RA at day 11 resulted in a marked decrease in ZnT4 mRNA (Figure 2C). For comparison, we also studied expression of ZnT6 that remained stable in expression throughout the experiment (Figure 2C). Other data showed no change in expression of ZIPs 3, 7 and 8 during the differentiation, while ZIPs 4, 6 and 14 decreased (data not shown).

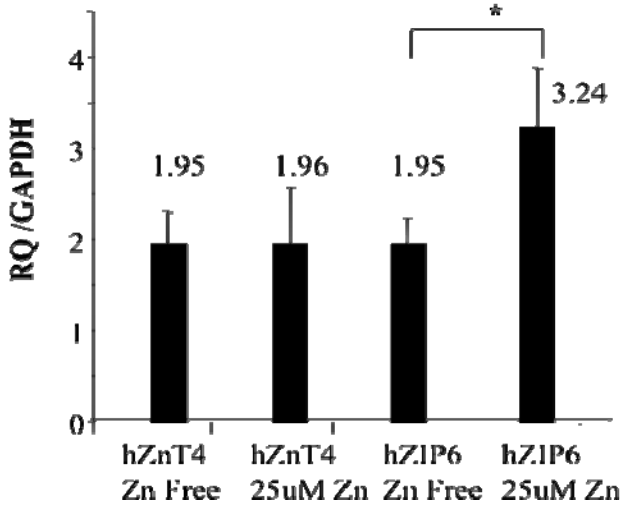
Figure 2. ZnT4 in air-liquid interface cultures of normal human bronchial/tracheal epithelial cells (NHBE). (**A**) Changes in transepithelial resistance (Trans-Epithelial Electrical Resistance (TEER), ohms × cm^2) plotted against days in culture on transwell filters (see methods). Note the steep increase in TEER up to day 9 of culture and loss of TEER after day 9 in the absence, but not presence, of retinoic acid (RA); (**B**) Transmission electron micrograph showing a typical tight junction formed in cultures exposed to RA; (**C**) ZnT4 mRNA relative to hypoxanthine phosphoribosyltransferase (HPRT) increased during culture on filters but was lost following addition of RA. For comparison, ZnT6 did not increase during culture.



To determine whether primary human AE also expresses ZnT4 mRNA, human nasal AEC were obtained from three donors by nasal brushing and ZnT4 mRNA was assayed by real time qPCR. ZIP6 was also determined for comparison since it was previously shown to be up-regulated by exogenous

zinc in the culture medium [35]. All three samples showed abundant expression of both transporters. Next, we determined whether levels of ZnT4 and ZIP6 were influenced by extracellular Zn. Human nasal AEC were cultured overnight in either Zn-depleted medium or Zn-depleted medium re-supplemented with 25 μM Zn sulphate. Figure 3 shows no change in ZnT4 mRNA levels. By contrast, there was a 1.7-fold increase in ZIP6 mRNA level in the Zn-supplemented cultures.

Figure 3. ZnT4 mRNA expression in human nasal AE was unaffected by extracellular Zn. ZnT4, unlike ZIP6, did not respond to an increase in extracellular Zn concentration (to 25 μM).



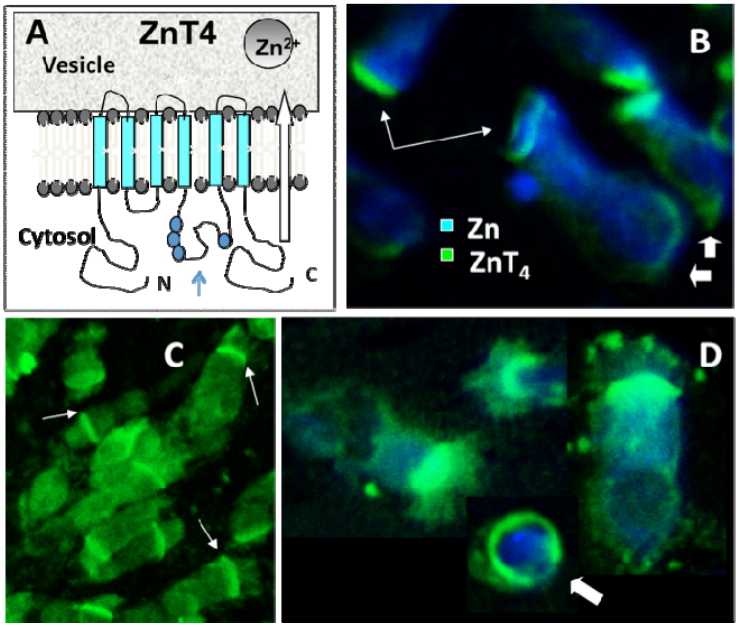
3.3. Localization of Vesicular Zn Transporter ZnT4 in Primary Human AEC by Immunofluorescence

A schematic representation of ZnT4 protein membrane topology is shown in (Figure 4A). The protein possesses 6 putative trans-membrane domains (light blue) and resides in the membranes of intracellular vesicles and other organelles [22,27]. Thin arrow in (Figure 4A) denotes the putative Zn-binding, histidine-rich loop protruding into the cytoplasm. The intracellular distribution of ZnT4 protein was studied in primary human AE cells (both nasal and bronchial), using a specific polyclonal antibody previously described [22]. Immunofluorescence studies showed a predominantly apical cytoplasmic distribution of ZnT4 protein in freshly isolated human airway epithelial cells (Figure 4B–D), similar to the distribution of labile Zn (Figure 1A). The apical ZnT4 fluorescence was predominantly in the region just below the cilia, although, in some cells, fluorescence was observed in the cilia, themselves (Figure 4D). Some cells also had a rim of fluorescence at the basolateral membrane (e.g., Figure 4B, thick arrows), occasionally extending around the entire plasma membrane, especially in rounded up cells (Figure 4D). Secondary antibody alone also did not result in significant fluorescence (not shown). In singly-labeled cells, the fluorescence intensities for Zn and ZnT4 were quantified by image analysis within different subcellular compartments (see Methods). The order from most intense staining to least intense for ZnT4 was apical, perinuclear, nuclear, membrane, cilia and basal. For zinc the order was apical, cilia, perinuclear, nuclear, membrane then basal.

Next, we attempted to study the co-localization of ZnT4 and Zn, using dual-labeled cells (anti-ZnT4 and Zinquin, (Figures 4B,D). There was considerable overlap of Zn (blue) and ZnT4 (green) fluorescence; however, the Zinquin fluorescence was often much more diffuse and lower in intensity than in cells stained with Zinquin alone. This was an artifact due to the effect of the

fixative/permeabilization technique (required for the immunofluorescence assay) on the labile Zn compartment.

Figure 4. Localization of vesicular Zn transporter ZnT4 in primary human airway epithelial cells (AEC) by immunofluorescence (**A**) ZnT4 with 6 putative trans-membrane domains penetrates the membranes of cytoplasmic endosomes, vesicles and secretory granules with both *N*- and *C*-terminal ends and the histidine-rich loop, thought to bind cytosolic Zn, protruding into the cytosol; the large arrow shows the presumed direction of Zn movement out of the cytosol and into the vesicle; (**B**–**D**) Immunofluorescence labeling of ZnT4 (green) in human bronchial epithelial cells showing a tight band of fluorescence in the far apical cytoplasm and in the cilia as well as occasional staining of basolateral membrane (B and C) and of the entire membrane in a non-columnar cell (arrow in D). Blue labeling in panels B and D depicts Zinquin fluorescence of dual-labeled human bronchial epithelial cells. The more diffuse blue staining of Zn ions is due to effects of fixative on subsequent Zinquin staining. Merged confocal images are shown. Thin arrows in B and C point to the apical staining while thick arrows in B point to the basal ends.



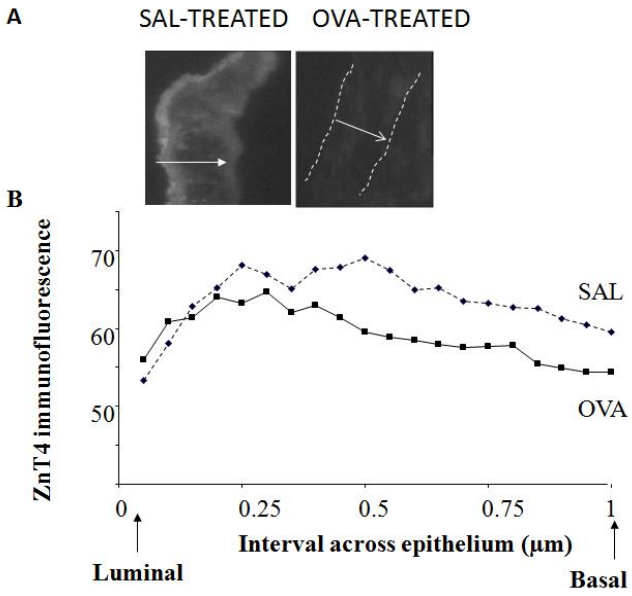
3.4. Comparisons between Nasal and Bronchial Human AEC

To determine whether the staining patterns for Zn and ZnT4 in nasal AEC were similar to those in lung AEC, staining patterns and fluorescence intensities for ZnT4 and Zn were compared for AEC derived by bronchial brushing (n = 5, obtained during routine bronchoscopies) with those for AEC derived by nasal brushing (n = 5). The patterns of Zn and ZnT4 distribution were similar in the two types of cells (not shown) and there were reasonably good correlations between levels of ZnT4 in nasal *versus* bronchial AEC (r^2 = 0.76) and between levels of labile Zn in nasal *versus* bronchial AEC (r^2 = 0.57). There was, however, a tendency for higher Zn values in the bronchus compared to the nose. The opposite was found with ZnT4 where there was significantly higher intensity (P = 0.008) in the nasal samples compared with the bronchial.

3.5. Down-Regulation of ZnT4 Protein in AE of Mice with Allergic Airway Inflammation

To confirm the presence of ZnT4 transporter protein in murine AE and its down-regulation during airway inflammation, ZnT4 protein was labeled by indirect immunofluorescence in cryo-sections of lung airways from control (SAL-treated) or airway-inflamed (OVA-treated) mice. (Figure 5A) shows typical images for ZnT4 in AE of SAL and OVA mice and (Figure 5B) shows the intensity across the epithelium from luminal (left) to basal (right) ends, averaged over 5–10 images for 10 SAL and 11 OVA mice (see Methods for detail). There was negligible fluorescence in AE stained with secondary antibody alone (not shown). For the entire epithelium, the mean ± SD ZnT4 fluorescence (arbitrary fluorescence units) for the SAL group was 63.8 ± 3.9 compared to the OVA group 59.2 ± 3.3. The difference was highly significant (P = 0.0002, Student t test, 2-tailed). For the apical side of the epithelium (intervals 1–7), the mean ± SD ZnT4 fluorescence (arbitrary fluorescence units) for the SAL group was 62.8 ± 5.3 compared to the OVA group 61.8 ± 2.9. The difference was not significant (P = 0.67, Student t test, 2-tailed). For the basal side of the epithelium (intervals 8–20), the mean ± SD ZnT4 fluorescence (arbitrary fluorescence units) for the SAL group was 64.3 ± 3.1 compared to the OVA group 57.8 ± 2.6. The difference was highly significant (P = 0.000006, Student t test, 2-tailed).

Figure 5. Loss of ZnT4 staining in AE of ovalbumin (OVA)-treated mice with airway inflammation (**A**) Figure shows ZnT4 immunofluorescence labeling in AE of a typical sham-treated control Balb/C mouse (left) and OVA-treated Balb/C mouse (right). In the latter photomicrograph, the dashed lines indicate the orientation of the epithelium. The arrow indicates the width of the epithelium from luminal (tail of arrow) to basal (head of arrow). Note the stronger ZnT4 fluorescence at the luminal (apical) end in the control section and the loss of fluorescence in the OVA-treated mouse AE; (**B**) Figure shows the loss of ZnT4 fluorescence averaged over a number of mice. Mean fluorescence intensity per interval across the epithelium is shown, from luminal end (left) to basal end (right). Means were from multiple images (5–10 per mouse) for a total of 10 SAL and 11 OVA mice. There was a significant loss of ZnT4 staining in the OVA-treated mice (P < 0.005), preferentially in the basal compartment.



In control mice, the expression of immuno-reactive ZnT4 was found throughout the AE but most pronounced at the luminal end. There was a significant and substantial decrease in the ZnT4 expression of the OVA mice especially towards the basal end but no significant decrease at the apical end.

4. Discussion

This study provides three major findings and an exciting new hypothesis: (1) EM tracing of Zn ions in clusters of vesicle-like organelles in the apical cytoplasm of AE and on the surface of the lower third of the cilia; (2) Immunohistochemical tracing of vesicular Zn transporter ZnT4 predominantly localized to the Zn-enriched apical cytoplasm but also at the basal end, suggesting its contribution to Zn homeostasis in this tissue; and (3) a parallel loss of ZnT4 protein and Zn ions in AE of mice with airway inflammation. Several models involving different animal species were used in this study, because of the specific techniques being used in the different experiments. Findings with the different species support the generality of the conclusions. We believe that these findings have important implications for the mechanisms of altered Zn homeostasis and Zn deficiency in chronic airway inflammatory disease.

We have previously shown striking losses of both airway epithelial Zn [13] and whole lung ZnT4 mRNA [25] in mice with allergic airway inflammation. In this study we have confirmed the ZnT4 gene expression in human AEC at the protein level. We have also found that the ZnT4 mRNA expression in AE cells was not affected by adding Zn ions to the medium while the ZIP6 was up-regulated. ZIP proteins are thought to be involved in cellular Zn uptake [24]. ZIP6 is one candidate for the plasma membrane Zn transporter that brings Zn ions into the AE cells, although other ZIP transporters are expressed in AE and require further study. Of particular interest are the recent elegant studies by Daren Knoell's group [36,37] showing a specific role for another ZIP transporter ZIP8 in mediating uptake of Zn by human airway epithelial cells (primary and BEAS-2B) in response to a pro-inflammatory stimulus TNFα and a role for ZIP8 in maintaining Zn levels in these cells required for epithelial cell monolayer integrity and cell survival as well as roles for ZIP8 in sepsis. Their studies showed that some of the imported zinc ended up in cytoplasmic vesicles in the airway epithelial cells [35]. It may be that ZIP8 and ZnT4 act in concert to maintain intracellular levels of Zn in airway epithelium, mediating plasma membrane uptake followed by vesicular localization of Zn, respectively. It is not clear whether AEC derive the bulk of their Zn from sub-epithelial capillaries or can absorb Zn across their apical membranes from epithelial secretions or plasma exudates. A study of the distribution of ZIP transporters through the basal-apical axis of AE cells followed by functional analysis needs to be carried out to answer this question.

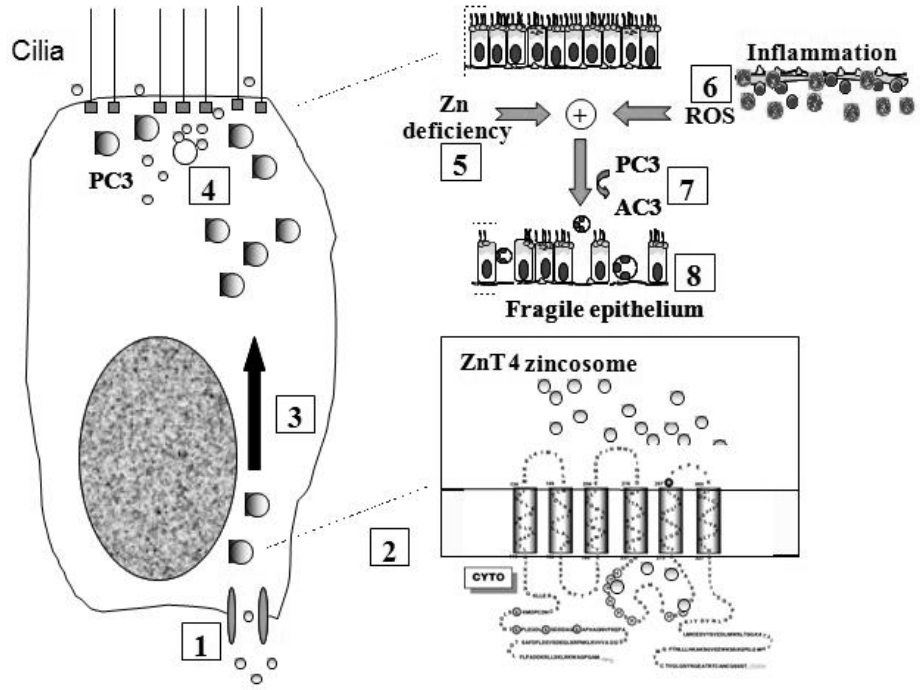
The experiments with the Se-AMG technique confirm the Zinquin findings of high levels of loosely bound or free Zn ions in the apical cytoplasm of AEC. Of particular interest from the EM studies was the presence of Zn-selenium nanoparticles in the apical vesicular-like structures. Similar apical Zn-rich structures have previously been reported in the prostate epithelium where they mediate secretion of Zn into the prostatic fluid and subsequently into ejaculated spermatozoa [38,39], in the synaptic vesicles of Zn-enriched (ZEN) neurons of the CNS and in the salivary glands of rats and multiple other secretory cells including Paneth cells and mast cells (22). The Zinquin fluorescence and ZnT4

immunofluorescence studies in both isolated human AEC and mouse AE cryosections suggest a prominent role for ZnT4 in redistribution of Zn within AE. The "starry sky" staining pattern of labile Zn in the apical cytoplasm and the coinciding patterns of labile Zn and ZnT4 indicate a sequestering role for ZnT4 in the membranes of cytoplasmic Zn vesicles (zincosomes) on the apical side of polarized epithelial cells. The term "zincosome" refers to vesicular pools of labile Zn in cells [40,41]. Free or loosely bound Zn ions seem to be involved in apoptosis when present in the cytoplasm under certain circumstances. They have been found to bind to and inactivate essential sulphydryls at the active sites of cytoplasmic enzymes [5]; however in most cases loosely bound or free Zn ions are beneficial and supportive (e.g., in wound healing and storage of proteins in vesicles such as insulin in the beta-cells of the pancreas).

The observation that ZnT4 is partially lost from AE during murine airway inflammation supports our previous findings that inflammation causes ZnT4 mRNA down-regulation [30] and loss of labile Zn staining [17]. There is evidence that some asthmatics have low systemic levels of Zn (serum/plasma and hair Zn). However, whether this is equivalent to clinical Zn deficiency has yet to be determined (discussed in [18]). Plasma Zn constitutes less than 1% of total body Zn [42] and estimations based on blood may therefore not be representative for the level of free or loosely bound levels of Zn in airway tissue. We have recently shown that the levels of Zn ions in induced sputum are decreased in older patients with asthma [43]. Measuring Zn levels in bronchial brushings presents a technical problem in that these brushings are not routinely performed in asthmatic subjects. Nasal epithelial cells, however, are more easily obtained by nasal brushing. Several studies have shown that nasal epithelial cells resemble bronchial epithelial cells from the same subject in a number of properties [44,45]. Here we have shown reasonably good correlations between bronchial and nasal Zn staining from the same subject, as well as between bronchial and nasal ZnT4 staining.

Figure 6 shows a model incorporating the specific role of ZnT4 in airway Zn homeostasis, the normal functions of apical vesicle Zn in AE and the abnormalities that accompany or promote airway inflammatory disease. We propose that Zn is transported into the AE cells by way of one or more ZIP transporters and stored in the apical vesicles by ZnT4. The finding that Zn repletion did not increase the transcription of ZnT4, but increased ZIP6 transcription, argues against a role for ZnT4 in the plasma membrane uptake of Zn. However, as our immunofluorescence studies suggested that some cells do appear to have plasma membrane ZnT4, a role also in Zn uptake cannot be entirely ruled out. The presence of a significant pool of ZnT4 at the basal end of the cells is consistent with the hypothesis that ZnT4 receives Zn as it is imported across the basal plasma membrane. If this is the case, the preferential depletion of the basal ZnT4 in the OVA-treated mice would be consistent with an interruption of the uptake and packaging of Zn during airway inflammation. The primary role of ZnT4 appears to be incorporation of cytosolic Zn ions into vesicles for transport to the apical region. Apical cytoplasmic Zn-regulated proteins include Cu/Zn superoxide dismutase, a major anti-oxidant in AE, and procaspase-3, a pro-apoptotic effector [21]. The presence of Zn ions in these enzymes is consistent with cytoprotective anti-oxidant and anti-apoptotic roles for labile Zn in AE [5].

Figure 6. Model for role of ZnT4 in AE Zn homeostasis and airway inflammation: Step **1**: Zn is taken up across the basolateral plasma membrane of AEC from sub-epithelial capillaries via ZIP6 or other ZIP transporter(s); Step **2**: Zn is incorporated into vesicles with the aid of ZnT_4 (see inset); Step **3**: These vesicles translocate to the apical cytoplasm; Step **4**: Apical cellular Zn may protect the cytoplasmic mucociliary apparatus (e.g., tubulin and basal bodies) from damage by oxidants and other toxins that would otherwise trigger pro-caspase-3 (PC3) activation. Vesicular Zn may also be secreted across the apical plasma membrane into the epithelial lining fluid, cilia and mucin; Step **5**: Apical cellular Zn is depleted in chronic inflammatory airway disease. Mechanisms may include abnormalities in ZnT_4, hyper-secretion of Zn or excessive loss of Zn by luminal shedding of dying AEC or exudation of inflammatory cells; Step **6**: Depletion of Zn renders AEC vulnerable to reactive oxygen species (ROS) released from inflammatory cells or mitochondria; Step **7**: This leads to premature activation of PC3 to active caspase-3 (AC3) and downstream events in apoptosis. Zn depletion also directly facilitates PC3 activation because this enzyme is inhibited by binding of Zn to an essential sulphydryl group [5]; Step **8**: The altered epithelium has increased apoptosis and epithelial sloughing, which contribute to the ongoing inflammation.



The protective role of Zn ions might also explain the Zn-selenium nanoparticles found adjacent to the bases of the cilia. These microtubule-rich organelles are vulnerable to oxidative stress [46] and Zn is known to protect tubulin from oxidation in cell-free extracts [47]. Impairment of the beating of cilia in Zn depleted AEC would be consistent with our previous studies showing substantial oxidative damage in the apical and ciliary membranes of Zn depleted human AEC [21]. Such damage might lead to paralysis of the cilia and, eventually, epithelial cell death. These processes would likely be facilitated in airway inflammatory disease where there is both Zn depletion in AE and oxidant release from infiltrating eosinophils and neutrophils. The observation of ZnT4 near the base of the cilia and

the images showing occasional cells with Zn-depleted cytoplasm and Zn-rich cilia (e.g., Figure 1D) suggest that some of the vesicular Zn is destined for secretion across the apical membrane and into the epithelial lining fluid. A role for ZnT4 in secretion of AE Zn is not unlikely, as it is involved also in Zn secretion into breast milk [32,33,37].

5. Conclusions

In summary, we have now characterized the distribution of a major Zn transporter ZnT4 in human airway epithelial cells and compared it at two sites: the nasal and bronchial mucosa. The subcellular distribution of ZnT4 supports the notion that Zn ions have significant roles in normal function of the mammalian airway epithelium, while the loss of ZnT4 in a murine asthma model implicates it as a factor in the mechanisms leading to aberrant Zn homeostasis, epithelial vulnerability and possibly ciliary dysfunction in airway inflammatory disease. However, further evidence including targeted up- or down-regulation of ZnT4 is required to prove a functional role for ZnT4 in maintaining zinc homeostasis within the airway epithelium while studies with other proinflammatory mediators (e.g., TNFα) will indicate the generality of the findings in other models of inflammation.

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Conflict of Interest

The authors declare no conflict of interest.

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Appendix 7: Copy of published manuscript

Tan N, Tran H, Roscioli E, Wormald P, Vreugde S. Prevention of false positive binding during immunofluorescence of *Staphylococcus aureus* infected tissue biopsies. Journal of Immunological Methods 2012; 384(1-2):111-7.

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Journal of Immunological Methods, v. 384(1-2), pp. 111-117

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