THE ROLE OF APOPTOSIS IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Abbreviations

7AAD	7-amino-actinomycin D
AEC	Airway epithelial cell
AM	Alveolar macrophage
AP-1	Activator protein-
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
Culture tubes	10ml conical polypropylene tubes
DIP	Desquamative interstitial pneumonia
DLCO	Diffusing capacity for carbon monoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERS	European Respiratory Society
Facs tube	Polystyrene tube (5 ml)
FADD	Fas associated death domain
FCS	Fetal calf serum
FL-1	Fluorescence channel 2
FEV-1	Forced expiratory volume in one second
FL-2	Fluorescence channel 2
FL3	Fluorescence channel 3
FITC	Fluorescein isothiocyanate
FSC	Forward light scatter
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony stimulating factor
GCS	Glucocorticosteroids
g	Gram
GOLD	Global Strategy for Chronic Obstructive Lung disease
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-	Interleukin
ILD	Interstitial lung disease
JAK	Janus kinase
JNK	Jun N-terminal kinase
Kda	Kilodalton
L	Litre
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein
MFI	Median fluorescence intensity
MRNA	Messenger ribonucleic acid
MAP	Mitogen activated protein
MIP-1	Macrophage inflammatory protein-1
Mab	Monoclonal antibody
MTG	Mitotracker green
MW	Molecular weight
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
Ng	Nanogram
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PHA	Phyto haemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PI	Propidium iodide
PMT	Photomulitiplier tube
PTS	Phosphatidyl-serine
RANTES	Regulated on activation, normal T expressed and secreted
Rh	Recombinant
Rb	Retinoblastoma gene product
RBILD	Respiratory bronchiolitis
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Culture Media
STAT	Signal-transduction-activated transcription factors
SD	Standard deviation
SEM	Standard error of the mean
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TRADD	TNF associated death domain
TRAF-	TNF-associated factor
TSP	Thrombospondin
μg	Microgram
μl	Microlitre
Wash buffer	0.5% BSA in Isoton II

Synopsis

COPD is a chronic disease of the airways, usually caused by cigarette smoking and characterised by chronic inflammation and tissue destruction. There are no effective treatments for this debilitating disease and the monetary cost to the Australian community is $800 million per year. In addition, there are devastating social consequences to those with COPD and their families. To date, however, there have been few studies on the cause of defective repair in COPD.

In COPD there is inadequate repair of the chronically damaged and inflamed airway epithelium. There is a school of thought that this inadequate repair may result from an accumulation of apoptotic material in the airways, resulting in secondary necrosis, tissue destruction and chronic inflammation. This thesis is principally an investigation of apoptosis and clearance of apoptotic cells in COPD using samples of peripheral blood, and bronchial brushings and bronchial lavage (BAL) obtained from the airways during fibreoptic bronchoscopy. Importantly, it describes the development of a number of new methods to investigate these biologic specimens.

Increased apoptosis of brushing-derived airway epithelial cells (AEC) and lymphocytes (from BAL) was observed in COPD. Activation of several apoptotic pathways was also increased in COPD, including Fas/Fas ligand, TNF-α/ TNF receptor and TGF-β/ TGF receptor. These results support the hypothesis that excess apoptosis and accumulation of apoptotic material in the airways are key factors in the pathogenesis of COPD.

Whether the increase in apoptotic material in the airways was a result of defective clearance by alveolar macrophages was subsequently investigated. A significantly reduced capacity for alveolar macrophages from COPD subjects to ingest apoptotic AEC was found. This deficiency appeared to be specific for apoptotic cells, as tests carried out in parallel using carboxylate-modified polystyrene microbeads revealed no significant difference between COPD patients and control subjects. The results

demonstrate that the failure to resolve epithelial damage in COPD may result, at least partially, from defects in recognition and/or clearance of apoptotic AEC by AM.

Based on the findings of increased apoptosis of lymphocytes in the airways in COPD as well as reports that lymphocytes from the airway re-enter the peripheral circulation, I investigated the hypothesis that, in COPD, there may be increased T-cell apoptosis in the peripheral blood. As in the airways, significantly increased apoptosis and upregulated TNF-α/TNFR-I, Fas and TGFR was observed in stimulated peripheral blood T-cells in COPD. Whether these findings represent a systemic effect of COPD on peripheral cells or, alternatively, whether these cells have re-entered the circulation after passing through the epithelium, requires further study. This study was then extended to show that the mechanisms by which T-cells in the peripheral blood undergo apoptosis include increased expression of the pro-apoptotic mediator p53, and decreased expression of the receptor for the anti-apoptotic cytokine, IL-7. Given these findings, it would be interesting to apply these techniques to brushing-derived AEC in COPD. Due to time constraints these were not carried out, but one can speculate that similar mechanisms may be involved in the airways.

In airway repair, TGF-β increases apoptosis and inhibits proliferation of AEC. A normal bronchial AEC line (16HBE) was applied as an in vitro model, to further investigate which mechanisms are responsible for control of TGF-β production once the repair process is complete. Both IL-4 and TNF-α, cytokines produced in response to inflammatory stimulus in the airways, were shown to inhibit production of TGF-β by AEC. In COPD, however, where there is increased production of TGF-β and ineffective repair, the inhibitory effects of TNF-α and IL4 on TGF-β production may be overwhelmed by other mechanisms.

An interesting cause for the increase in TGF-β in the airways in COPD was found. Apoptotic AEC were shown to produce cytokines, including TGF-β, *in vitro*. In the

normal lung, increased production of TGF-β by AEC undergoing apoptosis may contribute to inhibition of proliferation, airway repair and reduction of the inflammatory response following acute injury. In COPD, where there is already increased apoptosis and increased TGF-β, the added effect of more TGF-β production by apoptotic AEC may overwhelm the normal regulatory mechanisms.

The findings from this thesis indicate that failure to resolve epithelial damage and chronic inflammation in COPD may result, at least partially, from increased apoptosis, increased activation of apoptotic pathways and defective clearance of apoptotic material by AM. The new insights into the role of tissue defence mechanisms in COPD are relevant in relation to future experimental approaches that may help to design novel new treatment strategies for COPD.

Publications

Published manuscripts

Hodge S, Hodge G, Flower R, PN Reynolds, R Scicchitano, M Holmes (2003) Alveolar macrophages from subjects with COPD are deficient in their ability to phagocytose apoptotic airway epithelial cells. Immunology and Cell Biology (in press)

Hodge S, Hodge G, Flower R, PN Reynolds, R Scicchitano, M Holmes (2003) Increased production of TGF-β and apoptosis of T-lymphocytes in peripheral blood in COPD. Am J Physiol Lung Cell Mol Physiol, 285 (in press)

Hodge G, Hodge S, Lawrence A, Han P. (2003) A marked decrease in L-Selectin Expression by T and B lymphocytes in infants with B. Pertussis infection- Lymphocytosis explained? Respirology (in press)

Hodge G, Hodge S, Han P. (2002) Allium sativum (Garlic) suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. Cytometry, 48 (4), 209-215.

Hodge S, Hodge G, Flower R, PN Reynolds, R Scicchitano, M Holmes (2002) Up-regulation of production of TGF- β and IL-4 and down-regulation of IL-6 by apoptotic human bronchial epithelial cells. Immunol Cell Biol,80, 537-543

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Hodge G, Hodge S, Han P. (2000) Increased levels of apoptosis of leucocyte subsets in PBMCs compared to whole blood as shown by Annexin V binding- relevance to cytokine production. Cytokine, 12, 1763-1768.

Published abstracts

Hodge S, Hodge G, Reynolds P, Scicchitano R, Holmes. M. (2003) Apoptosis, phagocytosis and COPD. Respirology, 8 (Suppl.) A8

Hodge S, Hodge G, Reynolds P, Scicchitano R, Holmes. M. (2003) A flow cytometric assay to quantify phagocytosis of apoptotic epithelial cells by alveolar macrophages. Respirology, 8 (Suppl.) A65

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Hodge S, Hodge G, Flower R, Holmes M, Scicchitano R. (2001) IL-4 and TNF-α inhibit TGF-β production by human lung epithelial cells: relevance to COPD. Respirology, 5, 39A

Awards and external appointments

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External Appointments

2000 National Secretary, Australasian Flow Cytometry Group

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma 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.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

23/9/03

Sandra J. Hodge Dated

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Chapter One

Introduction and literature review

1-1 Introduction

Chronic obstructive pulmonary disease (COPD) arises as a result of noxious injury to the lungs, most commonly due to cigarette smoking. COPD is defined as "a disease state characterised by airflow limitation that is not fully reversible. The airway limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (US Department of Health and Human Services, 2001).

COPD is the fourth highest cause of mortality worldwide and costs the Australian community more than $800 million per year. Despite this high prevalence and the obvious burden to the national and international communities, the pathogeneseis of COPD, and therefore the rationale for therapy, is poorly understood and there are currently no satisfactory treatments for this important disease. New insights into the basis of COPD, which should lead to more effective treatments, are thus urgently required. However, to data, research in this area has been relatively neglected.

It is recognised that COPD is an inflammatory disease. Treatment has been based on traditional anti-inflammatory approaches, such as the use of corticosteroids (steroids). These have failed to have a significant impact on COPD progression as they do not control epithelial destruction nor alter the gradual decline of lung function associated with the disorder. Furthermore, the use of steroids is frequently

associated with adverse effects. Studies into the pathogenesis of COPD are of medical and scientific importance.

Airway epithelial cells (AEC) not only create a physical barrier between the airway lumen and the internal milieu, but are also an integral part of the bio-response of the lungs (eg, to noxious stimuli). These cells are an important source of cytokines, which play a role in the tightly controlled process of airway repair. In particular, the cytokine TGF-β facilitates airway repair by stimulating deposition of extracellular matrix components and controlling removal of unwanted cells by inducing apoptosis. Pertubations of the well- controlled repair process may be important in the pathogenesis of COPD.

There is inadequate repair of the chronically damaged and inflamed airway epithelium in COPD. It is possible that this inadequate repair may be the result of increased rates of apoptosis of AEC. Apoptosis contributes to immunoregulation and normal cell turnover in the lung. There is evidence from several studies, however, that excessive apoptosis and/or reduced clearance of apoptotic cells is associated with tissue damage and inflammation in the airways. These studies have investigated apoptosis in emphysema (Kasahara et al. 2000; Kasahara et al. 2001), pulmonary fibrosis (Hagimoto et al. 1997; Hagimoto et al. 1999), cystic fibrosis (Vandivier et al. 2002) and obliterative bronchiolitis in lung transplantation (Hansen et al. 2000).

Furthermore, tissue injury as a result of increased apoptosis has been demonstrated in Fas induced liver injury in mice (Ogasawara et al. 1993), chronic hepatitis C (Hiramatsu et al. 1994), eczema (Trautmann et al. 2000) and heart disease (Felzen et al. 1998). Uncleared apoptotic cells may undergo secondary necrosis with

discharge of injurious cell contents, tissue damage and chronic inflammation (Kuwano et al. 1999; Hagimoto et al. 1997). Cigarette smoking as well as some of the known changes associated with COPD (including production of oxidants, enzymes and disruption of the extracellular matrix) has the potential to cause apoptosis of AEC (D'Agostini et al. 2001; Jyonouchi et al. 1998; Vernooy et al. 2001). Based on these data I hypothesised that excess apoptosis, accumulation of apoptotic material and defective clearance of these cells in the airways are key factors in the pathogenesis of COPD.

The central theme of this study is an investigation of the role of apoptosis, and the cellular mechanisms and mediators involved in apoptosis and clearance of apoptotic cells, in the pathogenesis of COPD. New insights into the role of the airway epithelium and cellular interactions in the pathogenesis of COPD may lead to design of novel therapies for this debilitating disease.

1-2 The normal airway epithelium

The respiratory tract is lined with a continuous layer of AEC of various types (**Figure 1-1(a)**). The surface of the proximal airways comprises epithelial cells in a pseudo-stratified arrangement, in which the most prominent cell types are ciliated epithelial cells (approximately 70-80% of the surface epithelium) and goblet cells (approximately 15-25%) that reach from the basal lamina to the airway lumen. Other cell types, comprising less than 5% of the total numbers, include serous AEC, basal AEC, Clara cells and neuro-endocrine cells. The main functions of the ciliated epithelial cells are trans-epithelial electrolyte transport and mucociliary clearance. Goblet cells synthesise mucin. Basal cells are poorly differentiated, flattened,

pyramid shaped cells with the potential to differentiate into other cell types within the airways (Nettesheim et al. 1990).

In the bronchioles, Clara cells and basal cells predominate. Clara cells are responsible for surfactant production and may participate in the detoxification of inhaled noxious substances (Thompson et al. 1995). Cilia are not present in the distal airways, goblet cells are less numerous and the epithelium has a more columnar appearance.

In the alveolus, the columnar epithelium is replaced by a thin epithelium comprised of approximately 95% Type I cells, with smaller numbers of cuboidal-shape Type II cells (**Figure 1-1(b)**). The main function of Type I cells is gas exchange. The apical surface is covered with microvilli. The cytoplasm of Type II cells contains numerous lamellated inclusions containing lipids and proteins, which are secreted onto the apical surface to form surfactant (Thompson et al. 1995).

The connective tissue sub-strata (lamina propria) comprises fine capillaries, elastic tissue and a reticular connective tissue meshwork. The sub-mucosa contains denser connective tissue, submucosal glands, stromal cells, fibroblasts, smooth muscle cells and leucocytes.

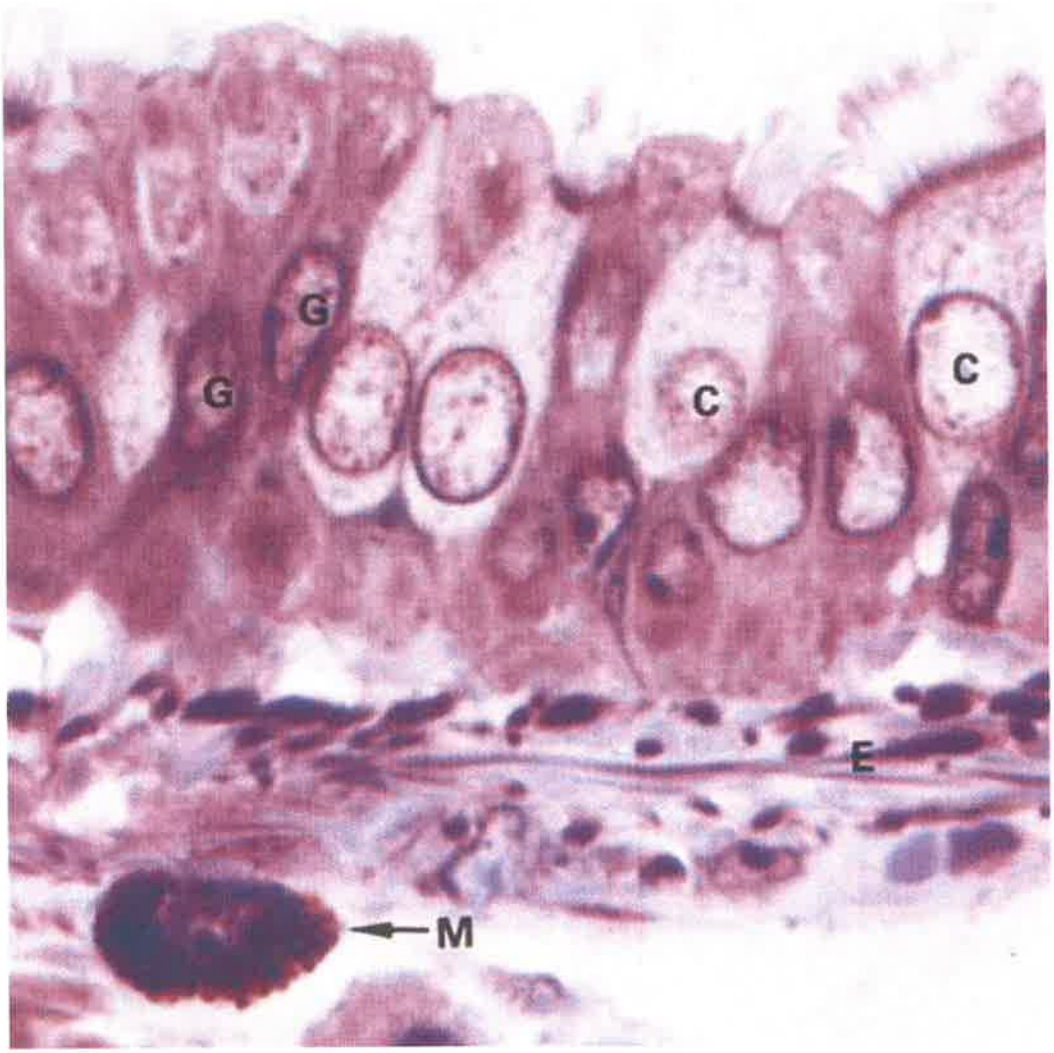

Figure 1-1(a)

Structure of pseudostratified airway epithelium. A micrograph shows that the bases of all cells extend down to the basement membrane although not all cells reach the luminal surface. G denotes goblet cell, C denotes ciliated AEC. The underlying lamina propria contains a large proportion of elastin (E). A mast cell (M) is shown.

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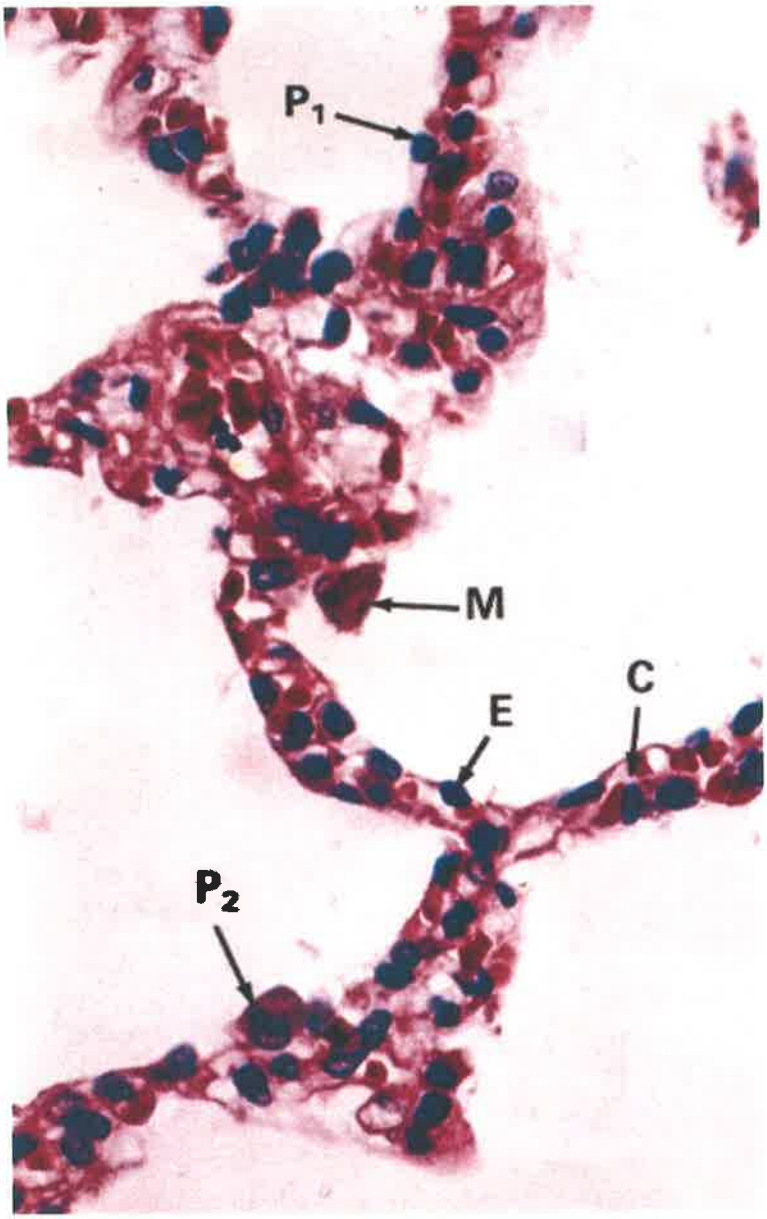


Figure 1-1(b)

Structure of alveolar wall. The micrograph shows that most of the surface area is covered by large Type I pneumocytes (P1). A second cell type, Type 11 pneumocytes (P2), represent some 60% of cells in the lining epithelium. Blood vessels, mainly capilliaries (C) 7-10 μm in diameter, form an extensive plexus around each alveolus. E denotes nuclei of capillary endothelial cell, M denotes AM, found in the alveolar wall or free in the alveolar space.

Reproduced with permission from Wheater's Functional Histology. Young B and Heath J;Churchill Livingstone 2000, p228.

1-3 AEC functions

The function and location of specific AEC types is presented in **Figure 1-2**.

AEC provide a morphological barrier for macromolecules, such as inhaled debris and infectious agents (**Figure 1-3**). This barrier is formed by arrangement of AEC into a continuous surface by tight junctions composed of specific proteins.

AEC also contribute to airway repair and inflammatory processes. AEC are target cells in airway inflammation and also effector cells, synthesising and releasing a variety of cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-4, IL-5, IL-6, IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF) and transforming growth factor (TGF)-β (Kwon et al. 1995; Striz et al. 1999; Christman et al. 1991; Magnan et al. 1994). A list of cytokines or mediators produced by AEC is given in **Table 1-1**.

The inflammatory and chemotactic cytokines listed above regulate recruitment and function of inflammatory cells in response to:

(a) Agents present in the lumen, such as oxidants

(b) inflammatory mediators

(c) irritants such as cigarette smoke

(d) other cytokines produced by cells in the airway or airway lumen.

AEC can interact with other inflammatory cells and regulate their activity via a number of mechanisms. Appropriate cytokine receptors are expressed that are responsive to cytokines produced by other cells such as AM (Takizawa et al. 1996;

Boussaud et al. 1998; de Boer et al. 1998; van der Velden et al. 1998) which are located directly in the surroundings of AEC. These cytokines can also direct inflammatory cell migration across the epithelium by modulating expression of cell surface adhesion molecules. Each one of these processes contributes to and can function to amplify an inflammatory response.

Thus, since AEC are an important cellular source of cytokines in the airways, these cells are likely to play an important role in the repair of injured epithelium, and the resolution of inflammation associated with acute lung injury.

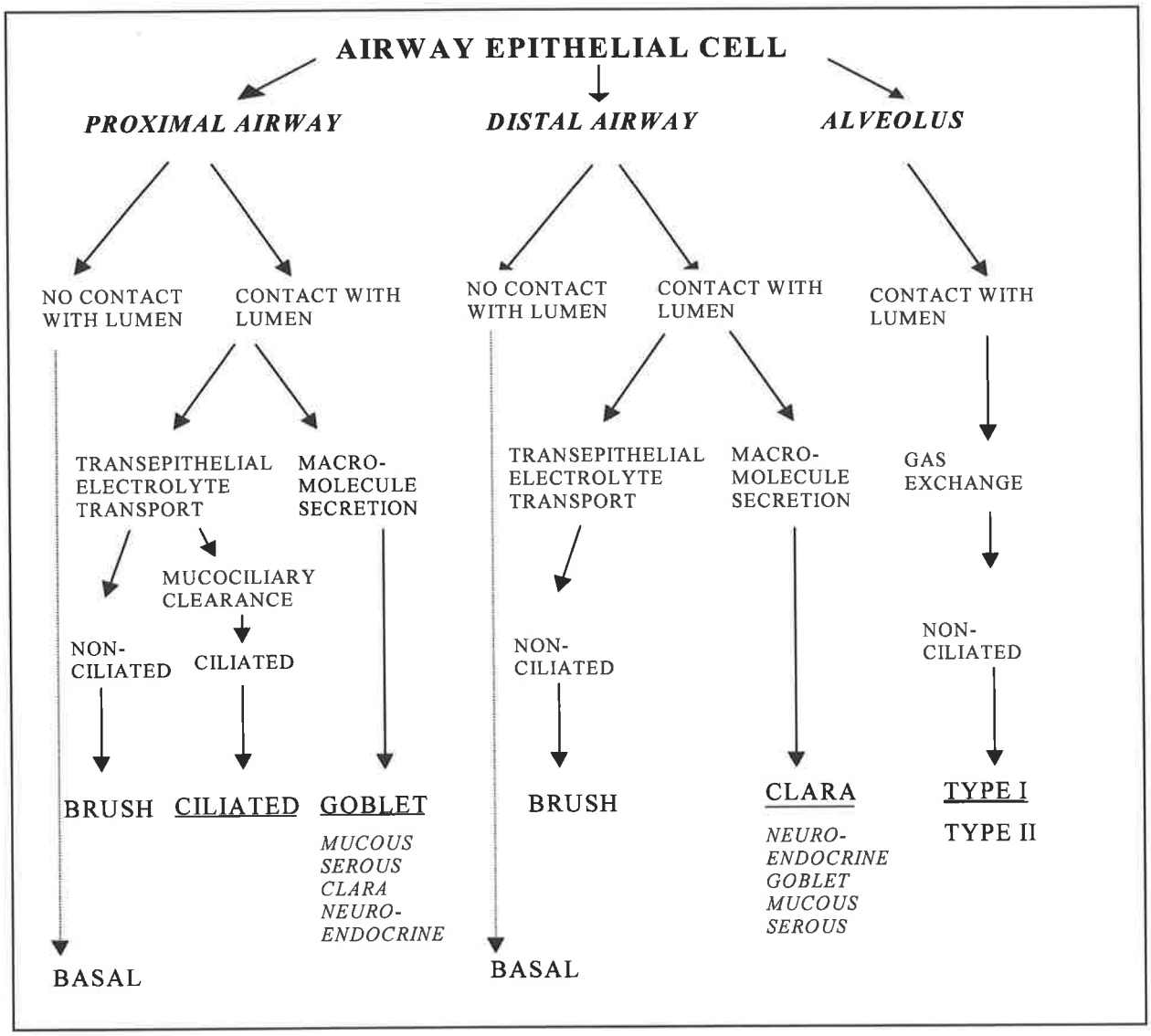
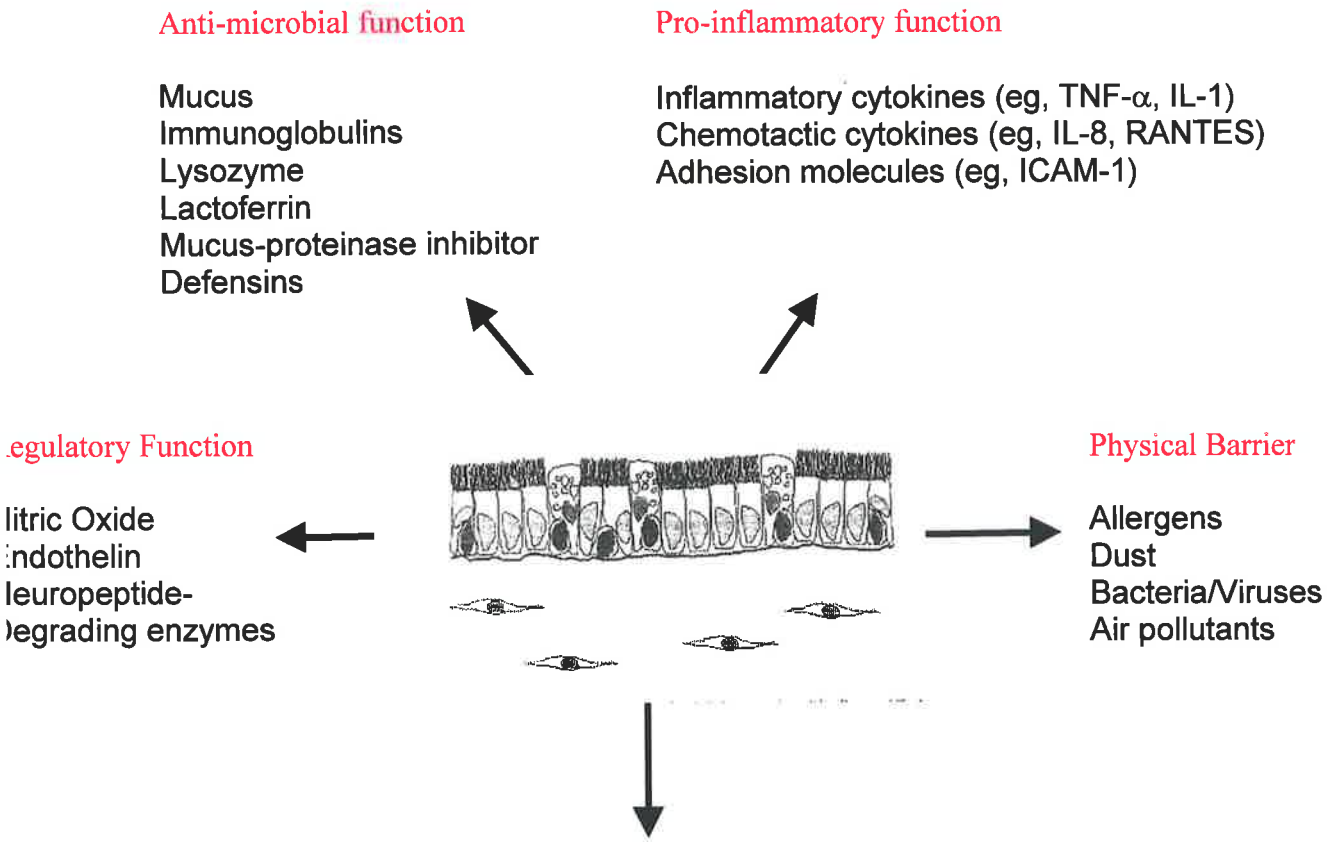


Figure 1-2

Function and location of major AEC types. The cell types most prevalent in the specified section of the airway is underlined.



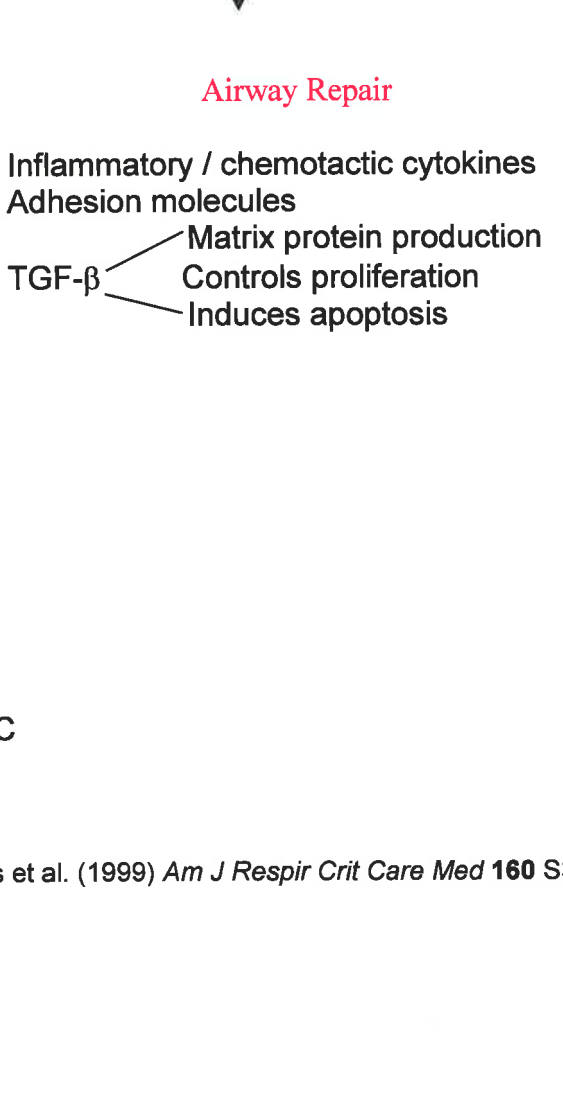


Figure 1-3

Function of AEC

Adapted from Mills et al. (1999) *Am J Respir Crit Care Med* **160** S38-40

Table 1-1. AEC production of cytokines and other mediators

Cytokine / mediator	Reference
TGF-β	Magnan et al, 1994
G-CSF	Levine et al, 1993, Takizawa et al,
GM- CSF	Kwon et al, 1994, Takizawa et al,
TNF-α	Striz et al, 1999
IFN-γ	Salvi et al, 2000
MCP-1	Lundien et al, 2002
IL-1β	Mills et al, 1999, Takizawa et al, 1998
IL-1RA	Levine et al, 1997
IL-2	Aoki et al, 1997
IL-3	Von Essen et al, 1994
IL-4	Christman et al, 1991
IL-5	Zhu et al, 2001, Salvi et al, 1999
IL-6	Ulich et al, 1991, Cromwell et al, 1992
IL-8	Bagglioni et al, 1992, Cromwell et
IL-10	Dosanjh et al, 2001
IL-11	Mills et al, 1999
IL-16	Cheng et al, 2001, Yoshida et al, 2001
IL-18	Cameron et al, 1999
RANTES	Wang et al, 1996
Eotaxin	Papadopolous et al, 2001
Endothelin-1	Endo et al, 1992, Nakano et al, 1994
Platelet derived growth factor [PDGF]	Fang et al, 2000
Nerve growth factor	Fox et al, 2001
Epidermal growth factor [EGF] (EGF)	Holgate S, 2000
Pre-protachykinin-A	Reynolds et al, 2001
PGE2 [Prostaglandin 2]	Christman et al, 1991
Leukotreine B4 (LTB4)	Koyama et al, 1991
Di-hydroxyeicosotetraenoic acids (di-HETES)	Koyama et al, 1991
Prostaglandin E2 (PGE2)	Rennard et al, 1995
Platelet activating factor PAF)	Rennard et al, 1995
Platelet derived growth factor (PDGF)	Rennard et al, 1995
Endothelin-1	Rennard et al, 1995
Calcitonin gene reactive protein (CGRP)	Rennard et al, 1995
Gro-gamma	Carter et al, 1994
Nitic Oxide (NO)	Robbins et al, 1993

1-4 Repair of injured airway epithelium in the normal lung

Acute injury to the lung epithelium, by noxious agents such as cigarette smoke, induces an inflammatory response (Mio et al. 1997). In the normal lung, downregulation of the processes involved in the inflammatory response is associated with resolution of injury. Therefore the reparative process includes removal of inflammatory stimuli, limitation of secretion of pro-inflammatory cytokine production by leucocytes and cessation of neutrophil and monocyte chemotaxis (Haslett, 1999)

Acute lung injury causes physical changes to the epithelium. Portions of the basement membrane are exposed as a result of loss of cells. A provisional matrix, derived from plasma proteins including fibrin and fibronectin, is formed rapidly. Residual basal cells release cytokines that initiate inflammatory responses (paracrine function). AEC respond to these cytokines by producing additional cytokines (eg, TGF-β), which upregulate AEC production of fibronectin and collagen (autocrine function).

Simultaneously, cytokines such as epidermal growth factor (EGF), MCP-1 and GMCSF induce de-differentiation and proliferation of AEC present at the wound margins (Holgate, 2000; Lundien et al. 2002; Huffman et al. 1996; Groves and Schmidt-Lucke, 2000). The AEC flatten, migrate across the provisional matrix to cover the wound, then the recruited proliferating AEC accumulate (epithelial cell hyperplasia) (Rennard, 1999). The accumulated de-differentiated AEC acquire differentiated phenotypes, restoring normal epithelial architecture. Although the cellular mechanisms for removal of excess AEC (as well as inflammatory cells, fibroblasts and endotheial cells) have not been elucidated, the process most likely

involves apoptosis followed by phagocytosis without disruption of neighbouring tissue (Bitterman et al. 1994; Polunovsky et al. 1993;Vandivier et al. 2002; Bardales et al. 1996). Various stages of airway repair are presented in **Figure 1-4**.

Without further injury, the repair process is rapid. In a study of mechanically injured tracheal epithelium, Erjefalt et al (1995) investigated epithelial repair using scanning- and transmission electron microscopy and light microscopy. Cell proliferation was analysed by [3H]-thymidine autoradiography. Immediately after epithelial removal, secretory and ciliated (and presumably basal) AEC at the wound margin dedifferentiated, flattened and migrated rapidly (2-3 microns/min) over the denuded basement membrane. Within 8-15 h a new, flattened epithelium covered the entire deepithelialised zone. At 30 h a tight epithelial barrier was established and after 5 days the epithelium was fully differentiated.

The processes that regulate AEC recruitment, proliferation and differentiation are not fully characterised. Chemotactic factors, such as fibronectin, initiate the recruitment stage (Shoji et al. 2001). Fibronectin is an important component of the extracellular matrix in the airways. Fibronectin is a multifunctional glycoprotein, produced by AEC in response to a variety of cytokines including TGF-β (Nakamura et al. 1995). Other extracellular matrix components, which are produced at increased rates in the presence of TGF-β, include laminin, fibrinogen, tenascin and collagens (Romberger et al. 1992; Ignotz and Massague, 1986). AEC express receptors for these matrix proteins (integrins) and their adherence to the extracellular matrix is important in maintaining epithelial integrity. TGF-β not only induces deposition of extracellular matrix but also increases expression of integrins on the AEC surface, thus facilitating adhesion of AEC to the matrix (Wang et al. 1996; Ignotz et al. 1989). Integrins are composed of one alpha and one beta subunit that are noncovalently

bound (eg, the fibronectin receptor, α5β1). **Table 1-2** presents a summary of these functionally important extracellular proteins and whether each is expressed on AEC.

The recruitment stage is followed by proliferation of AEC. Both mesenchymal fibroblasts and AEC themselves can release factors, including GM-CSF, MCP-1 and EGF, that promote AEC proliferation (Holgate, 2000; Huffman et al. 1996; Lundien et al. 2002). Further, *in vitro* studies have shown that AM can drive the proliferation of cultured AEC (Takizawa et al. 1990). In addition, both soluble and matrix components of the extracellular milieu promote proliferation (Rennard, 1999).

Table 1-2. Important extracellular matrix proteins, receptors, and their CD characterisation

Matrix protein	**Alpha/beta receptor subunit**	**CD-characterisation**	**Detectable on bronchial AEC?**
Laminin, collagen	α1β1	CD49A/ CD29	No
Laminin, collagen	α2β1	CD49B/ CD29	Yes
Laminin, collagen, fibronectin	α3β1	CD49C/ CD29	Yes
Fibronectin, VCAM-1	α4β1	CD49D/ CD29	No
Fibronectin	α5β1	CD49E/ CD29	Trace
Laminin	α6β1	CD49F/ CD29	Yes
Vitronectin	αvβ3	CD51/ CD61	CD51: Yes CD61: No
Fibronectin, tenascin, vitronectin	αvβ5	CD51/-	Yes
Fibronectin, tenascin, vitronectin	αvβ6	CD51/-	Yes

Adapted from Mette et al (1993), Sheppard et al (2001)

Figure 1-4

Stages of airway repair

a. Normal epithelium.
b. Following acute airway injury, portions of the basement membrane become stripped of cells. A provisional matrix derived from plasma proteins including fibrin and fibronectin is rapidly formed.
c. AEC drive the wound healing process. Remaining basal cells release cytokines which function by paracrine or autocrine means to drive an inflammatory response. AEC can respond to these cytokines by producing additional cytokines (eg, TGF-β, which drives production of fibronectin by AEC). These cytokines induce de-differentiation of AEC present at the wound margins. These cells migrate across the provisional matrix, restoring epithelial integrity.
d. The recruited AEC proliferate and accumulate (hyperplasia).
e. Over the course of days to weeks, the newly recruited AEC can differentiate and restore epithelial architecture, with resolution of inflammatory response. Unwanted cells undergo apoptosis and are removed by neighbouring AM without an inflammatory response.

Adapted from: Rennard S. (1999) *Am J Respir Crit Care Med* **160** S13

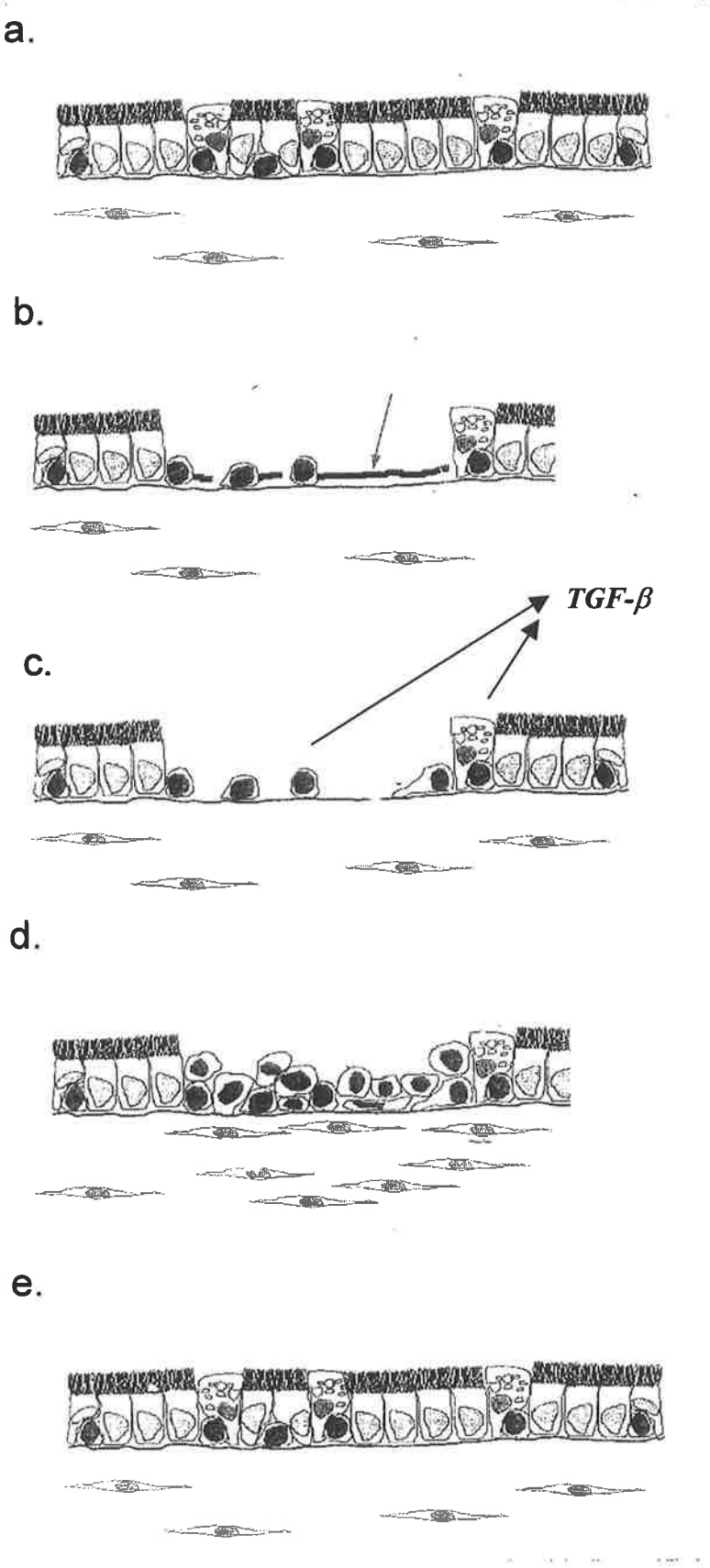


Figure 1-4

Stages of airway repair

1-5 TGF-β: role in repair of the normal airway

Transforming growth factor (TGF)-β is a homodimeric protein of 25 kDa produced by a wide variety of cell types, including AEC (Magnan et al. 1994). Activated TGF-β elicits a wide variety of biologic responses, depending on the target tissue (Kehrl, 1991). The TGF-β family comprises three isoforms, TGF-β1, β2 and β3. The latent form of TGF-β (L-TGF-β) is biologically inactive and unable to bind to TGF-β receptors until it has been activated (Blobe et al. 2000). Activation of TGF-β can occur in response to reactive oxygen species and also via a plasmin-mediated pathway that also involves thrombospondin-1 (TSP-1, a cell adhesion glycoprotein) the thrombospondin receptor (CD36) and integrin αvβ3 (Munger et al. 1999). Prior to activation, TGF-β is non-covalently associated with a latency-associated peptide (LAP). Once AM are activated, eg, as a result of acute lung injury, plasmin, increased L- TGF-β and increased TSP-1 are generated. TSP-1 associates with the LAP/ TGF-β complex which then associates with the cell surface of the macophage by the TSP receptor (CD36). In the presence of plasmin and the integrin αvβ3, TSP binds to LAP, releasing TGF-β in an activated state and able to bind to receptors on other cells (Yehualaeshet et al. 2000). The mechanisms for plasmin-mediated activation of latent TGF-β are presented in **Figure 1-5**. Another source of TGF-β is platelets, where TGF-β is stored in its activated form in intracellular granules that are released on platelet activation.

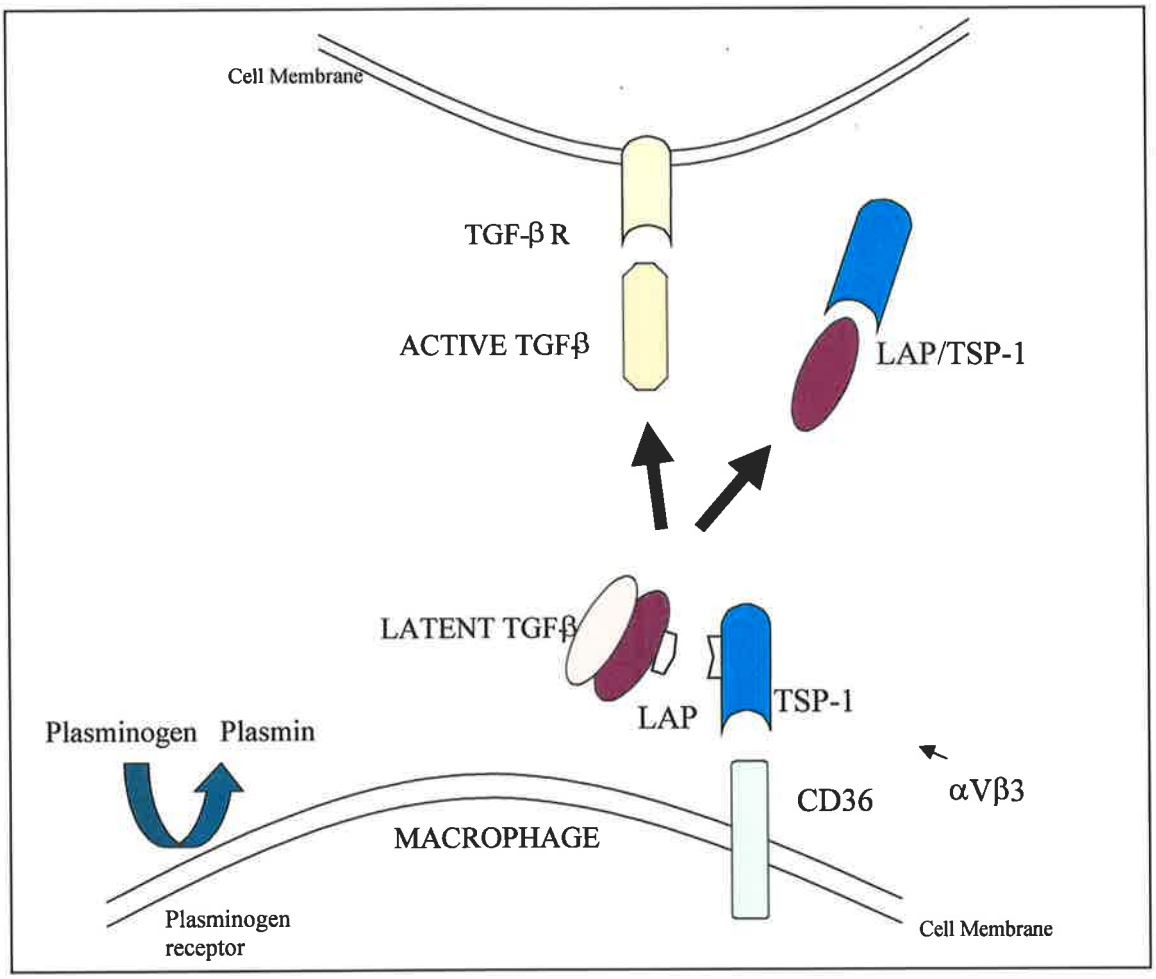


Figure 1-5 Activation of AM-derived latent (L-)TGF-β

Resting AM secrete small amounts of L-TGF-β and thrombospondin (TSP-1) but no plasmin. CD36 is expressed in small amounts on the cell surface but no active TGF-β is produced. Prior to activation, L-TGF-β is non-covalently associated with latency-associated peptide (LAP). Once AM are activated, eg, as a result of acute airway injury, increased plasmin, L- TGF-β and TSP are generated. TSP-1 associates with the LAP/ TGF-β complex which then associates with the cell surface of the macophage by the TSP receptor (CD36). In the presence of plasmin and the integrin αvβ3, TSP binds to LAP, releasing TGF-β in an activated state and able to bind to receptors on other cells.

Adapted from Yehualaeshet et al. (1999) *Am J Pathol* **155** p849

When activated, TGF-β is a key signal in initiation of the highly regulated processes of airway repair in the normal lung. Active TGF-β stimulates deposition of extracellular matrix components. Cells that migrate, de-differentiate and proliferate to cover denuded basement membrane adopt a highly flattened phenotype (Erjefalt et al. 1995) and TGF-β has been reported to induce these phenotypic changes in cultured AEC and may induce these changes *in vivo* (Yoshida et al. 1992). To complete the repair process, AEC proliferation must be controlled. It is likely that TGF-β plays a significant role in this process, as one of its activities is to impair proliferation of AEC, causing accumulation in the G0/G1 phase of the cell cycle (Blobe et al. 2000; Antoshina et al. 1997). Current concepts of the role of TGF -β in airway repair is presented in **Figure 1-6**.

TGF -β is a pleiotropic cytokine that impairs proliferation in some cell types (including AEC) while enhancing proliferation in other cell types (including fibroblasts). The mechanisms of signal transduction and cell cycle effects mediated by TGF -β are presented in **Figure 1-7**.

Table 1-3 Pleiotropic effects of TGF-β on different cell types

	T-cell	Macrophage	AEC	Fibroblast
Inhibits proliferation	+++		+++	
Enhances proliferation				+++
Induces apoptosis	+++		+++	
Impairs cytokine production	++	++	++	
Enhances IL-1 production		++		
Impairs cytolytic activity	+++			
Enhances chemotaxis		+++		
Deactivates oxidative burst		+++		

Adapted from Kehrl et al, (1991)*Int J Cell Cloning* **9** p438

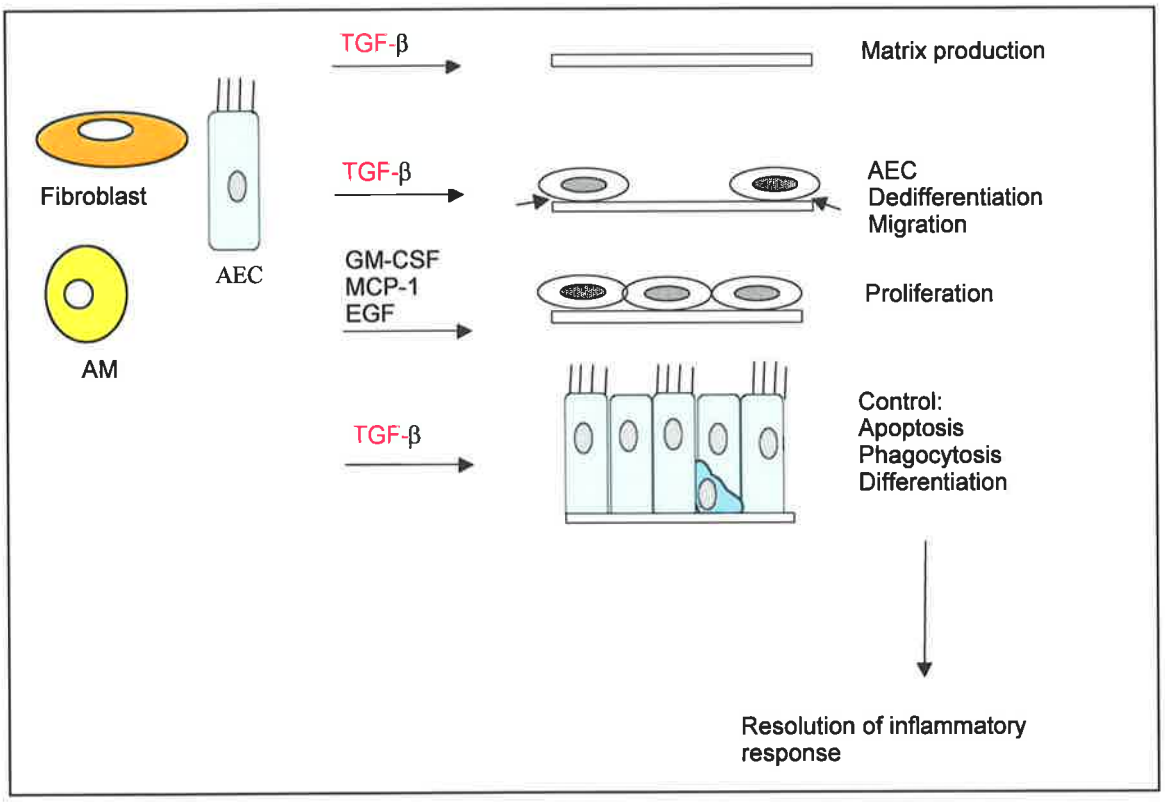


Figure 1-6

Role of TGF-β in airway repair

AEC loss is followed by proliferation (to cover the denuded area). Once repair is complete, excess AEC are removed by apoptosis and phagocytosis and inflammatory responses are ceased. TGF-β, produced by inflammatory cells and AEC, may play a role in repair by inducing apoptosis, controlling proliferation, and stimulating of extracellular matrix components.

Figure 1-7 Signal transduction and cell cycle effects mediated by TGF -β

a. The action of TGF -β is mediated through its binding to type I and type II receptors that form a complex. The membrane-bound proteoglycan betaglycan (BG) (also known as TGF-R type III) binds TGF -β and increases its affinity for the signalling receptors. Protein kinase activity is stimulated resulting in phosphorylation of transcription factors Smad 2, 3 and 4 and intracellular signalling (blocked by Smad 6 or Smad 7).

b. In cell types in which TGF -β enhances proliferation, a G1 specific retinoblastoma gene product (Rb kinase) is activated in middle G1 phase, resulting in phosphorylation of Rb (a pre-requisite for cell progression through G1 - S phase).

c. Cell types in which TGF -β inhibits proliferation, including AEC, also signal through the heteromeric receptor complex. In these cells TGF -β stimulates production of the cyclin-dependent protein kinase inhibitor, p15, via a functional cooperation and physical interaction of Smad 2, Smad 3 and Smad 4 with the transcriptional factor Sp 1. This prevents Rb phosphorylation allowing Rb to bind to and sequester members of the E2F family of transcription factors. Sequestered E2F is then unable to stimulate the expression of genes that regulate progression through the cell cycle, eg, *c-myc* and *b-myb*. Rb phosphorylation is also directly inhibited by p53 and indirectly by p16, p18 and p21. TGF -β also inhibits the function or production of cell cycle regulators, including cyclin-dependent protein kinases 2 and 4 and cyclins A and E. Since TGF -β does not reverse the phosphorylation of Rb kinase, TGF -β will not arrest cells at other points in the cell cycle.

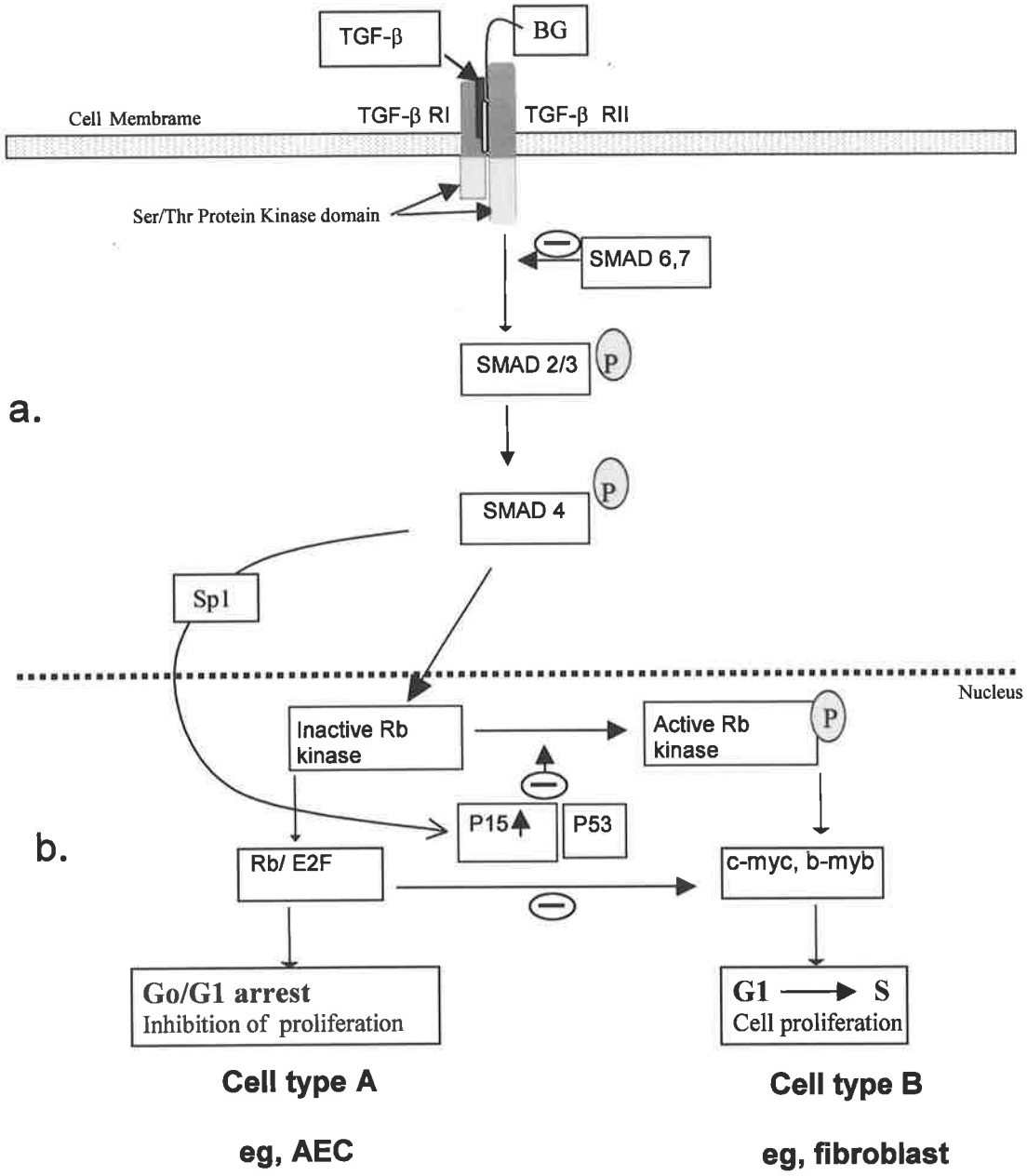


Figure 1-7

Signal transduction and cell cycle effects mediated by TGF -β

1-6 Apoptosis: role in repair of the normal airway

The mechanisms regulating the clearance of excess AEC after injury remain largely unknown. Inhibition of proliferation (by TGF-β) is most likely followed by apoptosis of AEC not anchored to the basement membrane, followed by removal of apoptotic fragments by neighbouring AM (Bardales et al. 1996). TGF-β has been shown to induce apoptosis of AEC *in vitro* (Hagimoto et al. 2002; Blobe et al. 2000; Antoshina and Ostrowski, 1997).

Apoptosis is active programmed cell death that provides a basis for efficient cell turnover and removal of cells. Apoptosis contributes to immunoregulation and normal cell turnover. With apoptotic cell loss and effective clearance of apoptotic cells there is minimal inflammation (Majo et al. 2001; Savill, 1997; Fadok, 1999).

In contrast, necrosis results in cellular swelling and disintegration with the potential for inflammation of surrounding tissues. Necrosis does not proceed in stages like apoptosis. Apoptosis is an active biochemical process and progresses through various stages. In early apoptosis, cells lose water and shrink, with changes in mitochondrial membrane potential ($\triangle\psi m$) (Darzynkiewicz et al. 1992). In the next phase, plasma membrane alteration occurs, with exposure of phospholipids (eg, phosphatidylserine (PTS)), which are normally present on the inner membrane (Darzynkiewicz et al. 1992). Cysteine proteases (caspases) are then synthesised first as inactive precursors (zymogens). Caspases are activated by proteolysis, usually initiated by caspases themselves. In this way, activated caspases can activate other caspases, leading to a 'caspase cascade' with controlled cleavage of vital macromalecular structures and initiation of the apoptotic program of cell death.

To date at least 15 caspases have been identified. Initiator caspases (caspases 2,8,9,10) are activated in response to the initial pro-apoptotic signal. Effector caspases (caspases 3,6,7) act downstream of initiator caspases and are activated by initiator caspases to cleave the cell into apoptotic bodies (Vermes et al. 2000). Final stages of apoptosis are characterised by dismantling of the nuclear membrane and cytoskeleton, activation of various degrading enzymes (including DNAses), loss of membrane pump function (Darzynkiewicz et al. 1992) and inability of the cell membrane to exclude dyes eg, 7-aminoactinomycin-D (7AAD) and propidium iodide (PI), from cells. Differences between apoptosis and necrosis are shown in **Figure 1-8**.

The mechanisms for TGF-β-induced apoptosis of AEC have not been fully characterised. In fetal hepatocytes (Fabregat et al. 1996; Sanchez et al. 1996; Inayat-Hussain et al. 1997; Herrera et al. 2001) proliferation was inhibited at low concentrations of TGF-β. When TGF-β was used at high concentrations it also induced apoptosis. Thus, the mechanisms for TGF-β-induced apoptosis of AEC may parallel those widely described for hepatocytes, where the process is preceded by an induction of reactive oxygen species (ROS) and a decrease in the intracellular glutathione content, indicating involvement of an oxidative process (Sanchez et al. 1996; Fabregat et al. 1996). Activation of caspase 3 was also demonstrated (Inayat-Hussain et al. 1997). More recent studies showed that the production of ROS preceded the loss of mitochondrial membrane potential, release of cytochrome c and activation of caspases 3, 8 and 9. TGF-β also caused a decrease in protein and mRNA levels of bcl-xL, an anti-apoptotic member of the Bcl-2 family (Herrera et al. 2001 (1); Herrara et al. 2001 (2)). These changes are summarised in **Figure 1-9**.

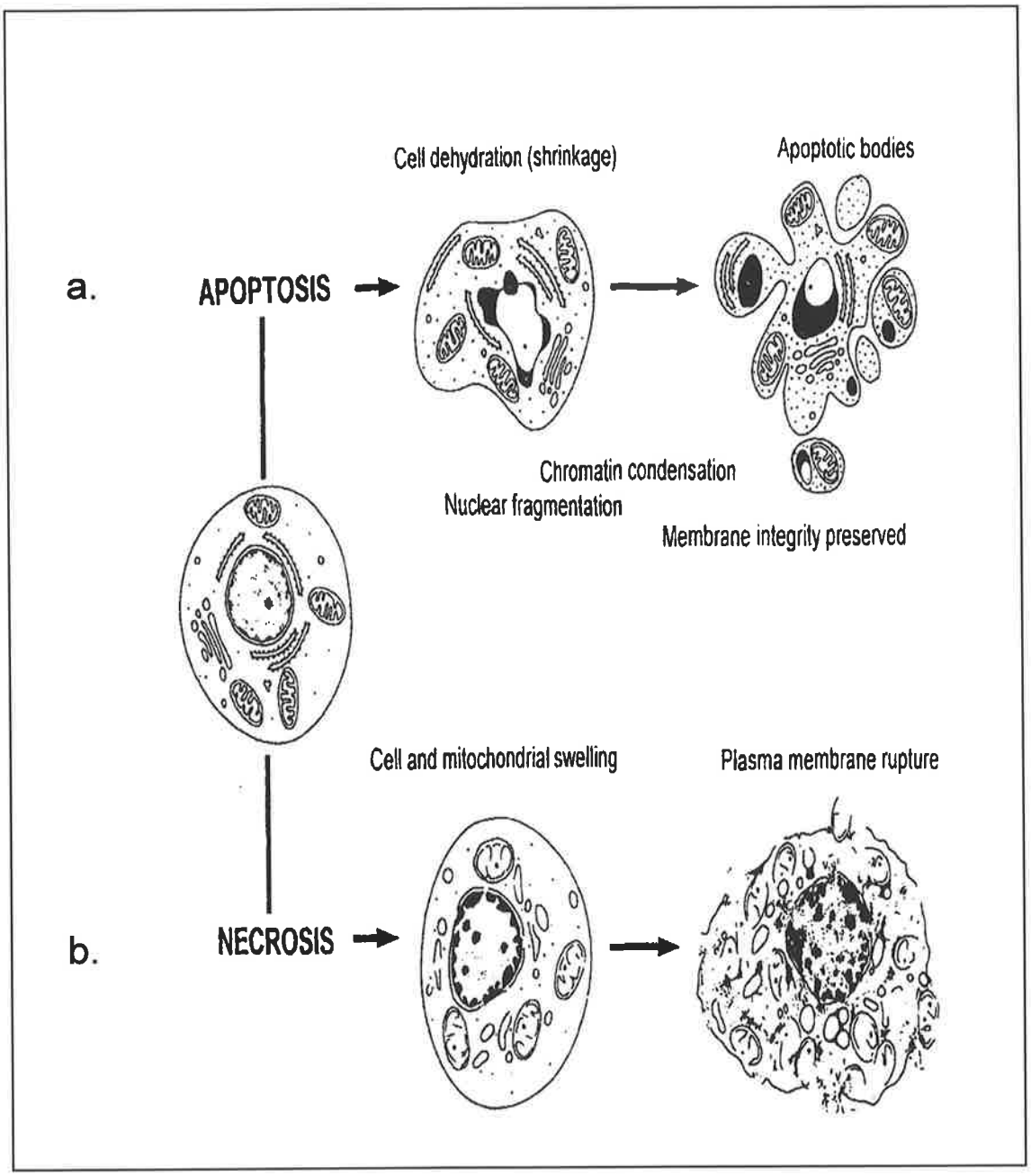


Figure 1-8

Differences between apoptosis and necrosis

a. Apoptosis (programmed cell death) showing cell shrinkage, chromatin condensation, nuclear fragmentation and production of apoptotic bodies with membrane integrity preserved

b. Necrosis showing cell swelling and plasma membrane rupture

Adapted from Darzynkiewicz et al. (1997) *Cytometry* **13** pp795-808

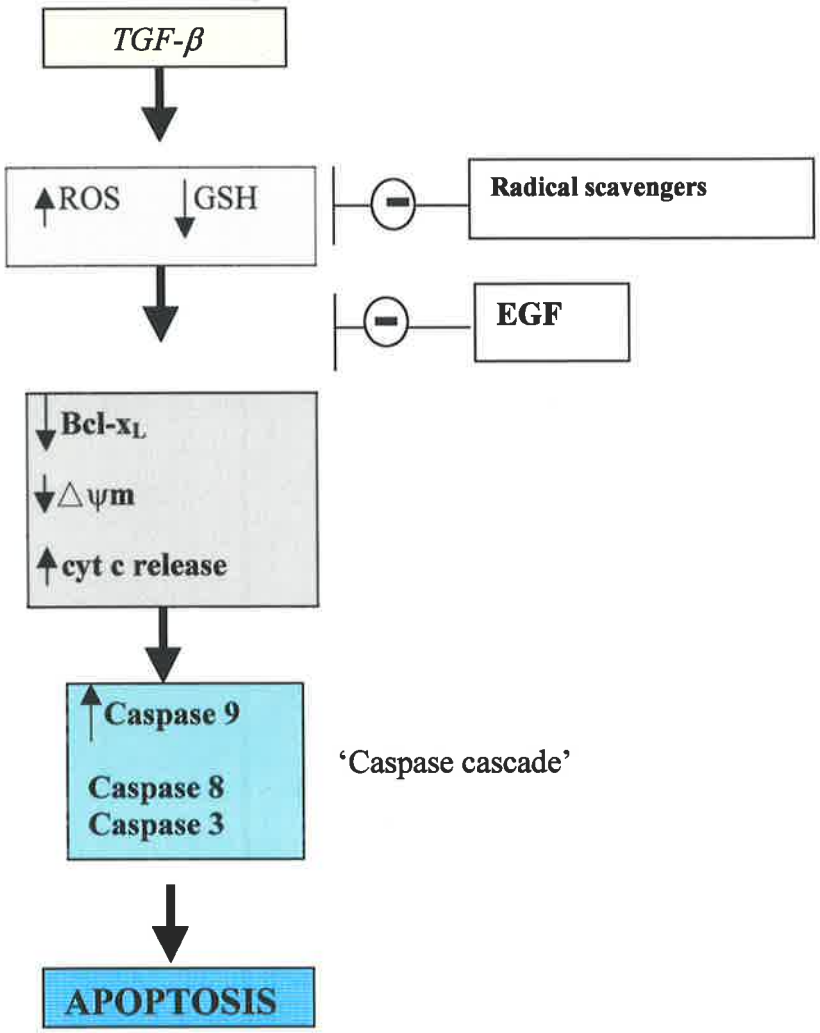


Figure 1-9

Likely mechanism for TGF-β-induced apoptosis of AEC (based on reported effects of TGF-β on fetal hepatocytes). TGF-β induces the transcription of redox-related genes, which activate the formation of ROS. The resulting oxidative stress contributes to the down-regulation of bcl-x_L (an anti-apoptotic protein) expression, loss of $\triangle\psi m$, release of cytochrome *c* and activation of the 'caspase cascade'. Factors that prevent down-regulation of bcl-x_L such as EGF and those that block production of ROS such as anti-oxidants and radical scavengers (eg, ascorbic acid) may prevent mitochondrial collapse and apoptosis.

Adapted from Antoshina et al. (1997) *In Vitro Cell Devel Biol Anim* **33**749

As represented in **Figure 1-10**, other extracellular signaling pathways may play a role in inducing apoptosis in the airways. Oxidative stress associated with the generation of free radicals, which induce phosphorylation of p53 (a nuclear protein that is upregulated in response to DNA damage (Hall et al. 1993; Di Leonardo et al. 1994), lead to increased expression of the protein, BAX. BAX generates pores in the mitochondrial membrane, allowing the release of pro-apoptotic protein, cytochrome *c,* and apoptosis-inducing-factor (AIF). These activate caspase 9 and caspase 3, leading to activation of the 'caspase cascade' and resulting in apoptosis.

Other apoptotic pathways that do not involve p53 include binding of glucocorticoids (GCS) to the GCS receptor, binding of Fas ligand (FasL) to Fas and TNF-α to TNFRI (Cohen et al. 1999; Hagimoto et al. 1997). Fas (CD95, Apo 1) is expressed on T-cells and epithelial cells, including AEC (Hamann et al. 1998). The Fas/Fas L system mediates deletion of autoreactive T-cells in the lung, and possibly contributes to regulation of AEC (Fine et al. 1997; Sayama et al. 1994). The Fas and TNF pathways use pre-existing molecules. The interaction of TNFα and TNF-RI leads to clustering of TRADD (TNF-associated death domain). The interaction of Fas and Fas ligand leads to clustering of FADD (Fas associated death domain). Both pathways result in activation of caspase 8, leading to a 'caspase cascade' and apoptosis (**Figure 1-10**). The remaining pathways require new gene expression.

Glucocorticosteroids (GCS) are also known to induce apoptosis of AEC by binding to the GCS receptor on the target cell (Dorscheid et al. 2001; White et al. 2002). This may be relevant because GCS are commonly used in treatment of airway disease.

IL-4 may also contribute to the process of epithelial repair of the normal lung by increasing AEC expression of various chemokines such as IL-8 (Striz et al. 1999)

and adhesion molecules such as ICAM-1 (Tosi et al. 1992) and VCAM-1 (Atsuta et al. 1997). These mediators increase AM chemotaxis to the site of injury, facilitating phagocytosis of apoptotic AEC. IL-4 has also been shown to directly increase AM phagocytosis (Raveh et al. 1998; Capsoni et al. 1995) and inhibit the production of inflammatory cytokines by monocytes (Banchereau J. and Rybak M, 2002).

Thus, cytokines, particularly TGF-β, directly influence the reparative capacity of the epithelium in response to injury. It is therefore likely that changed production or defective control of production of these cytokines may result in defective repair of the injured airway.

Figure 1-10

Extracellular signaling pathways to apoptosis in the airways

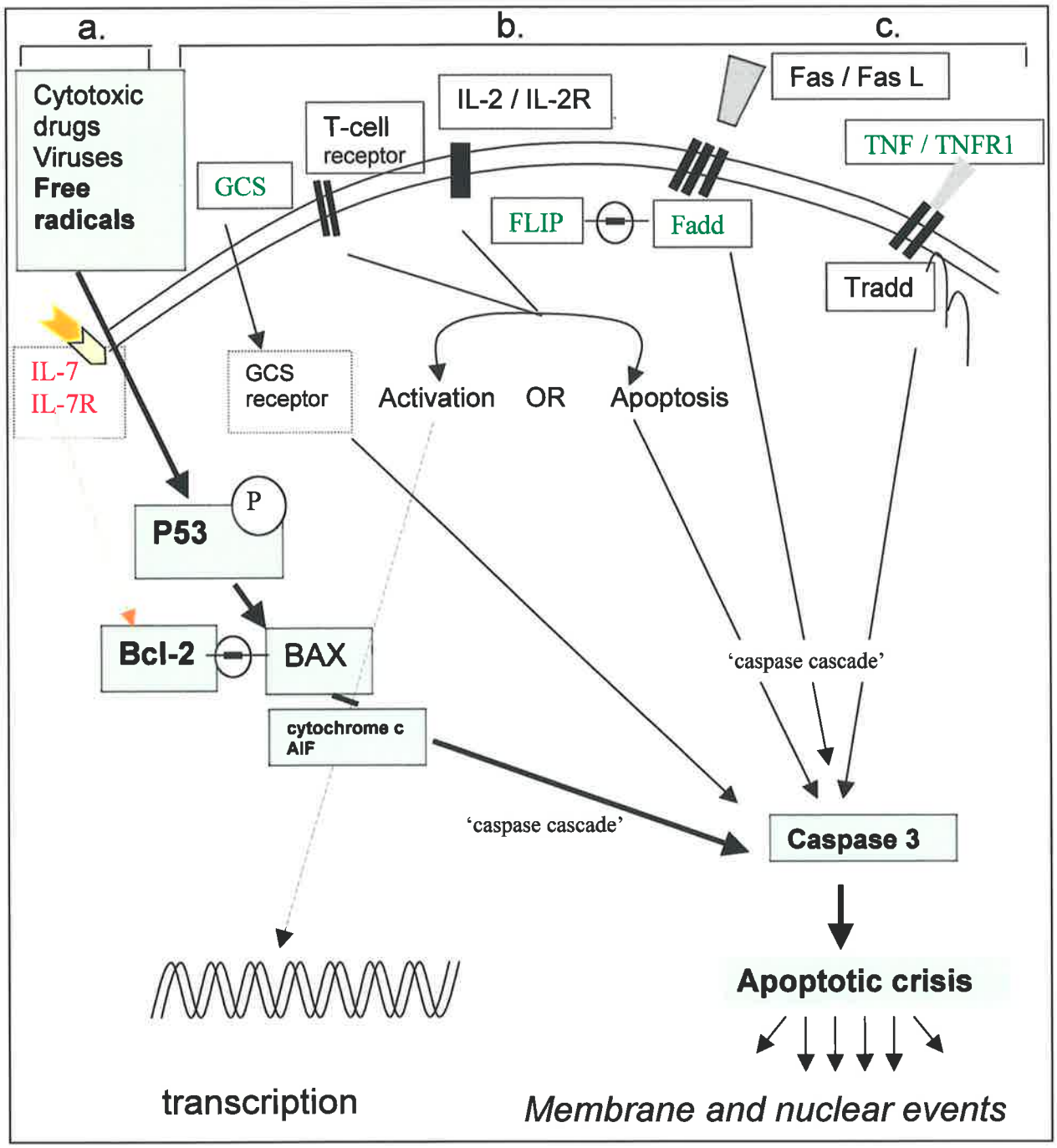
a. Cytotoxic drugs, viruses or free radicals induce phosphorylation of p53, leading to increased expression of the protein, BAX. BAX generates pores in the mitochondrial membrane, allowing the release of pro-apoptotic protein, cytochrome *c,* and apoptosis-inducing-factor (AIF). These activate caspase 9 and caspase 3, leading to activation of the 'caspase cascade' and resulting in apoptosis.

b. Other apoptotic pathways that do not involve p53 include binding of glucocorticoids (GCS) to the GCS receptor, binding of FasL to Fas and TNF-α to TNFRI. Activation through the T-cell receptor or binding of IL-2 to its high affinity receptor can either induce cell activation or program T-cells for apoptosis.

c. The Fas and TNF pathways use pre-existing molecules (eg, the interaction of TNFα and TNF-RI leads to clustering of death domains, TRADD (TNF-associated death domain) and FADD (Fas associated death domain), resulting inactivation of caspase 8 and activation of the 'caspace cascade' and apoptosis. The remaining pathways require new gene expression (induction pathways). Once the hypothetical "apoptotic crisis" is passed, the cell is committed to die.

Figure 1-10

Extracellular signalling pathways to apoptosis in the airways



Adapted from: Cohen et al. (1999) *J Allergy Clin Immunol* **103** p551

1-7 Phagocytosis: role in repair of the normal airway

Another critical component of the regulated process of epithelial repair in the normal lung is the rapid and effective removal of unwanted AEC and inflammatory leucocytes. This process most likely involves apoptosis followed by phagocytosis by neighouring AM (Bardales et al. 1996; Bitterman et al. 1994). Phagocytosis of apoptotic cells occur before cell lysis, thereby preventing secondary necrosis and release of potentially tissue damaging contents into the surrounding tissues. (Savill, 1997; Fadok, 1999).

A growing body of evidence indicates that a variety of diverse mechanisms are involved in recognition and removal of apoptotic cells by macrophages. These include:

(a) PTS on the apoptotic AEC surface combining with PTS receptor on the macrophage (Fadok et al. 2000)

(b) The vitronectin receptor ($\alpha V\beta 3$, CD51/CD61) combining with the thrombospondin receptor (CD36) and the adhesive glycoprotein, thrombospondin (TSP), on the macrophage. This "molecular bridge" binds to an incompletely characterised ligand on the apoptotic AEC (**Figure 1-11**) (Savill et al. 1992). Changes reported to occur on apoptotic cells include expression of immature sugars on apoptotic thymocytes and hepatocytes (Dini et al, 1992) and changed expression of ICAM-3 on apoptotic B cells (Moffatt et al. 1999). In recent studies, the $\alpha V\beta 3$/CD36/TSP complex on human monocyte-derived macrophages has been shown to bind to a CD36/TSP complex on apoptotic fibroblasts (Moodley et al, 2003). In addition, apoptotic fibroblasts actively release TSP to recruit and

activate phagocytes (Moodley et al, 2003). It is possible that similar mechanisms apply in recognition of apoptotic AEC by AM.

(c) Other receptors on macrophages may be involved with recognition and phagocytosis of apoptotic cells include CD14 (Devitt et al. 1998), the class A scavenger receptor SR-A (Platt et al, 1996), the ATP-binding cassette transporter, ABC-1 (Luciani et al. 1996), the receptor tyrosine kinase, MER (Scott et al. 2001) and the α-2-macroglobulin receptor, CD91 (Ogden et al. 2001). Additional molecules, including Clq, mannose binding lectin and calreticulin may serve as intermediates that bridge apoptotic cell/macrophage cell surfaces (Ogden et al. 2001). However, the relative importance of these molecules remains to be established.

1-8 Apoptosis: role in controlling inflammation

There is evidence that apoptosis not only avoids the pro-inflammatory effect of necrosis, but also has an active anti-inflammatory role (Fadok, 1999). Apoptosis is followed by rapid phagocytosis of apoptotic cells by macrophages/monocytes with minimal inflammatory response. Several positive feedback mechanisms accelerate the removal of apoptotic leucocytes in the airways. Macrophages and activated T-cells express cell surface Fas ligand (Brown and Savill, 1999). This enables triggering of apoptosis in other leucocytes expressing Fas. Importantly, phagocytosis of apoptotic neutrophils by AM has been reported to trigger AM release of Fas ligand, and to induce apoptosis of bystander leucocytes (Brown and Savill, 1999).

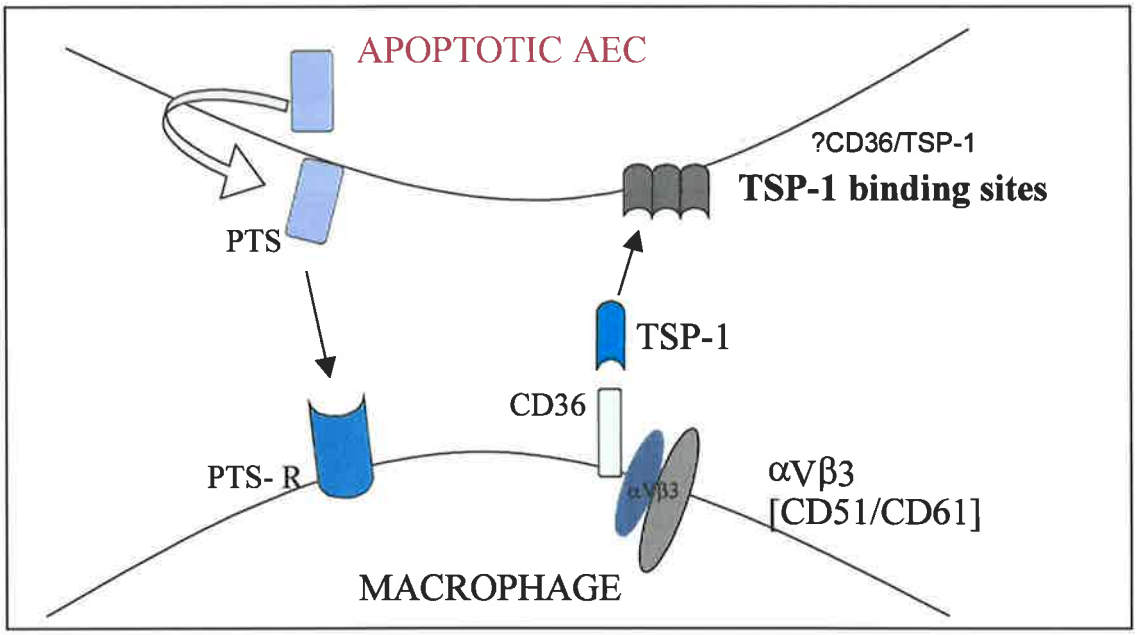


Figure 1-11

Recognition and removal of apoptotic AEC by AM

a. Expression of phosphotidyl serine (PTS) on the surface of the apoptotic cell.

b. Cooperation of the vitronectin receptor (αVβ3 integrin, CD51/CD61) with the thrombospondin receptor (CD36) and the adhesive glycoprotein, thrombospondin (TSP-1), on the macrophage. This "molecular bridge" binds to an incompletely characterised ligand on the apoptotic AEC

It has been recently shown that macrophages ingesting apoptotic cells produce regulatory cytokines IL-10 and TGF-β (Fadok et al. 1998; Ronchetti et al. 1999). These findings suggest that, in the normal airway, the phagocytic process may directly contribute to cessation of inflammatory responses following acute lung injury by induction of regulatory cytokines.

It has also recently been demonstrated that apoptotic cells themselves have the ability to produce cytokines, which may further contribute to an autocrine anti-inflammatory function of the apoptotic cell. Lymphocytes undergoing FasL induced apoptosis have been shown to produce the anti-inflammatory cytokine, IL-10 (Gao et al. 1998). IL-4 production by apoptotic mononuclear and tumour cells has also been described (Stein et al. 2000).

It is therefore possible that, in the human lung, apoptotic epithelial cells produce cytokines, such as TGF-β and IL-4, which could act in an autocrine manner to control epithelial cell hyperplasia and limit inflammatory responses.

1-9 Chronic Obstructive Pulmonary Disease (COPD)

1-9-1 Morbidity and mortality

Chronic obstructive pulmonary disease (COPD) is a complex, chronic disease of the airways usually caused by cigarette smoking. COPD is defined as "a disease state characterised by airflow limitation that is not fully reversible. The airway limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (US Department of Health and Human Services, 2001). There is defective repair of injured epithelium and loss of alveolar structures (emphysema).

COPD is a major cause of morbidity and mortality in adults, and has important health economic consequences. The World Health Report of 1998 stated that 2.9 million adults die each year of COPD, and that COPD was ranked as the fifth cause of mortality worldwide (just after ischaemic heart disease, cerebrovascular disease, acute lower respiratory infection, and tuberculosis). In Australia, COPD is the 4th most common cause of death, accounting for over 5,000 deaths per year and causing serious disability in a much larger number of patients. Extensive public health campaigns and pharmaceutical approaches to promote smoking cessation have been implemented, but while these are important public health measures, their success has been limited, and a large percentage of the adult population continues to smoke and develop COPD. Furthermore, many patients stop smoking only once COPD is established and therefore need effective ongoing treatment. It has been estimated that COPD costs the Australian community more than $800 million per year (McKenzie et al. 2003).

1-9-2 Pathophysiology and pathology

Clinically, COPD is a heterogenous disease, characterised by mostly irreversible chronic airflow limitation (reduced capacity of the airways to remain open during expiration). There may be a small reversible component.

Pathological changes in COPD from smoking-related lung tissue damage and chronic inflammation result in mucus hypersecretion, airway oedema and dysfunction of the normal cilliary clearance mechanisms. These changes lead to chronic cough and sputum production ('chronic bronchitis').

Inflammation initiated by cigarette smoking may lead to repeated cycles of injury and repair of the airway walls leading to remodelling (fibrosis and narrowing) of the small airways. The irreversible component of airflow limitation is primarily due to remodelling (Cosio et al. 1978), thought to result from a mixture of small airways disease (obstructive bronchiolitis) and destruction of the lung parenchyma (emphysema) with loss of alveolar attachments to the small airways and decrease in elastic recoil. In turn, these pathological changes contribute to airflow limitation and other physiological abnormalities characteristic of COPD. Hypoxemia may result from inequality in the ventilation/perfusion ratio (V_A/Q). A significant correlation has been shown between bronchiolar inflammation and the distribution of ventilation (V_A/Q mismatching) (Rodriguez-Roisin et al. 1998). In the parenchyma, destruction of the lung surface area by emphysema reduces the diffusing capacity and interferes with gas exchange. This is reflected by good correlation between the diffusing capacity of carbon monoxide per litre of alveolar volume ($DLCO/V_A$) and the severity of emphysema (McLean et al. 1992). Pulmonary hypertension may develop late in the course of COPD, usually after the development of severe hyoxemia. Vasoconstriction, remodeling of pulmonary arteries (thickening of the vessel walls with reduction in the lumen), and destruction of the pulmonary capillary bed by

emphysema (increasing the pressure required to perfuse the pulmonary vascular bed) may contribute to pulmonary hypertension.

Other effects associated with COPD include systemic oxidative stress and skeletal dysfunction with loss of skeletal muscle mass. Infections are also a problem in COPD. In moderate to severe COPD, an average of 1.5 acute exacerbations occurs per patient per year and 80% of these are infectious in origin. One third of these are respiratory tract viruses, 5-10% are caused by *Chlamydia pneumoniae* and the rest by other bacteria (enterobacteriacae, *Pseudomonas aeruginosa*, streptococcus sp, *Mycoplasma catarrhalis* and *Haemophilus influenzae* (Sethi, 2000).

1-9-3 Classification of severity

Airflow limitation assessed by pulmonary function testing helps to establish the diagnosis of COPD. The forced expiratory volume in one second (FEV1) and the ratio of the FEV1 to the forced vital capacity (FVC) are reduced. The following Table presents guidelines for classification of COPD by severity. Most individuals do not present with symptoms until FEV1 is less than 50% predicted. Repeat spirometric testing following medications (ie, with inhaled steroids) determine to what extent the disease is reversible. COPD is frequently associated with an increase in total lung capacity (due to loss of elastic recoil) and residual volume (gas trapping due to airway closure), and a reduction in DLCO/V_A.

Table 1-4 Classification of COPD by Severity

	Stage	Characteristics
0:	At risk	Normal spirometry Chronic symptoms
1:	Mild COPD	FEV1 ≥ 80% predicted FEV1/FVC < 70% ± chronic symptoms (cough, sputum production)
2:	Moderate COPD	FEV1 30% - 80% predicted (2A: FEV1 50%-80% predicted) (2B: FEV1 30%-50% predicted FEV1/FVC < 70% ±chronic symptoms (cough, sputum production, dyspnea)
3.	Severe COPD	FEV1 < 30% predicted or < 50% predicted plus respiratory failure or clinical signs of right heart failure FEV1/FVC < 70%

Adapted from 'GOLD' guidelines, 2001

Individual variation in susceptibility to cigarette smoke has also been investigated by Fletcher and Preto (1997). Their well-known model of decline of FEV1 over time is presented in **Figure 1-12 (a)**.

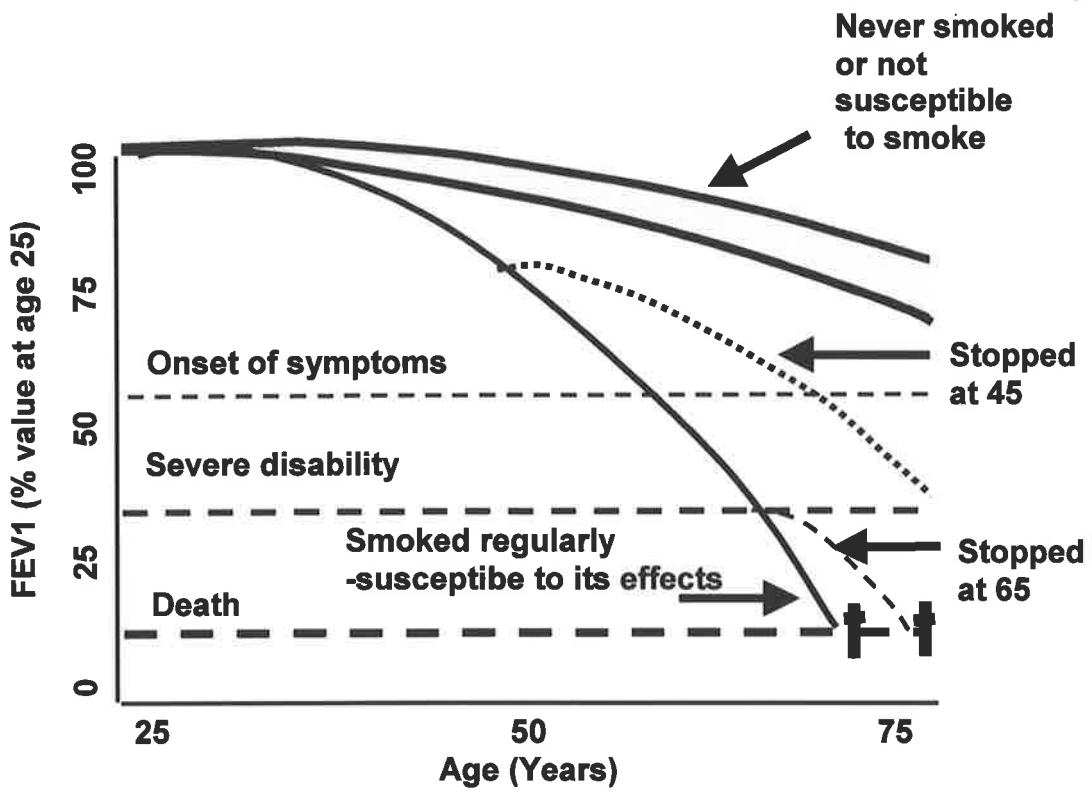


Figure 1-12 (a)

Influence of smoking on lung function

Age (X axis) versus FEV 1 (% of value at age 25; Y axis) showing increased mortality and morbidity in long term smokers

Adapted from Fletcher C, Peto R. (1977) *BMJ* **1** 1645

However, not all individuals with COPD follow this classical model, which represents the mean of many individual courses. Examples of individual courses followed by four patients with COPD are represented in **Figure 1-12 (b).** While total cumulative dose of tobacco smoke is important, genetically determined susceptibility may also play a role in individual susceptibility to cigarette smoke.

The causes for variation in individual susceptibility to cigarette smoke have not been determined, and are therefore an important area for research.

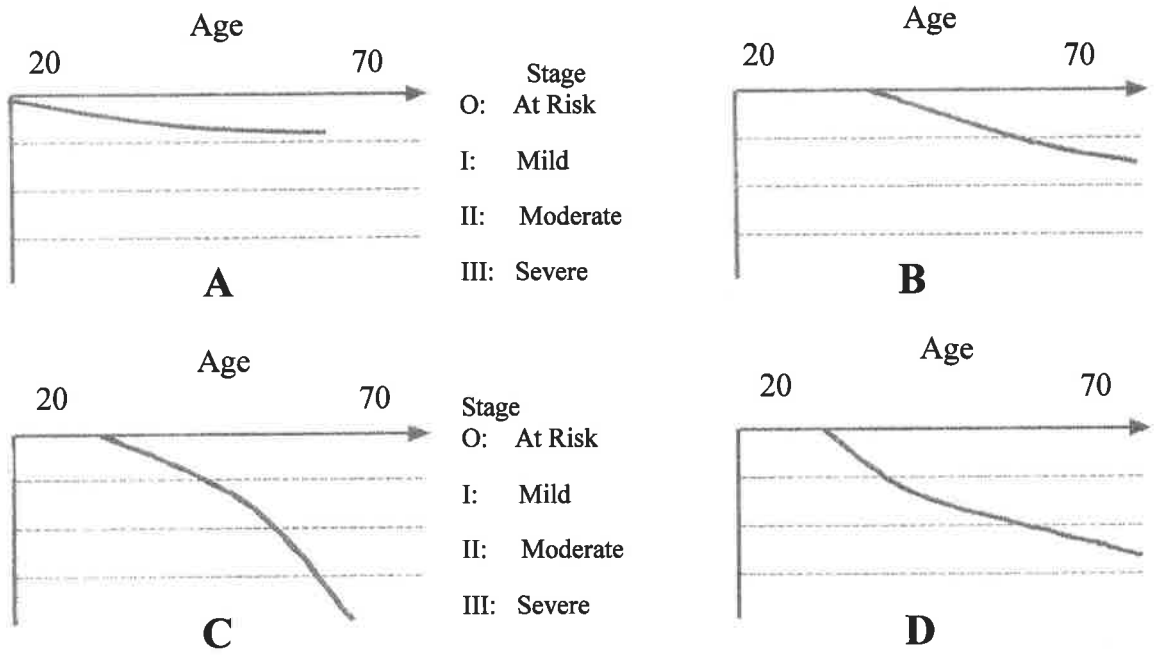


Figure 1-12 (b)

Examples of individual COPD patient histories

A. Cough and sputum production: never develops abnormal lung function

B. Abnormal lung function: patient may never come to diagnosis

C. Abnormal lung function (age 50); progressively deteriorates for 15 years; dies of respiratory failure age 65

D. Abnormal lung function aged around 30, continues to deteriorate gradually but does not die as a result of COPD

Adapted from 'GOLD' workshop report, 2001

The causative link between cigarette smoking and COPD is proven (US Department of Health Services Report, 1989). Patients have usually been smoking at least 20 cigarettes per day for 20 or more years (20 pack years). They commonly present in their 5th decade with productive cough or acute chest illness. Dyspnea on effort usually does not occur until the 6th or 7th decade. There is a dose-dependent relationship between the amount smoked and the rate of decline of FEV1 (Burrows et al. 1983). The common belief that only 20% of smokers develop clinically significant COPD is misleading, as a much higher proportion develop abnormal lung function if they continue to smoke (US Department of Health and Human Services, 2001).

COPD can co-exist with asthma, however, in asthma airflow limitation is usually reversible and variable whereas in COPD it is usually irreversible. In some cases of asthma, where the airflow obstruction does not completely reverse, the patient may be classified as having the asthmatic form of COPD. Some patients in this group also have chronic bronchitis and emphysema. The overlap between conditions is represented in **Figure 1-13**.

Patients with asthma whose airway limitation is completely reversible are not considered to have COPD. There is, however, evidence that long-standing asthma on its own can lead to airway remodelling and partly irreversible airflow limitation (US Department of Health and Human Services, 2001).

There is an overlap between persons with chronic bronchitis and emphysema who have partially reversible airway limitation. Chronic bronchitis, airway narrowing and emphysema are independent effects of cigarette smoking and may occur in various combinations in COPD. There is therefore a need for further study into the

pathogenesis of COPD to identify specific markers that can be used to differentiate between COPD and asthma in individual patients.

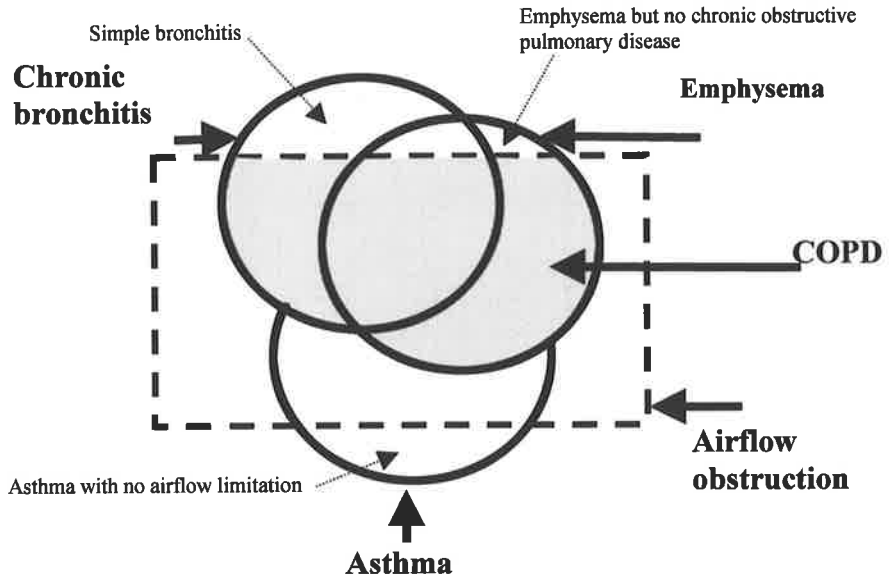


Figure 1-13

Non-proportional Venn diagram showing the overlap of chronic bronchitis, emphysema and asthma within COPD

Adapted from ATS statement. (1987) *Am Rev Respir Dis* **136** 225

1-9-4 Pathogenesis

Despite the high prevalence of COPD and the clear need for effective therapies, the development of new treatment strategies is hampered by a limited understanding of the pathogenesis of the disease. In asthma, inflammatory cells, cytokines and bronchial mucosa have been studied widely (Del Prete et al. 1993; Dutoit et al. 1987; Krouwels et al. 1996). A paradigm shift in the management of asthma occurred after the realisation that asthma is a disease characterised by a specific type of inflammation.

1-9-4(a) Elevated leucocyte numbers in the airways

Elevated numbers of AM and neutrophils are thought to be central factors in the inflammation associated with COPD (Saetta et al. 1993; O'Shaughnessy et al. 1997; Di Stefano et al. 1998). These increases are brought about by increased recruitment, activation or survival of these cells. Components of cigarette smoke, including nicotine, have been shown to stimulate the recruitment and activation of neutrophils and macrophages (Mio et al. 1997; Masubuchi et al. 1998).

Many studies have shown a correlation between the number of inflammatory cells of different types in the lung and COPD. However, the predominant cell type has varied between publications, and most studies have been performed on bronchial biopsies. It is possible that the inflammatory processes present in the airway lumen (investigated using BAL) may not reflect the inflammatory processes in the airway wall (investigated using biopsy). This is supported by a study that showed no correlation between inflammatory cell numbers in BAL and biopsy (Maestrelli et al.

1995; Rutgers et al. 2000). Therefore, there is still a need for further studies to more clearly define the role of inflammatory cells in COPD.

In studies of bronchial biopsies, AM were increased in both mild and severe COPD (Saetta et al. 1993; O'Shaughnessy et al. 1997). Others have studied BAL and have reported no significant change in AM numbers in COPD (Lacoste et al. 1993; Thompson et al. 1989). In contrast to mild COPD, an increase in neutrophil numbers (studied using bronchial biopsies) in severe COPD correlated with the degree of airflow limitation (Saetta, 1999; Maestrelli et al. 2001).

Studies of bronchial biopsies from patients with COPD have shown an increase in T-cells, especially CD8+ T-cells in COPD (Finkelstein et al. 1995). CD8+ T-cells can release perforin, granzyme B and cytokines including TNF-α. These mediators have the potential to cause apoptosis or cytolysis of AEC, thus contributing to the persistence of inflammation (Liu et al. 1999).

Therefore, there is still a need for accurate characterisation and clarification of cellular infiltrates in BAL from the airway lumen in COPD. Current techniques to enumerate cell types in BAL rely on manual differential counting methods that do not allow for further analysis of cell characteristics, such as activation status, cytokine production, receptor expression and accurate evaluation of apoptosis for different cell types.

1-9-4(b) Pathological effects of increased leucocytes in the airways

Persistent inflammation in the lungs leads to the pathological changes characteristic of COPD (US Department of Health and Human Services, 2001). In this regard,

neutrophils can injure lung tissue by release of contents, such as proteases and oxygen radicals (Haslett et al. 1989). Released contents promote further inflammation by degradation of matrix proteins into chemotactic fragments (Vartio et al. 1981).

1-9-4(c) Mediators and cytokines

Increased production of cytokines TGF -β and GM-CSF have been reported in COPD (Vignola et al. 1997; de Boer et al. 1998; Takizawa et al. 2001; Balbi et al. 1997; Hoshi et al. 1995). These cytokines can enhance neutrophil survival in vitro (Lagraoui and Gagnon, 1997; Brach et al. 1992) and such enhanced survival may further contribute to release of potentially damaging contents and tissue damage.

A variety of other mediators have been found at increased levels in the airways in COPD, including IL-4, Macrophage chemotactic factor (MCP)-1, Macrophage inflammatory protein (MIP)-1β, MIP-1α, Leukotreine B4 (LTB4), Il-8 and TNF-α (Jeffery, 1994; Capelli et al. 1999; Di Stefano et al. 1998; Hill et al. 1999; Keatings et al. 1996; Pesci et al. 1998; Yamamoto et al. 1997). LTB4, TNF-α and IL-8 can damage airway structure and potentiate the inflammatory response by stimulating the release of chemotactic factors (including MCP-1 and IL-8) (Senior et al. 1980; Kwon et al. 1994).

1-9-4(d) TGF-β, chronic inflammation and inadequate airway repair

In COPD, ciliary abnormalities, such as decreased ciliary length and decreased numbers of ciliary cells, combined with loss of mucociliary clearance due to alterations in the flow and adhesive properties of mucus, result in pooled secretions

which support the growth of bacteria. The presence of goblet cells in small bronchi and bronchioles less than 2mm diameter where goblet cells are normally absent or sparse, may occur. Squamous cell metaplasia may be also be present (Cosio et al. 1978). TGF-β, which induces squamous differentiation of tracheobronchial epithelial cells in vitro (Jetten, 1987) is likely to be involved in these changes in COPD.

Once airway repair is complete it is essential that production of TGF-β is shut down rapidly. A failure to shut down production by ongoing injury or by a defect in TGF-β regulation may result in a vicious circle, with increased apoptosis of AEC, defective clearance, secondary necrosis and chronic inflammation. Because of its role as a potent chemotactic cytokine for monocytes/macrophages (Wahl et al. 1987) increased release of TGF-β by de-granulating platelets, leucocytes or AEC in chronic inflammation may lead to increased macrophage infiltration in the airways in COPD.

There have been several reports of increased TGF-β production in the airways in COPD (Vignola et al. 1997; de Boer et al. 1998; Takizawa et al. 2001). Others have reported no change in production of TGF-β in COPD (Aubert et al. 1994; Buhling et al. 1999). All of these studies have utilised lung tissues and histochemical, ELISA or in situ hybridisation techniques for investigating TGF-β production. The conflicting data may therefore result from limitations of the methods that have not been able to identify the types of cell producing TGF-β.

Further comprehensive studies are, therefore, required to clarify the possible role of this important cytokine in COPD. Of particular relevance would be the quantitation of TGF-β production by the various cell types that are involved in the pathogenesis of COPD.

1-9-5 AEC apoptosis

In the normal lung, cell turnover, repair of injured epithelium and resolution of inflammation are highly regulated processes whereby cells and their potentially damaging contents are removed by phagocytosis of apoptotic cells by neighbouring AM once the repair process is complete. During an inflammatory response, various cytokines are produced which affect both susceptibility to and protection from, apoptosis. Excess apoptosis may result in inappropriate cell loss, tissue destruction and further inflammation; uncleared apoptotic cells may undergo secondary necrosis with discharge of injurious cell contents (Hagimoto et al. 1997; Kuwano et al, 1999). This accumulation of apoptotic material may arise due to excess apoptosis per se, thereby overwhelming normal clearance mechanisms (principally macrophage phagocytosis), defects in macrophage function, or a combination of these processes.

There is evidence that in COPD, apoptosis of AEC and lymphocytes is increased (Vignola et al. 1999; Kasahara et al. 2000; Kasahara et al. 2001; Segura-Valdez et al. 2000). In studies of biopsies, increased epithelial and endothelial apoptosis has been reported in the alveolar septa in COPD (Kasahara et al. 2000; Kasahara et al. 2001). Increased apoptosis of AEC and lymphocytes has been described in a recent study of bronchial biopsies from patients with COPD (Segura-Valdez et al. 2000). In contrast, Vignola et al (1999) reported that few T-cells were apoptotic in mucosal biopsies from patients with COPD.

Cigarette smoke is known to increase apoptosis of AEC and AM both *in vitro* and *in vivo* (D'Agostino et al. 2001). One of the main mediators of apoptosis found in cigarette smoke is acrolein, a toxic unsaturated aldehyde that induces apoptosis in

AEC (Nardini et al. 2002). In contrast, acrolein inhibits apoptosis of neutrophils by preventing activation of caspase 3 (Finkelstein et al. 2001). Cigarette smoke also generates reactive oxygen intermediates (ROI), such as hydrogen peroxide, which induce apoptosis in a variety of cell types. Nitric oxide also induces apoptosis in AEC (Persinger et al, 2001). Bacterial lipopolysaccharide (LPS), a contaminant in cigarette smoke, is a further apoptotic stimulus that may play a role in COPD (Hasday et al. 1999). Smoking of one cigarette delivers approximately 0.2μg LPS to the lung (Hasday et al. 1999) and LPS has been shown to induce apoptosis in AEC (Bingisser et al. 1996; Vernooy et al. 2001).

There is also evidence to suggest that some other changes associated with COPD have the potential to cause increased rates of apoptosis of AEC. For example, in COPD, there is increased production of matrix-degrading enzymes, including serine proteinases and matrix metalloproteinases in the airways (Shapiro S. 2002). The resulting disruption of epithelial cell/ matrix interactions causes apoptotsis of AEC (Frisch and Francis, 1994). The presence of neutrophils has also been reported to induce apoptosis in AEC after ozone exposure *in vitro* (McDonald and Usachencko, 1999).

Furthermore, there is new evidence linking apoptosis of pulmonary endothelial cells with the development of emphysema. VEGR, an endothelial cell mitogen, also exerts a protective effect, inhibiting apoptosis in vascular endothelial cells (Spyridopolous et al. 1996). In a rat model, specific inhibition of vascular endothelial growth factor receptors (VEGFR) led to endothelial apoptosis and loss of alveolar walls (Kasahara et al. 2000). Studies using resected specimens of human lung have also shown an association between endothelial apoptosis, emphysema and downregulation of VEGFR2 (Kasahara et al. 2001). There may be a direct link between this

phenomenon and smoking, because cigarette smoke extract decreases the expression of VEGFR in cultured cells and triggers apoptosis of pulmonary endothelial cells.

Taken together, these studies point to a key, central role for excess apoptosis in the pathogenesis of COPD and broaden the concepts of pathogenesis in this disease beyond the traditional protease/anti-protease considerations.
Further studies to better characterise apoptosis in COPD are thus warranted, and may lead to the development of novel anti-apoptotic therapies for this disease.
Further studies are also needed to determine if changes in mediator release in COPD are the result of transcriptional regulation of their gene products.
Quantification of key pro-apoptotic genes (such as Bax and p53) as well as anti-apoptotic genes (eg, Bcl2, NFκB) and cytokines (eg, IL-7 and IL-2) (Gupta, 2001) may detect phenotypic differences in COPD.

1-9-6 Phagocytic clearance of apoptotic material

In addition to an increase in pro-apoptotic mechanisms, impaired capacity for phagocytic clearance of apoptotic material may be important in COPD (Prieto et al. 2001). Normally, cells undergoing apoptosis are quickly removed *in vivo* by non-inflammatory engulfment by phagocytosis (Bitterman et al. 1994; Vandivier et al. 2002; Bardales et al. 1996)

There have been few studies on phagocytic ability of AM in COPD (Ferrara et al. 1996; Meloni et al. 1996; Prieto et al. 2001). These studies have reported decreased phagocytic capacity using opsonised yeast, *E. coli* and *C. albicans* as the targets for phagocytosis. Further studies to characterise more fully the phagocytic

capacity of AM in COPD are warranted; the use of apoptotic AEC as phagocytic targets would be more physiologically relevant for the study than previous methods. Measurement of phagocytosis by flow cytometry (FACS analysis) (examining thousands of cells in a short time) may be more objective than manual counting methods.

There are two major recognition signals involved in phagocytosis. The first involves the thrombospondin receptor, CD36, and the integrin $\alpha V\beta 3$ (vitronectin receptor, CD51/CD61) which form a complex that binds to thrombospondin, and in turn to an unidentified ligand on the apoptotic cell (Fadok et al. 1998). Interestingly, these molecules, as discussed earlier, are also are important for activation of latent TGF-β. A second system involves exposure of PTS (present on the inner layer of the plasma membrane in viable cells) on the outer leaflet of the plasma membrane of apoptotic cells. A receptor for PTS has been recently identified (Fadok et al. 2002). CD36 is also an important co-factor in this system (Fadok et al. 1998). Despite the obvious importance of these molecules for efficient removal of apoptotic cells by AM, there have been no studies of expression of these receptors on AM in COPD.

It is possible that the failure to resolve epithelial damage in COPD may result from defects in apoptosis or the mechanisms that induce apoptosis, or defective recognition or clearance by AM. The balance between the processes of repair and destruction in COPD may be altered so that there is failure to resolve the inflammatory reaction rapidly.

1-9-7 Peripheral blood involvement

Activated T-cells are increased in the airways in COPD and are thought to play an important role in the pathogenesis of the disease. Further, their numbers in the alveolar wall correlate with the extent of emphysema (Finklestein et al. 1995; Saetta et al. 1993).

In the peripheral blood, as in the airways, activated T-cells must be removed by apoptosis at the end of an immune response in order to maintain cellular homeostasis (Lenardo et al. 1999). However, excessive rates of apoptosis of activated T-cells may result in unbalanced homeostasis and defective clearance by macrophages/monocytes (Ogasawara et al. 1993). In view of the known trafficking of lymphocytes between the airways and the peripheral blood (Lehmann et al. 2001; Schuster et al. 2000), circulating T-cells in COPD may have an increased propensity to undergo apoptosis.

Several extrinsic pathways have been reported to induce apoptosis of T-cells. These include TGF -β/TGF-R1, TNF-α/TNF-R1 and Fas/Fas ligand (Lenardo et al. 1999; Sillett et al. 2001; Siegmund et al. 2001). Increased activation of these extrinsic pathways in the peripheral blood could contribute to increased T-cell apoptosis not only in the peripheral blood but also in the airways, thus playing a direct role in the pathogenesis of COPD.

Alterations in lymphocyte subsets in the peripheral blood and airways of patients with COPD have been reported (Majo et al. 2001; Saetta et al. 1998). The CD4: CD8 ratio is significantly decreased with the percentage of CD8+ lymphocytes increased. The CD8+ T-cells have been reported to be cytotoxic cells (O'Shaughnessy et al. 1997). These findings further suggest that lymphocytes may

play an important role in the pathogenesis of COPD, both in the peripheral blood and the airways.

Reasons for the changed CD4: CD8 ratio in both airways and peripheral blood in COPD have not been determined. It is possible that there may be increased apoptosis of CD4+ T-cells relative to CD8+ T-cells or changed expression of associated mediators of apoptosis (TNF-α and Fas) for CD4+ T-cells in COPD. As TGF-β and IL-4 enhance proliferation of CD8+ T-cells (Lee and Rich, 1993), it is also possible that increased T-cell production of these cytokines or expression of their receptors may be detectable in peripheral blood in COPD.

In view of the evidence that potentially significant changes occur in the peripheral blood in COPD, studies concerning the role of apoptosis should be extended to this compartment.

1-10 Conclusion

Repair processes in the lung are initiated as part of inflammatory responses to factors such as cigarette smoke. If there are no underlying structural changes, lung function can be restored if these repair processes can restore normal lung epithelium. Efforts at repair, however, in the presence of chronic inflammatory stimulus, may result in disruption of the balance of cytokines involved in airway repair, and disruption of normal tissue regeneration. There is evidence that in COPD, both in the airways and alveolar structures, tissue dysfunction likely results from altered structure due to ineffective repair processes. Thus it appears that the chronic inflammation in COPD may result from an imbalance between the load of inflammatory agents and their secretions, and tissue defense mechanisms, such as apoptosis and phagocytosis. However studies to date have been minimal and conflicting. Therefore, investigations were performed to evaluate apoptosis and clearance of apoptotic cells using samples obtained from the airways during fibreoptic bronchoscopy and peripheral blood from patients with COPD.

In addition, to better understand the processes of cell recruitment, activation, death and clearance in the human airways, *in vitro* studies were undertaken using a human bronchial epithelial cell line as a model. In this manner, I resolved to provide important data that would contribute to the design of rational interventions that could act at a variety of stages along the pathogenesis pathway and prevent further irreversible changes in lung function in COPD.

1-11 Questions addressed and hypothesis

1-11-1 Specific questions addressed in the following chapters

1) Is there evidence that excess apoptosis and accumulation of apoptotic material in the airways and peripheral blood are key factors in the pathogenesis of COPD?

2) If so, does the increase in apoptotic material in the airway in COPD result from defective phagocytosis by alveolar macrophages?

3) Is there evidence that cytokines (in particular TGF-β) and/or cytokine receptors and adhesion molecules play a role in the pathogenesis of COPD?

4) Do cytokines produced by airway epithelial cells play a role in controlling TGF-β production in the lung?

5) Is there a direct link between the apoptotic process and generation of cytokines that modulate the inflammatory response?

1-11-2 Hypothesis

The pathogenesis of COPD is associated with excess activation of apoptotic pathways and defective clearance of apoptotic cells by alveolar macrophages. The resulting accumulation of apoptotic material may lead to secondary necrosis and have a direct pro-inflammatory effect in the airways leading in turn to more apoptosis, further damage, and perpetuation of airway injury and progression of disease pathology.

Chapter Two

Materials and methods used in this thesis

2-1 Flow cytometry

Specific details of methods are provided in relevant chapters.

Specific details of suppliers of reagents and monoclonal antibodies (Mabs) are presented in Appendix.

2-1-1 Flow cytometric analysis

Flow cytometry was performed using a FacsCalibur flow cytometer (Becton Dickinson. Ca, USA (BD)) equipped with an air-cooled 488 nm argon ion laser. Data analysis was performed by collecting at least 10,000 events in list mode format and a Cell Quest analysis program (BD). The experiments were carried out under consistent instrument settings and results expressed as a percentage of cells exhibiting positive fluorescence.

A flow cytometer measures and analyses optical properties of single cells passing through an argon laser beam. Analysis of hundreds of cells per second provides a picture of the sample's physical and biochemical make-up.

When cells pass through the laser beam, they disrupt and scatter the incident light path. Detectors measure scatter in two directions. Narrow angle scatter, recorded from less than 1 degree to 10 degrees, is termed forward scatter (FSC) and related

to cell size. Wide angle scatter, collected 90^0 from the incident path, is termed side scatter (SSC) and provides a measure of internal cell complexity.

In addition to scatter, the flow cytometer can measure up to three fluorescent parameters (*Fluorescence channel* 1 (FL-1), channel 2 (FL-2) and channel 3 (FL-3). Fluorescent dyes absorb the blue (488nm) light produced by the argon laser, and emit a portion of this light in different regions of the spectrum. With the use of optical filters, the emission signals are separated by the flow cytometer. Light emitted in the green region of the spectrum is measured in FL-1, light emitted in the orange region in FL-2 and light emitted in the far red in FL-3. The most common fluorochromes for cell phenotyping are f*luorescein isothiocyanate (FITC)* and *Phycoerythrin (PE).* PE is one of several phycobilliprotein- based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping. PE has a molecular weight of 240,000 kD. Absorption and emission wavelengths of fluorochromes used in this thesis are listed below.

Table 2-1 Absorption and emission wavelengths of fluorochromes

Flourochrome	Absorption (nm)	Emission (nm)
Fluorescein isothiocyanate (FITC),	490	530 (green)
Phycoerythrin	480	578 (orange)
Propidium iodide (PI)	493	630 (red)
Peridin chlorophyll protein (PerCP)	480	660-697 (red)
PE-CY5	480	660-697 (red)
7-amimo-actinomycin D (7AAD)	480	670 (red)

2-1-2 Definition of terms

Cluster Differentiation (CD) system *(refer* ***Table 2-2****):* The identification of monoclonal antibodies with similar patterns of reactivity with human cells has been the focus of international workshops. Each group of antibodies has been assigned a CD number. Not all antibodies in a CD group react with identical portions (epitopes) of their target antigen. An antigen recognised by a given cluster of antibodies (eg, CD4) is referred to as a 'CD antigen' (eg, CD4 antigen).

Monoclonal antibodies (Mabs): Highly specific antibodies obtained by fusing mouse immune B-cells from the spleen with tumour (myeloma) cells to produce hybridomas, each of which will then secrete a single antibody. Such antibodies can then be labelled with fluorescent dyes. The e*pitope* (antigenic determinant) is that portion of an antigen against which the specific binding region of a monoclonal antibody reagent is directed. Epitopes may be linear sequences of as few as six amino acid sections of the antigen; each antigen typically contains multiple epitopes.

Immunophenotyping*:* Discrimination among subsets of leucocytes utilising fluorescence labelled Mabs that recognise membrane associated molecules.

Data display: Data is displayed as dual or single parameter displays. *Dual parameter displays* (dot plot, contour plot, density plot) are graphic representations of data in which corrected intensities for two different parameters are measured on the same cell and plotted on an x,y grid (eg, log FITC and log PE immunofluorescence). From this display, percentages showing positive fluorescence for a particular marker could be calculated.

A *single parameter display* (histogram) shows frequency distribution of measured signal intensities observed for cells within a population.

Table 2-2

CD and other antibodies employed in this study

CD antigen	Function
CD3	T-cell marker
CD4	T-cell subset (helper/suppressor)
CD8	T-cell subset (precursor/cytotoxic)
CD14	(LPS receptor) Monocyte / macrophage marker
CD33	Monocyte / macrophage/ myeloid marker
CD36	Thrombospondin receptor
CD45	Common leucocyte antigen
CD49 (a-f)	Receptors for extracellular matrix proteins
CD51	αunit of αv/α3 integrin
CD61	β unit of αv/β3 integrin
CD95	Fas receptor
CD122	β subunit of IL-2 receptor
CD124	IL-4 receptor
CD127	IL-7 receptor
CD132	γ subunit of IL-2 receptor
ECA	Epithelial cell antigen
aTNF-RI	TNF-α receptor I
aTNF-RII	TNF-α receptor II
aTGF-R	TGF-β receptor
aBcl-2	Anti-apoptotic nuclear protein
aP53	Pro-apoptotic nuclear protein

Forward Angle Light Scatter (FSC): Measurement of light at a low radial angle relative to the incident light source. Measured values are a function of the cross-sectional area of a cell or particle and the wavelength used for measurement. It is commonly used as a measure of the relative size of a cell.

Side scatter (SSC, Ninety Degree Light Scatter): Measurement of light at right angles to the incident light source. This measurement is related to cytoplasmic granularity, membrane irregularity and/or shape of a cell nucleus.

Gate, gating: A set of parameters used to define a specific subset of the cell population, which then allows the measurement of additional parameters within that subset. Typically, a region of interest was defined based on one set of parameters (eg, FSC vs. 90^{0} SSC) and other properties (eg, positive staining with fluorescent antibodies) are evaluated only for cells within the defined region.

Autofluorescence : The intrinsic fluorescence of unstained cells generally caused by pyrimidines and flavin nucleotides. The level of autofluorescence varies with the cell type being analysed and/or the state of cellular activation. Cultured cell lines and monocytes/macrophages usually demonstrate high levels of autofluorescence.

Quadrant markers: Background staining occurs due to a combination of natural *autofluorescence* of cells and non-specific binding of Mabs to cells. This staining is defined using IgG mouse antibodies directed to *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in humans. The *dual parameter display* (eg, dot plot) that is obtained after staining with IgG mouse antibodies is divided into rectangular quadrants by the use of two perpendicular boundaries (quadrant markers). These markers are then set so that background readings of less than 2% are obtained.

Resolution: The ability to discriminate between cells having different signal intensities. Such ability is a function of biological factors (heterogeneity of signal within each population, difference in signal intensities between populations) as well as instrumental factors (sensitivity).

Sensitivity: The ability to distinguish signals of interest from background 'noise'. 'Noise' may be instrumental (optical or electronic signals arising when no fluorochrome or cell is present) or biological (autofluorescence, non-specific reagent binding).

Colour compensation: Electronic subtraction of a fraction of one signal from a second, typically used in correcting for overlapping fluorescence from one fluorochrome in the wavelength region to the second.

Threshold: Level of signal above which a measured value is considered to be significantly different to background 'noise'. Threshold for instrument fluorescence sensitivity is the level of signal found for what is considered to be a non-fluorescent object. Threshold for determination of positive antibody staining is the level of signal found for cells not believed to react specifically with a given antibody reagent.

Linear amplification: A linear amplifier (amp) produces a signal output proportional to the input signal amplitude. For example, a linear amp could have output varying from 1 to 5 volts as the input signal varies from 0.01 to 0.05 volts.

Logarithmic amplification: A logarithmic amplifier produces a signal output proportional to the logarithm of the input signal amplitude. For example, a three decade log amp will have an output varying from 0 to 10 volts as the input signal varies by a factor of 1000. Log amps are useful when analysing samples containing cells whose measured parameters differ by orders of magnitude

2-1-3 Controls

Sub-class control: Subclass refers to variations in immunoglobulin heavy and light chains. Most monoclonal antibodies used in flow-cytometry are either IgM, or various subclasses of IgG (IgG1, IgG2a, IgG2b, or IgG3). Thus, a subclass control is an immunoglobulin of the same isotype (class or subclass) as the monoclonal antibody of interest but without specificity for any known human antigens.

Autofluorescence control: To determine the background autofluorescence of the cells, the appropriate control is a tube containing unstained cells.

Positive controls: A peripheral blood specimen from a "normal" adult, used to verify the performance of reagents and staining procedures and to test new reagent lots. Variability for new lots of reagents compared to current lots is accepted when it is no greater than the variability found for replicate samples of the current lot.

2-1-4 Setting up flow cytometer for optimal performance

Weekly alignment of the flow cytometer was carried out using Auto Comp Software (BD), to ensure that the cytometer recorded intensity of events consistently. Auto Comp settings were stored in a file, and retrieved every time an alignment was carried out. The software is a menu-driven program that adjusts the photo-electric components of the FACSCalibur according to known standards- gain settings and fluorescence compensation values were adjusted to standard samples of CaliBRITE beads (plastic microspheres) (BD). The beads included (a) unlabelled, (b) labelled with FITC and (c) labelled with PE. Three FACSCalibur adjustments were performed.

(i) ***Gating of single events*** - upper and lower gates for FSC intensity were set to minimise the effects of doublets and debris, and eliminate data which did not fall within the set light scatter limits. By acquisition and analysis of 5,000 events from unlabelled beads, the program automatically set the upper and lower gates by statistical analysis of the resulting frequency histogram.

(ii) ***Adjustment of PMT voltage*** - variable voltage settings of photomultiplier (PMT) tubes influence their detection sensitivity so that increasing the voltage results in greater amplification of signal and alteration in channel distribution. The PMT was automatically set so that unlabelled CaliBRITE beads had a mean within 2 channels of target value. The FSC photodiode does not have variable voltage setting so was unaffected by this procedure.

(iii) ***Fluorescence compensation*** - FITC and PE have some overlap of emission spectra. Despite the use of emission filters in the flow cytometer which minimise the overlap, some FITC radiation reaches the PE detector and visa-versa. AutoCOMP compensated for this by electronically subtracting the unwanted signal from the signal of interest.

Method:

(i) One drop of unlabelled beads was added to 1 ml Isoton II (Coulter.Immunotech, USA).

(ii) One drop of each of unlabelled and FITC and PE labelled beads were added to 3 ml Isoton.

(iii)To adjust the PMT voltage, the tubes containing the beads were put on the FACSCalibur, making sure that the fluidics Mode Selector was set to HIGH. Fluorescence compensation and sensitivity testing was then automatically performed and results printed.

2-1-5 Detection of cell surface antigens and cytokines

When fluorochromes are coupled to Mabs directed against cell surface antigens, flow cytometry (FACS analysis) can determine the relative amount of dye on individual cells, thus generating information on the molecular properties of the cells.

Anti-cytokine Mabs provide a tool for multi-parametric flow cytometric analysis of individual *cytokine-producing cells* within unseparated cell populations in samples such as whole blood or BAL. Cell samples can be stained for surface antigens as well as intracellular cytokines. This protocol can be used to identify the phenotype and frequency of cell types defined by membrane antigens and intracellular cytokines.

Staining of *intracellular cytokines* by flow cytometry depends on identification of cytokine-specific Mabs compatible with a fixation-permeabilisation procedure. Paraformaldehyde fixation allows preservation of cell morphology and intracellular antigenicity, while also enabling the cell to withstand subsequent permeabilisation. Permeabilisation by detergents such as saponin allows the cytokine-specific monoclonal antibody to penetrate the cell membrane, cytosol, and membranes of the endoplasmic reticulum and Golgi apparatus.

During in vitro stimulation, intracellular transport inhibitors are necessary to block intracellular transport processes, which therefoe results in the accumulation of most cytokine proteins in the Golgi complex, thereby enhancing the ability to detect cytokine- producing cells. This study used brefeldin A (a fungal metabolite produced by *Penicillium brefeldianum*) as a Golgi block.

2-2 Culture and stimulation of cells

Some form of stimulation is genererally required for detection of cytokine production by flow cytometry (propensity of a cell to produce cytokine). Various *in vitro* models are available to specifically or non-specifically induce cell activation, resulting in cytokine production, cytokine receptor expression and cell proliferation. Mitogens are agents capable of inducing activation and cell division in a high percentage cells. Unlike an immunogen, which only activates a cell bearing receptors specific for that immunogen, a mitogen can activate many cell types, irrespective of their antigen specificity (polyclonal activators). Polyclonal activators include pharmacological agents (phorbol 12-myristate 13 acetate (PMA, which directly activates protein-kinase C), calcium ionophore/ionomycin, phytohaemagglutinin (PHA), enterotoxins (SEB), anti-CD3/TCR, anti CD28 and lectins. T-cells can be non-specifically triggered by antibodies to a variety of cell surface molecules- full activation usually requires an additional TCR/CD3 mediated signal. *E-coli* lipoolysaccharide (LPS), a component of the gram- negative bacterial cell wall, has been widely used as a patho-physiological stimuli for monocytes. Details of experiments undertaken to determine the optimal stimuli for various cell types investigated in this study are given in the appendix.

PHA has been widely reported for studies of the potential of T-cells to undergo apoptosis when stimulated (Frassanito et al. 1998; Kaser et al. 1999; Alcouffe et al. 1999; Chen et al. 1998; McLeod et al. 1998; Novelli et al. 1997; Ito et al. 1997). The present study therefore utilised PHA stimulation of peripheral blood-derived T-cells for investigation of apoptosis in these cells.

For all studies described in this thesis (unless otherwise specified), cells were stimulated with appropriate stimuli for 24h at 37°C/5% CO_2 in10 ml culture tubes.

Table 2-3 Stimuli used in this study

Cell type	Mediator	Stimulus	Concentration
16HBE AEC	Cytokine	LPS	1μg/ml
16HBE AEC	Surface	None	
16HBE AEC	Apoptosis	None or	
		AFAS or	500 ng/ml
		Dexamethasone	10^{-5} M
Brushing-derived AEC	Cytokine	LPS	1μg/ml
Brushing-derived AEC	Surface	None	
Brushing-derived AEC	Apoptosis	None	
BAL-derived AM	Cytokine	LPS	1μg/ml
BAL-derived AM	Apoptosis	None	
BAL-derived AM	Surface	None	
Blood-derived lymphocyte	Cytokine	PMA	25ng/ml
Blood-derived lymphocyte	Apoptosis	PHA+	5μg/ml+
		Ionomycin	1ug/ml
Blood-derived monocyte	Cytokine	LPS	1μg/ml

Chapter Three

Differential cell counting by flow cytometry

3-1 Introduction

Several studies have shown that neutrophils, lymphocytes and AM are involved in the inflammatory process in COPD (Finkelstein et al. 1995; O'Shaughnessy et al. 1997; Keatings et al. 1996; Lacoste et al. 1993; Balbi et al. 1997; Di Stefano et al. 1998; Saetta et al. 1998), as discussed in Chapter 1. There is a correlation between the numbers of these cells in the lungs and the severity of COPD (US Department of Health and Human Services, 2001). There is, however, variation in the predominant cell types reported, possibly resulting from a difference in inflammatory cell type numbers in various parts of the lung (Maestrelli et al. 1995; Rutgers et al. 2000). As most studies have been performed on bronchial biopsies, a need for accurate characterisation and clarification of cellular populations in bronchioalveolar lavage (BAL) from the airway lumen in COPD remains. Current techniques to enumerate cell types in BAL rely on manual differential counting methods that do not allow for analysis of additional characteristics of infiltrating cells, such as cytokine production, receptor expression and an accurate evaluation of apoptosis for different cell types. The initial studies described in this chapter were therefore designed to develop and apply flow cytometric methods to identify various cell types in BAL. Comparisons of cell counts obtained using flow cytometric and manual counting methods are also described.

For these studies, samples of bronchial brushings and BAL were obtained directly from human subjects undergoing fibre-optic bronchoscopy.
Because brushing-derived cells are greater than 98% AEC, for the purposes of this thesis these cells will be henceforth referred to as AEC.

3-2 Methods

3-2-1 Subject population

Patients undergoing fibre-optic bronchoscopy for diagnostic purposes were invited to participate in the study. Potential subjects were supplied with a written information sheet. If they consented, written documentation was obtained. Examples of the information sheet and consent form are supplied in the Appendix. Clinical judgement was used to assess the suitability of patients and those considered high risk based on the nature of their underlying disease were not included in the study. These included immunocompromised patients and those with serious infectious disease (particularly AIDS, Hepatitis B or C, or tuberculosis), limited pulmonary reserve (FEV1 less than 1.2 L), coagulopathy or sensitivity to local anaesthetics. The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital following the guidelines of the declaration of Helsinki. Subjects were supplied with contact information for the principle researchers (S. Hodge, M. Holmes, R. Scicchitano and P. Reynolds, as well as the Chairperson of the ethics committee), so that any questions or concerns that arose after the study could be addressed. Subjects were not financially compensated for their involvement in the study. Patients underwent pulmonary function tests (spirometry and diffusing capacity measurement) as part of their routine clinical assessment.

BAL and bronchial brushings were collected from 16 patients with COPD. For 10 non-smokers undergoing bronchoscopy for clinically indicated reasons and with no history of COPD, asthma or allergy, specimens were obtained and used as controls (**Table 3-1**). The diagnosis of COPD was established using the European Respiratory Society criteria (Siafakas et al. 1995). Eight COPD patients were categorised as mild COPD (FEV1 ≥ 70% predicted, with clinical correlation, and 8 as moderate – severe COPD (FEV1 < 70% predicted) There was no exacerbation of disease for 6 weeks prior to involvement in the study.

Table 3-1 - Demographic characteristics of the population studied

Subjects	Control group	COPD (total group)	Mild COPD	Moderate-severe COPD
No. of subjects	10	16	8	8
Age (y)	63 (± 17)	66 (± 9)	69 (± 9)	63 (± 8)
Smoking, pack yr	0	67 (± 26)	56 (± 20)	83 (± 26)
FEV1, % pred	94.7 (± 10.4	68.1 (± 21.1)	82.5 (± 6.7)	54 (± 18.5)
FVC, % pred	97.8 (± 9.7)	82.5 (± 18.8)	94.0 (± 17.0)	76 (± 13.7)
FEV1, % FVC	81.7 (± 15.8)	71 (± 13.7)	74.0 (± 5.0)	64 (± 19.2)
DLCO	97.6 (± 2.3)	67 (± 22.4)	69.0 (± 38.3)	64 (± 4.3)

Results are expressed as mean values (± SD)

3-2-2 Bronchoscopy procedure

Brushings and BAL were obtained from the right-sided airways unless there was pathology in this area, when the left-sided airways were sampled. The patients were pre-medicated with 0.6mg of i.m. atropine (Astra Pharmaceuticals, Sydney, NSW, Australia) then sedated with titrated doses of i.v. midazolam (Roche Products Pty Ltd., Sydney, NSW, Aust.) and fentanyl (Astra). Transnasal fibreoptic bronchscopy was performed using either an Olympus 1T2000 or BF240 bronchoscope (Olympus Optical Co. Ltd., Tokyo, Japan). Topical xylocaine (Astra) was applied to the nostrils, pharynx, larynx and airways. Particular attention was given to use a minimal amount of xylocaine (100mg) in the airways in view of the adverse effect it has on cell

viability (Kelsen et al. 1992). Bronchial brushings were obtained by positioning the tip of the bronchoscope in a subsegmental airway, then gently advancing a standard cytology brush (Fuginon Inc., Wayne, NJ, USA) into four to six distal airways. AEC were obtained with several gentle passages of the brush into each airway so as to avoid bleeding. Cells were deposited by washing the brush in 10ml RPMI 1640 media (Gibco, BRL, Germany), in a 10ml conical polypropylene tubes (Johns Professional Products, Sydney, Australia; hereinafter referred to as 'culture tubes'), and kept on ice. BAL was performed by wedging the tip of the bronchoscope into a subsegmental airway. A 50 ml aliquot of sterile normal saline (at room temperature) was instilled into the airway with a syringe then aspirated using low suction into a 50ml plastic suction trap. Two further aliquots of saline were instilled and aspirated in the same way into two further traps. To avoid contamination with airway mucus, the first aliquot was discarded. Aspirated specimens were immediately transferred to 50ml polypropylene tubes (to avoid attachment of cells to the polystyrene plastic trap) and kept on ice. Cells were processed within 1h of collection.

3-2-3 Preparation of ex vivo samples

For each collection from an individual patient, BAL specimens 2 and 3 were pooled. Cells were pelleted by centrifuging at 500×g for 5 min. Supernatant was discarded and cells re-suspended to a cell count of 4 x 10^5 cells / ml with RPMI 1640 media. AEC from bronchial brushing specimens were pelleted by centrifuging at 500 × g for 5 min, supernatant was discarded, and cells re-suspended to to a cell count of 4 x 10^5 cells / ml with RPMI 1640 media. Prior to processing for flow cytometry, morphology of cells derived from BAL and bronchial brushings was assessed on cytospin preparations and cells counted.

3-2-4 Identification of cell types by manual counting

Manual cell counting and differential were carried out by a diagnostic laboratory (Histopathology Department, IMVS, Adelaide). Cytospin preparations were stained by Papanicolaou and Giemsa methods. For Papanicolaou staining, slides were washed in tap water followed by staining with haematoxylin for 2 min. Slides were washed again in water then immersed six times in acid-alcohol followed by further washing in water. Treatment with sodium bicarbonate for 2 min was followed by further washing in water. Alcohol treatment for 1 min (70% alcohol then 96% alcohol), OG-6 for 2 min, alcohol for 1 min (96% alcohol x 2), EA-50 for 5 min and alcohol for 2 min (absolute alcohol x 2) was followed by final treatment with Xylene substitute for 2 min. Giemsa staining was carried out using an automated staining machine (Shandon Veristat Southern Products, Astmore, UK).

3-2-5 Identification of cell types by flow cytometry

3-2-5 (a) Reagents

Further details of monoclonal antibodies (Mabs) used in the studies are supplied in the Appendix. Phycoerythrin (PE) conjugated Mabs to CD45 (a common leucocyte marker), fluorescein isothyocyanate (FITC) conjugated MoAbs to CD33 (a macrophage/monocyte marker), epithelial cell antigen (ECA). Phycoerythrin cyanocobalamine (PE-CY5) conjugated Mabs to CD14 (a macrophage/monocyte marker) and CD3 (a T-cell marker) were also included.

3-2-5(b) Cell surface staining with Mabs

Cell surface antigens were stained with Mabs as shown in **Figure 3-1.**

3-2-5(c) Identification of bronchial brushing - derived AEC

AEC were stained as described above with fluorescent-conjugated Mabs to identify AEC (FITC-conjugated -epithelial cell antigen (ECA)) and contaminating leucocytes (PE-conjugated CD45, a common leucoctye antigen marker).

A region (R1) was drawn to exclude debris and red blood cells based on known forward (FSC) and side (SSC) scatter characteristics. All subsequent analysis was carried out on cells gated in R1 (**Figure 3-2(a)**).

Leucocyte contamination of bronchial brushing was excluded from a further region (R2) based on bright staining with CD45 and negative staining with ECA (**Figure 3-2(b)**). All subsequent analysis of surface staining was carried out on cells from R1 and R2.

Figure 3-1

Procedure for surface staining of BAL- and bronchial brushing- derived cells by flow cytometry. 200μl aliquots of BAL and 100μl aliquots of bronchial brushings were added to labelled FACS tubes. To block Fc receptors and reduce non-specific binding, 20μl of normal human immunoglobulin (60g/l, Intragam, (CSL)) was added to each tube for 20 min at room temp. After a further incubation for 20 min, in the dark, with directly conjugated Mabs to surface markers of interest, cells were washed with 0.5% bovine serum albumin (hereinafter refered to as 'wash buffer'), centrifuged at 1500 x g for 90 sec, and the supernatant discarded. Twenty microlitres of wash buffer was added, and events acquired immediately by flow cytometry. For AEC from bronchial brushings, 10,000 events were collected. For BAL, 50,000 events were collected.

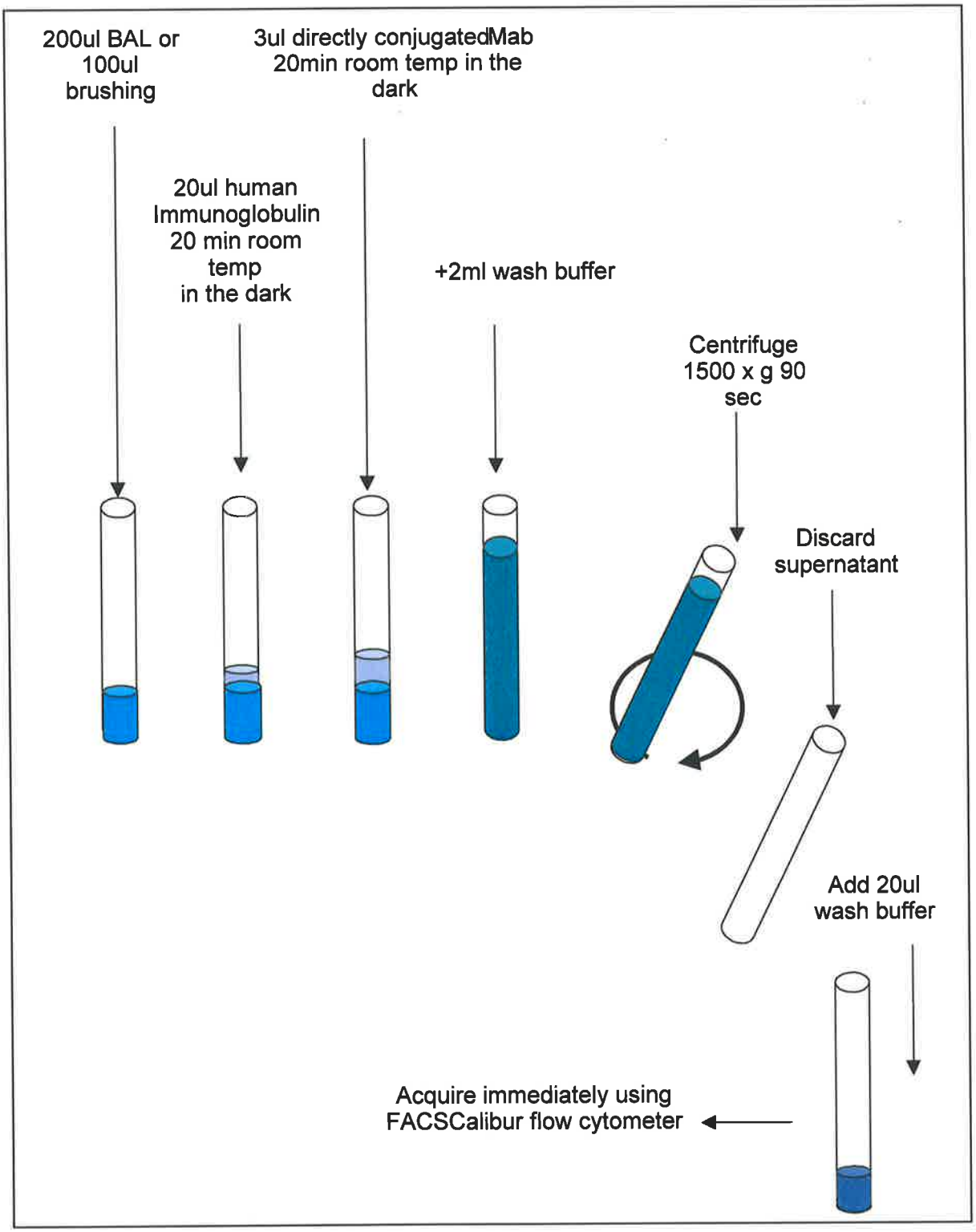


Figure 3-1

Staining of cell surface antigens for flow cytometry

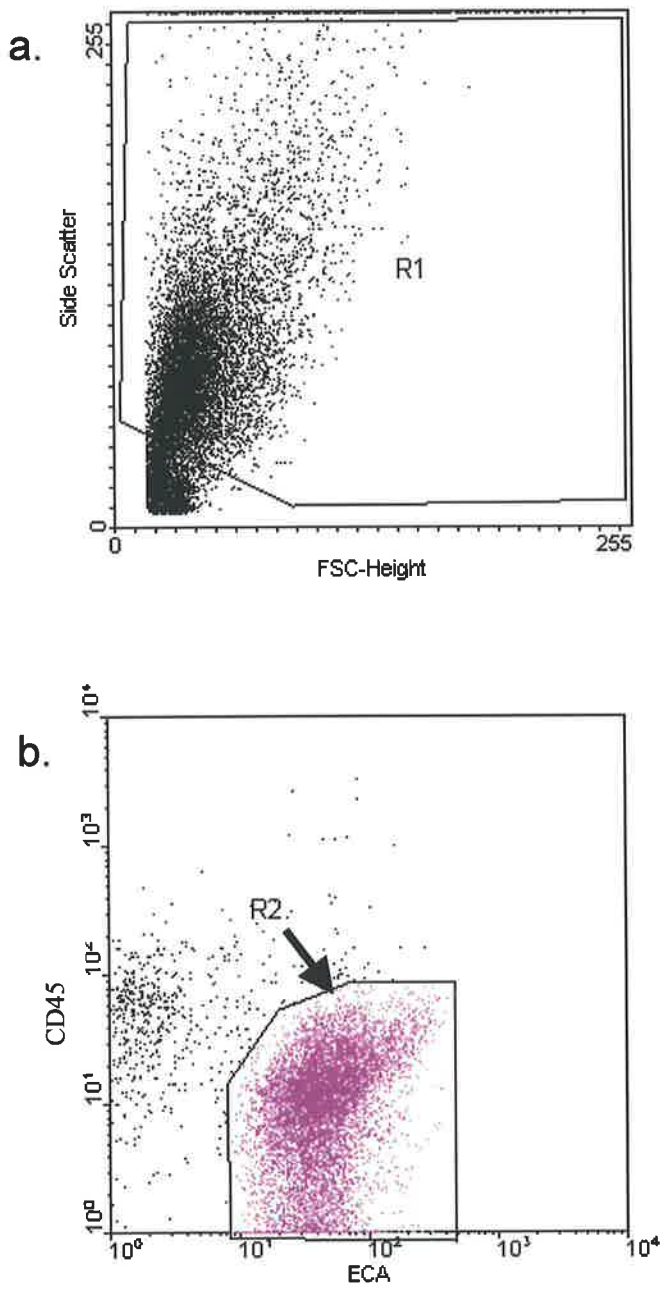


Figure 3-2

Identification of brushing- derived AEC by flow cytometry (a) Debris and red blood cells excluded from Region 1 (R1) based on low FSC and SSC. All subsequent analyses were carried out on cells from R1 (b) AEC gated in R2 based on bright staining with epithelial cell antigen and dim staining with CD45, a common leucocyte antigen marker. All subsequent analyses were carried out on cells from R1 and R2.

3-2-5(d) Identification and differential counting of BAL- derived cells

BAL – derived cells were stained as described above using fluorescent-conjugated Mabs to identify the various cell types:

Tube 1 -Epithelial cell antigen FITC/CD45 PE (to quantify AEC)

Tube 2 -CD 33 FITC/CD45 PE (to quantify AM, lymphocytes and neutrophils)

Tube 3. -CD 33 FITC/CD45 PE/CD14 PE-Cy5 (to verify results)

Details of flow cytometric analysis to identify the various cell types are given in **Figure 3-3**. A quenching strategy to reduce autofluorescence of alveolar macrophages was also used (see 3-2-6).

The gated populations were confirmed by sorting the cell populations from the BAL sample, and preparing a stained preparation of these cells on a microscope slide followed by Giemsa staining as previously described in this report (**Figure 3-4**).

Figure 3-3

Identification and differential counting of BAL – derived cells using flow cytometry

a. Forward Scatter (FSC) vs Side Scatter (SSC) characteristics of BAL.

b. Region 1 (R1) was drawn to define leucocytes identified based on bright staining with CD45 (debris and red blood cells were excluded). All subsequent analyses were carried out on cells from R1. R2 defines lymphocytes, based on low side scatter and bright staining with CD45.

c. R3 defines AM based on high side scatter and dim staining with epithelial cell antigen (ECA).

R4 defines AEC based on bright staining with ECA and low SSC.

d-f. Identification of neutrophils and confirmation of AM gating:

Neutrophils were considered to be any cells not gated in the previous regions. These cells demonstrated dim staining with CD33 and CD14 (monocyte/macrophage markers), bright staining with CD45 and lower SSC characteristics than AM. These gating strategies were confirmed by examining stained preparations of sorted cell populations (refer **Figure 3-4**).

A small population of cells considered blood monocytes were gated in R5 (CD45 bright, medium SSC) and excluded from all subsequent analysis.

Note: red arrows indicate a sub-population of neutrophils considered apoptotic (we have previously shown that apoptotic neutrophils lose some CD45 expression and therefore stain CD45 dim with low forward and side scatter characteristics (Hodge et al, 1998))

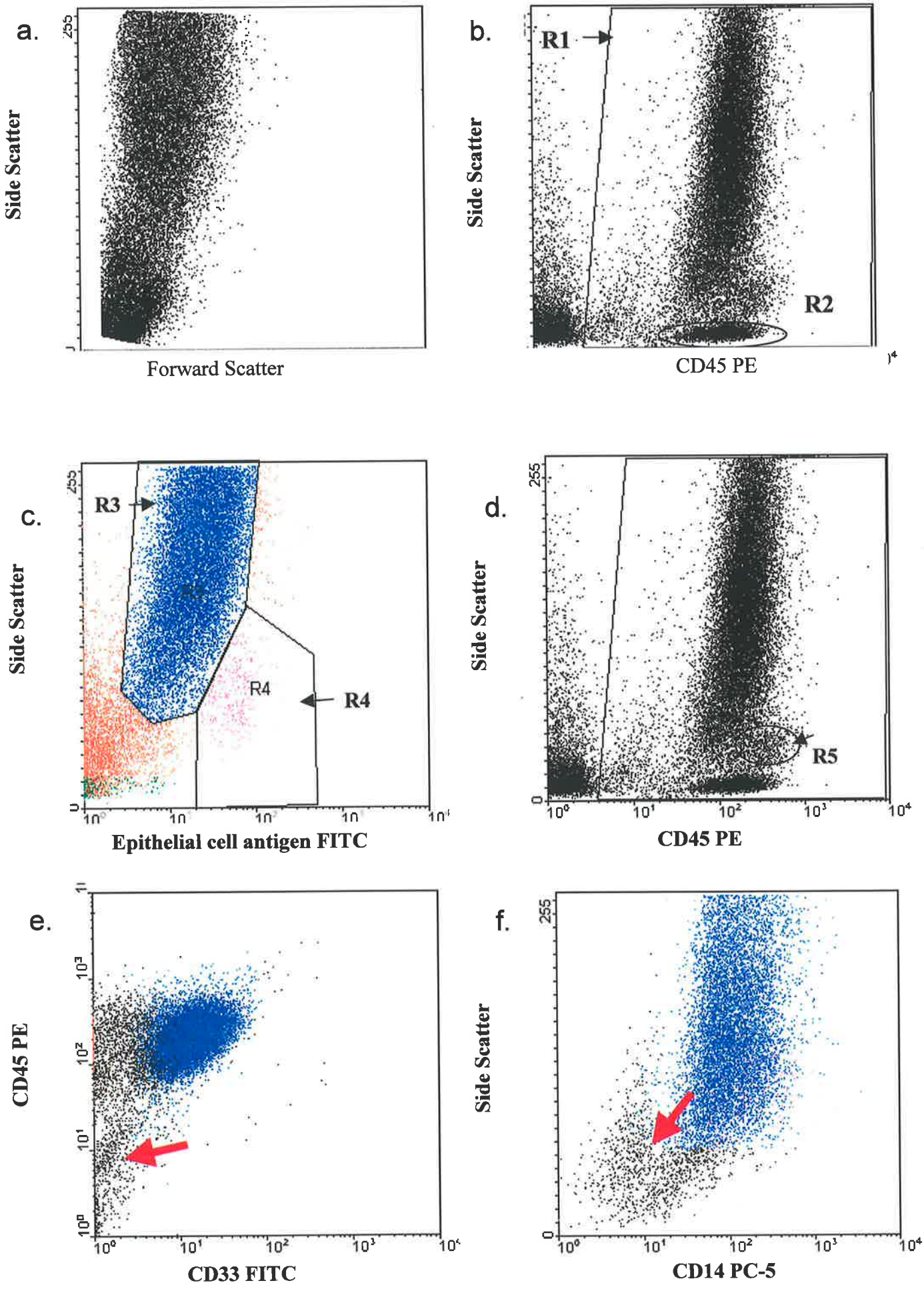
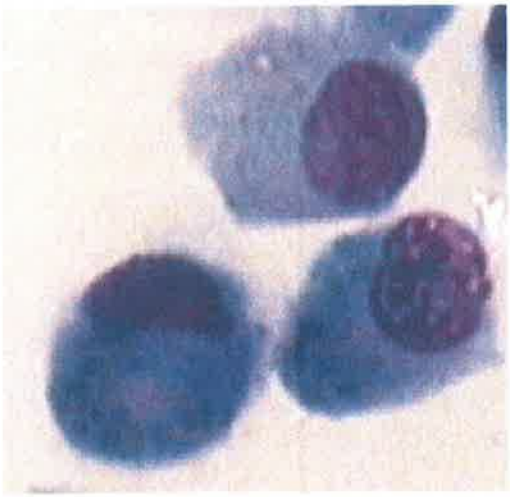

a.
Side Scatter
Forward Scatter
b.
R1
R2
Side Scatter
CD45 PE
c.
R3
R4
R4
Side Scatter
Epithelial cell antigen FITC
d.
R5
Side Scatter
CD45 PE
e.
CD45 PE
CD33 FITC
f.
Side Scatter
CD14 PC-5

Figure 3-3

a.



b.

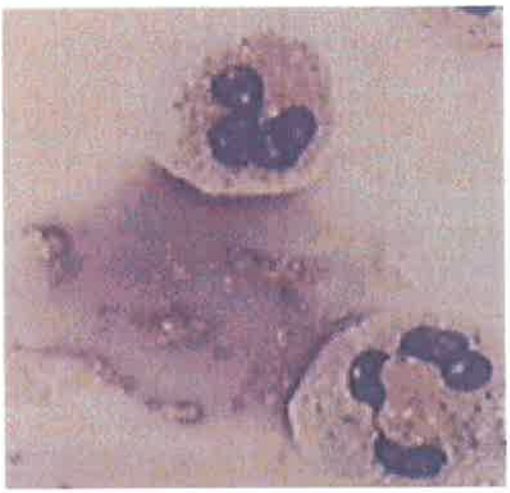


Figure 3-4

BAL: Cytospin preparations of sorted cell populations. Cell populations identified by flow cytometry were verified by sorting [FACStar plus] and examining stained cytospin preparations (A) AM (B) Neutrophils

3-2-6 Autofluorescence of AM: Effect of quenching

BAL- derived AM, especially those obtained from smokers, have greatly increased autofluorescence properties that hinder analysis by flow cytometry due to spectral overlap between AM autofluorescence and fluorochrome emission spectra. Several dyes have been utilised to quench autofluorescence of BAL- derived AM (Umino et al. 1999; Hallden et al. 1991; Hed et al. 1987). Furthermore, permeabilisation of the cell membrane with η-Octyl β-D-galacto-pyranoside has been reported to increase the efficiency of quenching with crystal violet (Hed et al. 1987). To establish optimal quenching methods, I investigated the effects of a range of quenching agents (**Table 3-2**). BAL-derived cells were stained for surface antigens as described above, re-suspended in 100 μl quenching agent for 1 min, washed, then acquired and analysed by flow cytometry. For some experiments, permeabilisation of the cell membrane was carried out using a commercial reagent, FACSPerm (BD). Cells were stained for surface antigens, washed then left at room temperature in the presence of 500 μl FACSPerm for 10 minutes, before washing and quenching.

Optimal quenching was determined by observation of the reduction in autofluorescence and non-specific binding in dot plots of:

a. Fluorescence channel 1 (FL1) vs fluorescence channel 2 (FL2)

b. Epithelial cell antigen (FITC) vs CD45 (PE) (**Figure 3-5 (a) and (b)**)

c. CD14 (FITC) vs CD45 (PE) (**Figure 3-5 (c) and (d)**)

Crystal violet was determined to be the most successful quenching agent, with cell permeabilisation by FACSPerm or 0.1% η-Octyl β-D-galacto-pyranoside marginally improving the effects. Therefore crystal violet was thereafter routinely applied for analysis of BAL.

Table 3-2

Quenching of autofluorescence of BAL-derived AM: Agents investigated

1	0.2% Crystal Violet (CV) /PBS pH7.4	7	Non fat milk (Milk) (1% in PBS)
2	0.1% η-Octyl β-D-galacto-pyranoside/PBS	8	Milk + CV
3	Galacto- pyranoside then CV	9	Milk then galacto-pyranoside then CV
3	Facsperm	1	Tween (0.1% in PBS)
5	Facsperm then CV	1	Tween + CV
6	Facsperm, galacto-pyranoside then CV	1	Tween, galacto-pyranoside then CV

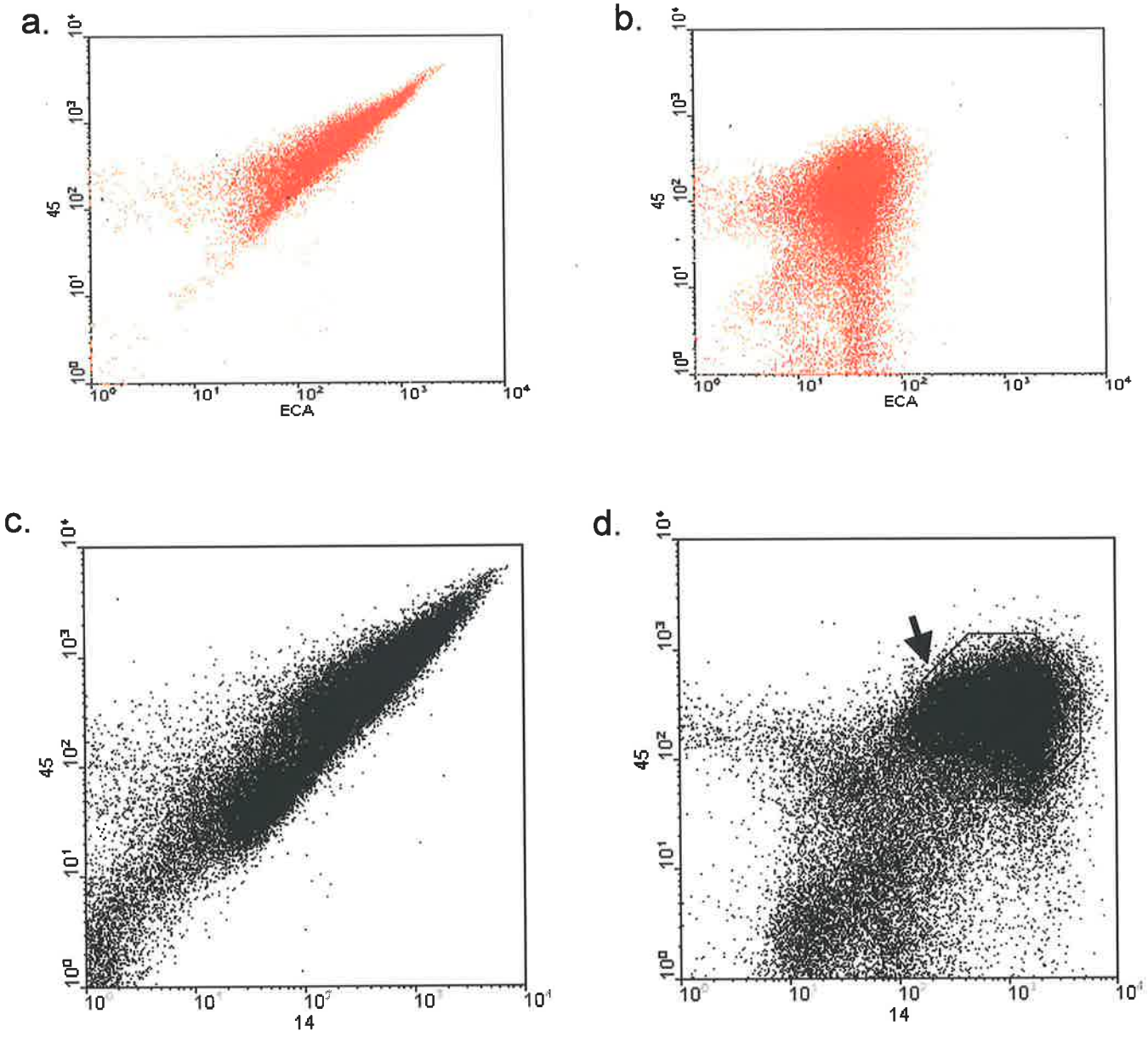


Figure 3-5 Autofluorescence of AM: Effect of quenching.

Representative dot plots of AM before and after quenching with η-Octyl β-D-galacto-pyranoside then 0.2% Crystal Violet/PBS pH7.4 (a) Epithelial antigen (ECA) FITC vs CD45 PE showing marked autofuorescence of AM before quenching (b) The same preparation after quenching. Note well differentiated positive staining with CD45 and dim staining with epithelial cell antigen (c) CD14 FITC vs CD45 PE showing marked autofluorescence of AM before quenching (d) After quenching, note well differentiated positive staining of AM with CD45 and CD14

3-3 Results

3-3-1 Identification of brushing-derived AEC

For bronchial brushings, manual differential cell counting and flow cytometry showed 95-97% of the cells to be of epithelial type. The AEC demonstrated well preserved nuclear and cytoplasmic architecture.

3-3-2 Correlation between manual and flow cytometric differential cell counts

Observation of stained cytospin preparations of BAL revealed cells that were difficult to identify by manual counting methods (cell death and disintegration) (**Figure 3-6**).

Correlation between lymphocyte and neutrophil counts using manual counting or flow cytometric techniques was satisfactory (R^2=0.75 for lymphocytes and R^2=0.8 for neutrophils). Correlation between AM counts using the two methods was less satisfactory (R^2=0.5) with manual counting providing higher estimates of the percentage of macrophages compared to flow cytometry (**Figure 3-7**). AEC contamination was less than 2% for both methods.

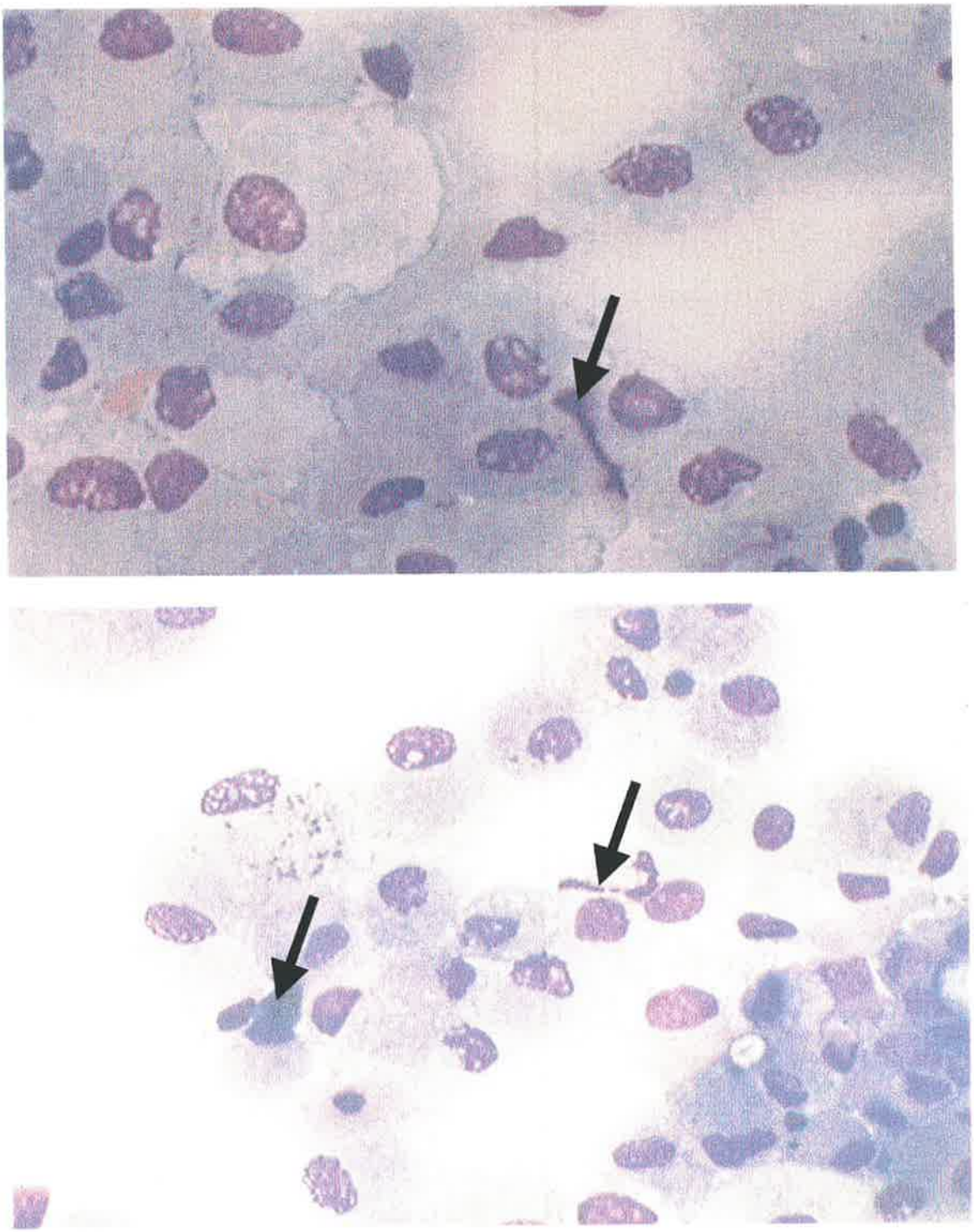


Figure 3-6

Cytospin preparations of BAL stained with May Grunwald Giemsa: Arrows denote examples of cells which are hard to identify by manual methods [due to cell death] but which may still retain surface staining properties which can be identified by flow cytometry [CD markers].

Figure 3-7

Correlation between maual and flow cytometric differential cell counts

Analysis of BAL- derived cells using flow cytometry (x axis) and manual counting of Papanicolou- stained cytospin preparations (y axis)

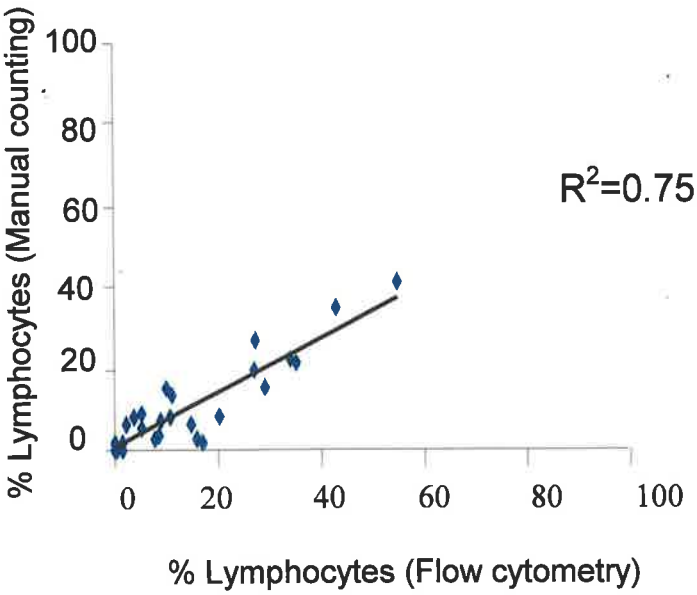
a. Lymphocytes

b. AM

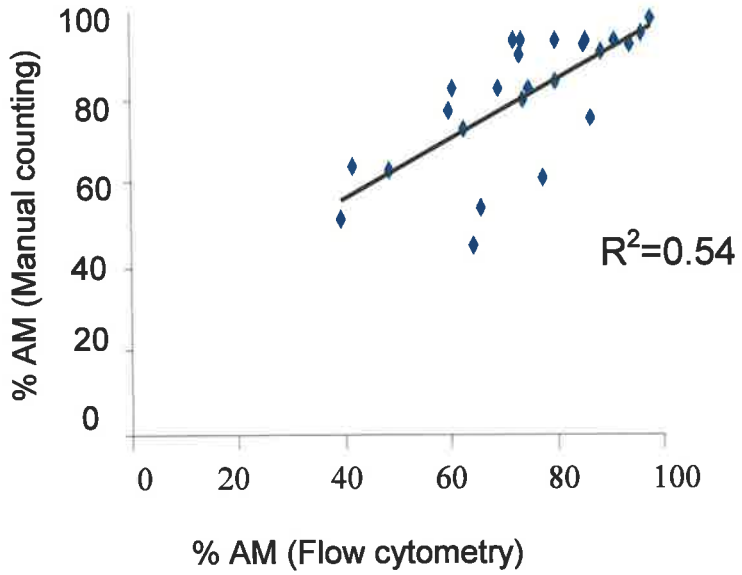
c. Neutrophils

Note good correlation between manual and flow cytometric counting methods for lymphocytes (R^2=0.75) and neutrophils (R^2=0.80) but less satisfactory correlation between methods for AM (R^2=0.54); N = 25

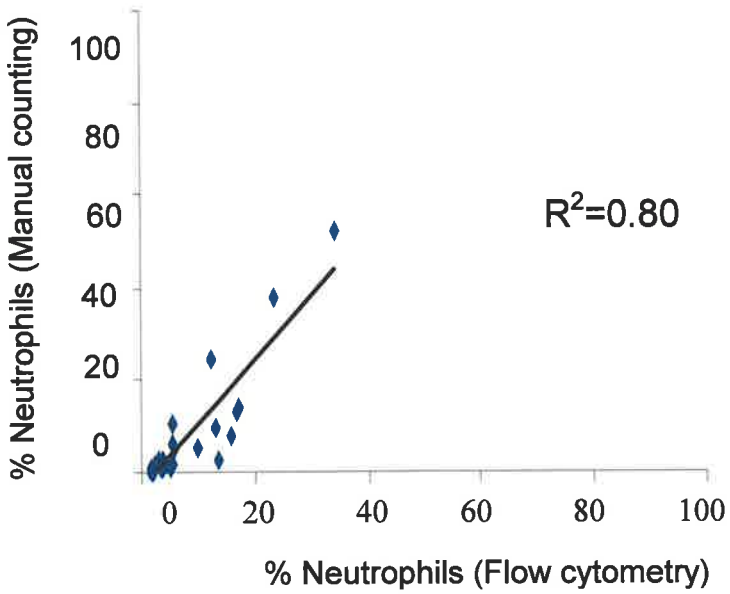
a.


% Lymphocytes (Manual counting)
100
80
60
40
20
0
0
20
40
60
80
100
% Lymphocytes (Flow cytometry)
R2=0.75

b.


% AM (Manual counting)
100
80
60
40
20
0
0
20
40
60
80
100
% AM (Flow cytometry)
R2=0.54

c.


% Neutrophils (Manual counting)
100
80
60
40
20
0
0
20
40
60
80
100
% Neutrophils (Flow cytometry)
R2=0.80

3-3-3 Identification and differential counting of BAL-derived cells

For BAL, leucocyte cell types ranged from 0-55% [lymphocytes], 47-97% [AM] and 0-38% [neutrophils]. AEC contamination was less than 2%. The percentage of AM or lymphocytes was not significantly higher in BAL from COPD patients than from control subjects (**Figure 3-8**). The percentage of neutrophils was higher in BAL from both moderate-severe and mild COPD patients compared with control subjects, although the difference was only significant for the moderate-severe COPD group ($p=0.04$) (**Figure 3-8**). Absolute numbers of lymphocytes were not significantly different between COPD and controls (COPD total 23 ± 18 vs control 22 ± 18, $p=0.5$; COPD mild 18 ± 18 vs 22 ± 18, $p=0.3$; COPD moderate-severe 29 ± 19, $p=0.2$). Similarly, absolute numbers of and macrophages were not significantly different between COPD and controls (COPD total 207 ± 174 vs control 125 ± 50, $p=0.08$; COPD mild 186 ± 144 vs 126 ± 50, $p=0.1$; COPD moderate-severe 228 ± 210 vs 126 ± 50, $p=0.1$). As observed for percentage expression, neutrophil numbers were increased in COPD, although only reaching significance in the moderate-severe group (COPD total 19 ± 22 vs control 6 ± 12, $p=0.051$; COPD mild 16 ± 21 vs 6 ± 12, $p=0.1$; COPD moderate-severe 19 ± 23, $p=0.03$).

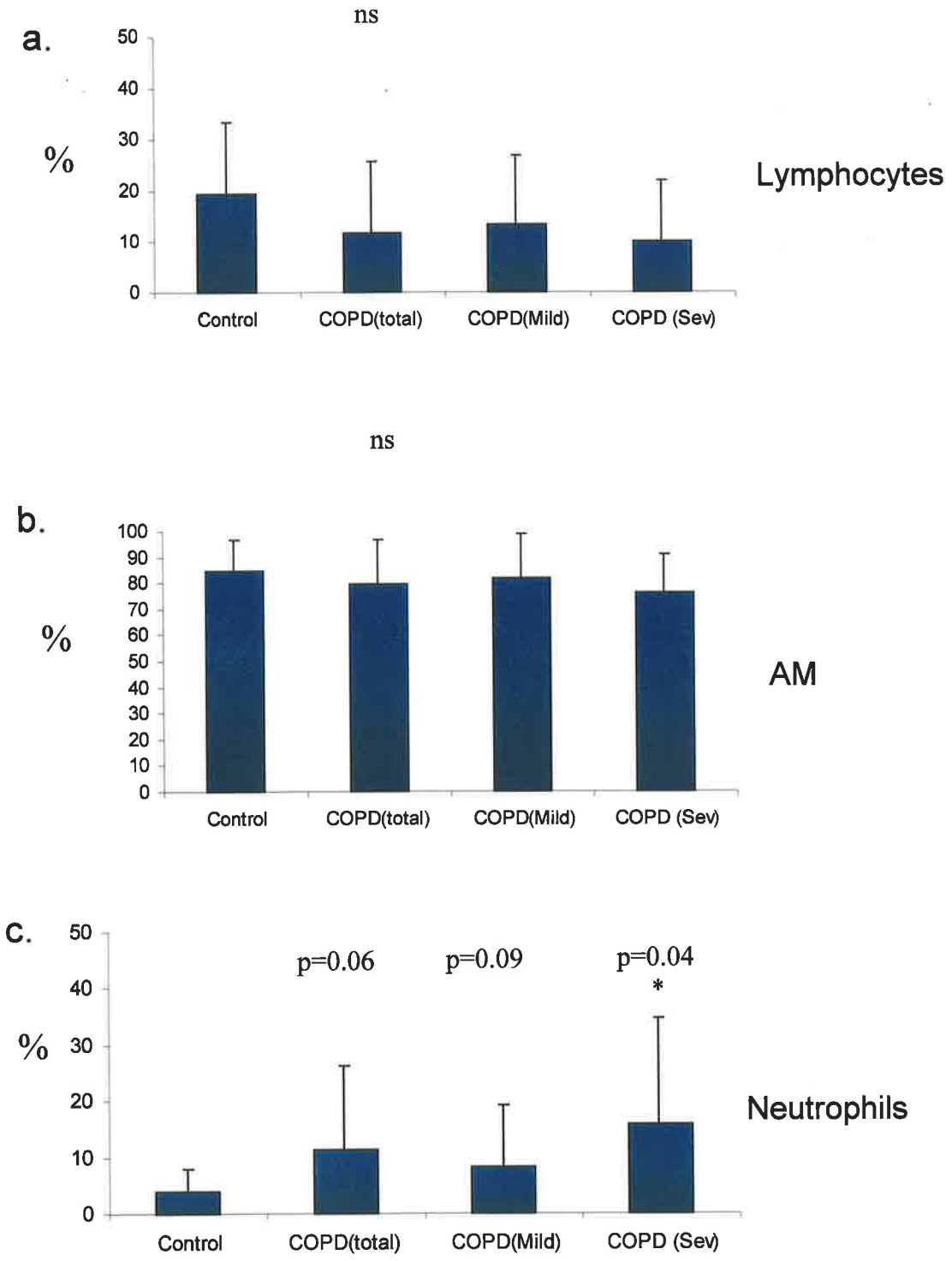


Figure 3-8 Differential cell counting of BAL-derived leucocytes from total (n=16), mild (n=8) and moderate-severe (n=8) COPD and control subjects (n=10)) (a) Lymphocytes (b) AM (c) Neutrophils. Results are expressed as mean values ± 2 SD. Note significantly increased numbers of neutrophils in BAL from moderate – severe COPD. * denotes significant p value ≤.05.

3-4 Discussion

A flow cytometric method for differential counting of cells from heterogeneous BAL and brushing-derived cell populations was applied. BAL- derived AM have increased autofluorescence properties that hinder analysis by flow cytometry due to spectral overlap between AM autofluorescence and fluorochrome emission spectra. Crystal violet was determined to be the most successful quenching agent with cell permeabilisation by FACSPerm or 0.1% η-Octyl β-D-galacto-pyranoside marginally improving the effects. Crystal violet is a dye that will get into cells fairly readily, and which, being violet, absorbs green light. This allows non-radiative energy transfer to occur between the green autofluorescence material and the crystal violet. However, since crystal violet is non-fluorescent, the transferred energy is lost non-radiatively. This method enabled cell type identification in BAL from smokers, and also allowed further staining for surface markers and cytokines, described in the next chapters.

Differential cell counts by manual counting and flow cytometry were compared. While there was good correlation between methods for lymphocyte and neutrophil counts, the correlation for AM counts was less satisfactory. Manual counting overestimated AM numbers compared with flow cytometry. Stained preparations of BAL showed numerous cells with necrotic changes, making cell type identification difficult. It is possible that some necrotic neutrophils or lymphocytes may have been classed as 'AM' on examination of stained BAL, due to the loss of obvious 'segmented nuclear' appearance of some necrotic neutrophils and loss of cell structure, as we have reported (Hodge et al, 1999). Manual counting marginally under-estimated the percentage of lymphoctes compared to flow cytometry. It is also possible that manual counting of 500 cells may underestimate cells present in small numbers (vs flow cytometric count of 50,000 events).

Consistent with reports by others (Lacoste et al. 1993; Thompson et al. 1989) I found no significant change in the precentage of AM or absolute AM numbers in the groups tested. These results are in contrast to reports of increased AM numbers in bronchial biopsy in COPD (O'Shaughnessy et al. 1997; Saetta et al. 1993).

Similarly, as found by others (Lacoste et al. 1993; Thompson et al. 1989), there were no significant changes in the percentage of lymphocytes or absolute numbers of lymphocytes in the present study.

COPD is associated with increased neutrophil numbers in BAL (Thompson et al. 1989). My results support these findings- both percentage and absolute numbers of neutrophils were higher in BAL from both moderate-severe and mild COPD patients compared with control subjects. However, the difference was only significant for moderate-severe COPD, In contrast to these findings in BAL, studies of bronchial biopsies in COPD have reported no significant changes in neutrophil numbers (Saetta et al. 1993). It thus appears that the inflammatory processes present in the airway lumen (investigated using BAL) do not reflect those in the airway wall (investigated using biopsy), with a higher percentage of neutrophils being present in the BAL and a predominance of AM and lymphocytes in the airway wall. This is supported by studies that showed no correlation between inflammatory cell numbers in BAL and biopsy (Maestrelli et al. 1995; Rutgers et al. 2000). A possible explanation for the discrepancy is the rapid migration of neutrophils across the tissue into the airway lumen, making increased neutrophil numbers undetectable by tissue analysis but detectable by BAL. It is also possible that cytokines, including GM-CSF and TGF-β, released by neighbouring AEC and AM into the airway lumen, may augment the prolonged survival of neutrophils in COPD (Lagraoui and Gagnon, 1997), and the production of TGF-β in COPD is evaluated in Chapter Five.

Chapter Four

Apoptosis in the airways in COPD

4-1 Introduction

Inflammation in COPD, initiated by factors including cigarette smoke, leads to recurring cycles of injury and repair of the peripheral airway walls. Disorders in the repair process can lead to tissue remodelling with altered structure and function (US Department of Health and Human Services, 2001).

Apoptosis contributes to immunoregulation and normal cell turnover in the lung. There is evidence, however, from studies of emphysema (Kasahara et al. 2001), pulmonary fibrosis (Hagimoto et al. 1997; Hagimoto et al. 1997), cystic fibrosis (Vandivier et al. 2002) and obliterative bronchiolitis in lung transplantation (Hansen et al. 2000), that excessive apoptosis and/or reduced clearance of apoptotic cells is associated with tissue damage and inflammation in the airways. Furthermore, tissue injury as a result of increased apoptosis has been demonstrated in Fas induced liver injury in mice (Ogasawara et al. 1993), chronic hepatitis C (Hiramatsu et al. 1994), eczma (Trautmann et al. 2000) and heart disease (Felzen et al. 1998).

Some factors associated with COPD have been shown to cause apoptosis of AEC. These include cigarette smoke, LPS (a contaminant of cigarette smoke), increased production of oxidants and activation of apoptotic pathways including Fas/Fas ligand

and TNFα/TNF receptor type I (Jyonouchi et al. 1998; D'Agostini et al. 2001; Vernooy et al. 2001).

There have been limited studies of apoptosis in COPD (Vignola et al. 1999; Segura-Valdez et al. 2000; Kasahara et al. 2001; Majo et al. 2001). Increased AEC and endothelial apoptosis has been reported in the alveolar septa in COPD, possibly contributing to the pathogenesis of emphysema (Kasahara et al. 2001). Increased apoptosis of AEC and lymphocytes has been described in a recent study of lung tissue from patients with COPD (Segura-Valdez et al. 2000). These studies have investigated apoptosis using airway tissue sections and immunohistochemical techniques. To my knowledge there have been no studies of apoptosis for discrete cell types from heterogeneous cell populations obtained from BAL and bronchial brushing in COPD.

I hypothesised that increased apoptosis would be detected in AEC from these heterogeneous cell populations in COPD.

4-2 Methods

4-2-1 Subject population

Samples of bronchial brushings and bronchial lavage (BAL) were obtained directly from human subjects undergoing fibre-optic bronchoscopy. The subject population, bronchoscopy procedure and preparation of ex vivo samples are described in the previous chapter.

4-2-2 Quantitation of apoptosis

The first stage of the study used three methods for the investigation of apoptosis of brushing- derived AEC. Cell type identification was performed as described in the previous chapter.

4-2-2(a) Annexin V staining

Flow cytometry was used as a sensitive, rapid method to study changes in cells associated with apoptosis.

In viable cells, phosphatidylserine (PTS) is only present on the inner layer of the plasma membrane. Early in apoptosis it is translocated to the outer membrane and can be identified by binding of Annexin V, which is a ligand for PTS. Its binding can be measured flow-cytometrically using FITC - conjugated Annexin V. Three ml of HEPES buffer (refer A-3-4) were added to 200μl aliquots of BAL and 100μl aliquots of bronchial brushing. Specimens were centrifuged at 500 × g for 5 min. FITC conjugated Annexin V (Pharmingen/BD, Ca, USA) (1μl) was added and the cells left for 15 min at room temperature in the dark, then washed again in 3ml HEPES buffer prior to aquisition by flow cytometry.

4-2-2(b) 7-AAD staining

Staining with 7-amino-actinomycin D (7-AAD) was used to quantify apoptosis. The plasma membrane of apoptotic cells is permeable to the dye, 7AAD, due to loss of membrane pump action. 7AAD staining can be measured by flow cytometry. Three ml of HEPES buffer was added to 200 μl aliquots of BAL and 100 μl aliquots of

bronchial brushing. Specimens were centrifuged at 500 × g for 5 min and cells stained for cell type identification as described in the previous chapter. After washing and discarding the supernatant, cells were re-suspended in 100 μl 2 μg/ml 7-AAD (Sigma, Australia) in HEPES buffer. 100,000 events from BAL and 20,000 events from the bronchial brushing were analysed by flow cytometry.

4-2-2(c) PI staining

Necrotic cells or cells late in apoptosis are characterised by fragmentation of DNA due to activation of endonucleases which cleave the DNA at nucleosomal sites. The plasma membrane of these cells is totally permeable to propidium iodide [PI], which intercalates between DNA base pairs (Darzynkiewicz et al. 1992). Cell staining can be analysed using flow cytometry by collecting FL2 data as PI emits in this (and FL-3) wavelengths. In contrast, the membrane of cells early in apoptosis, while still expressing PTS in the outer membrane is impermeable to PI. For staining with PI, cells were firstly stained with Mabs to surface markers as described in the previous section (using Mabs conjugated to FITC) and washed. After discarding the supernatant, cells were re-suspended in 5μg/ml PI (Sigma) in HEPES buffer and analysed by flow cytometry.

These three methods were compared using bronchial brushing-derived AEC from three subjects on three separate occasions.

To further compare Annexin V and 7AAD staining *in vitro*, AEC from an immortal epithelial cell line (16HBE) were prepared as described in the next chapter and stained for apoptosis using a combination of 7AAD and Annexin V. Experiments were performed using unstimulated cells (n=9) or those cultured in the presence of brefeldin A (n=5) (refer Chapter 5) or aFAS (500ng/ml, n=6) (refer Chapter 10).

4-2-3 Investigation of cell membrane damage during the bronchoscopy procedure

To investigate the possibility that removal of AEC from the basement membrane caused membrane damage during the bronchoscopy procedure, it was important to establish alternative methods for assessing apoptosis that did not rely on changes to the cell membrane. Two methods were used to detect these changes and results compared with those obtained using Annexin V and 7AAD staining.

Four normal controls were recruited to undergo bronchoscopy for this phase of the study (three females, one male, mean age 40, FEV1 and FVC greater than 90%). Written consent was obtained and bronchoscopy undertaken as described in 3-2-1 and 3-2-2.

4-2-3 (a) Staining of apoptotic cells with Mabs to single-stranded DNA

Antibody MAB3299 (F7-26, Chemicon, CA, USA) is specifically reactive with single-stranded DNA (ie, does not recognise DNA in double stranded conformations and is independent of internucleosomal DNA fragmentation). The antibody reacts specifically with deoxycytidine and requires a length of ssDNA of at least 25-30 bases in length for the binding. Importantly, Mabs to ssDNA are specific for apoptotic cell death and do not identify necrotic cells.

For preparation of working concentration of F7-26 Mab, 4.5 ml of 5% fetal calf serum in PBS was added to the vial containing 100μg (0.5 ml) of F7-26. The diluted antibody was aliquoted and stored at -20^0C. For testing, cell pellets were re-suspended in 1 ml cold PBS and 6 ml methanol (pre-cooled to –20°C) added, while

vortexing. Fixed cells (4 x 10^5) were transferred to 10ml culture tubes, centrifuged at 200 g for 5 minutes and supernatant removed. The fixed cells were stored at -20^0 for 1-3 days before staining. The pellet was re-suspended in 0.25 ml of 50% formamide (v/v distilled H_2O) and left for 5 minutes at room temperature. The rack containing the tubes was then immersed into a circulating water bath pre-heated to 75^0C for 10 min. After the heating, the rack was immediately transferred into room temperature water. Non-fat dry milk (2 ml of 1%) in PBS was added to the tubes containing formamide, followed by vortexing and standing at room temperature for 15 min. Following centrifugation and removal of supernatant, the pellet was re-suspended in 100μl of Mab F7-26 diluted to 1:10 and incubated at room temp for 15 min.
PBS (1 ml) was then added followed by centrifugation, re-suspension of the cell pellet in 100 μl of fluorescein-conjugated goat anti-mouse IgM (diluted 1:50 in 1% non-fat milk in PBS and frozen in aliquots at -20^0), and incubation for 15 min at room temp.
Prior to acquisition by flow cytometry, 1 ml PBS was added, tubes centrifuged and cell pellet re-suspended in 0.5 ml PBS.

4-2-3 (b) Staining of apoptotic cells with Mab to active Caspases

Sequential activation of caspases plays a critical role in the induction of apoptosis, as discussed in Chapter One. CaspACE FITC-VAD-FMK (Promega, WI, USA) is a FITC-conjugate of the cell permeable caspase inhibitor VAD-FMK. This structure allows delivery of the inhibitor into the cell where it binds to activated caspase, serving as a marker for apoptosis.

Washed cells (4 x 10^5/ml) were stained with 100μl 10μM CaspACE FITC-VAD-FMK In Situ Marker for 20 min in a 37^0C 5% CO_2 incubator. Cells were washed with *wash*

buffer then stained with surface markers to enable cell type identification as described in the previous chapter, then acquired by flow cytometry.

4-3 Results

4-3-1 Optimisation of techniques

4-3-1(a) Annexin V and 7AAD staining of AEC

There was good correlation between Annexin V staining and 7AAD staining for the detection of apoptosis of brushing- derived AEC (n=3) (**Figures 4-1**) and AEC from an immortal cell line, 16HBE (n=19 , R^2=0.8) (**Figure 4-2**).

Flow cytometry was thereafter applied to measure apoptosis of various cell types from BAL and bronchial brushing based on staining with 7AAD. This method was chosen for two reasons. Firstly, there was good correlation between 7AAD and Annexin V staining (**Figures 4-1, 4-2**). Secondly, 7AAD fluoresces in FL3 (unlike Annexin, which fluoresces in FL1). This property enabled FITC-conjugated markers (FL1) and PE-conjugated markers (FL2) to be concurrently used for cell subset identification.

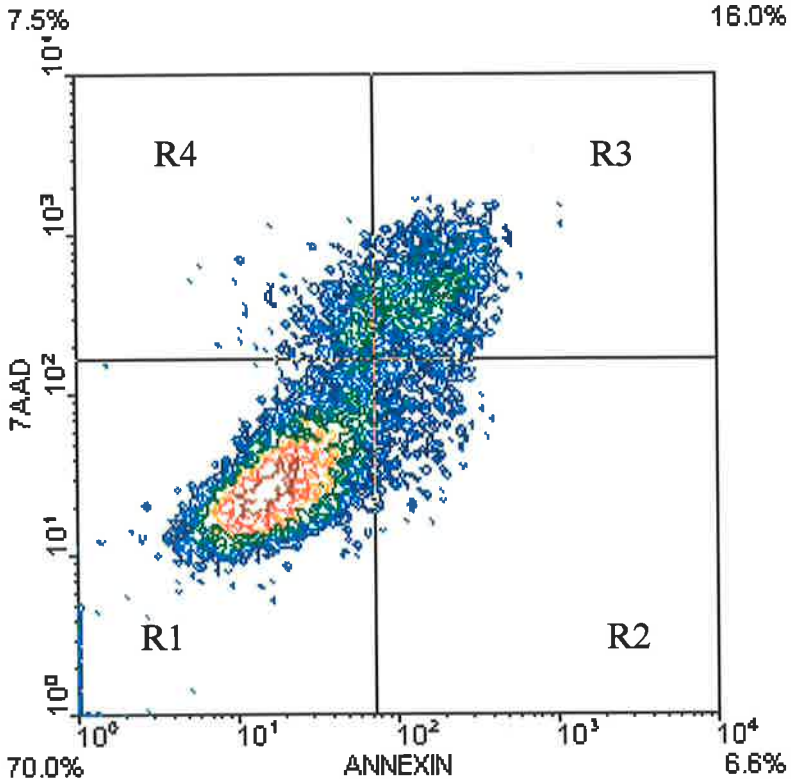


Figure 4-1

Validation of 7AAD staining of brushing-derived AEC.

Representative contour plot showing

(a) **Annexin V staining** (x axis) R1 = Viable cells (Annexin V negative) 70%; R2 + R3= apoptotic AEC (Annexin V bright) 22.6% (6.6 + 16%)

(b) **7AAD staining** (y axis) R5= R1 = Viable cells (7AAD negative) 70%; R3+R4= apoptotic AEC (7AAD bright) 23.5% (16 + 7.5%)

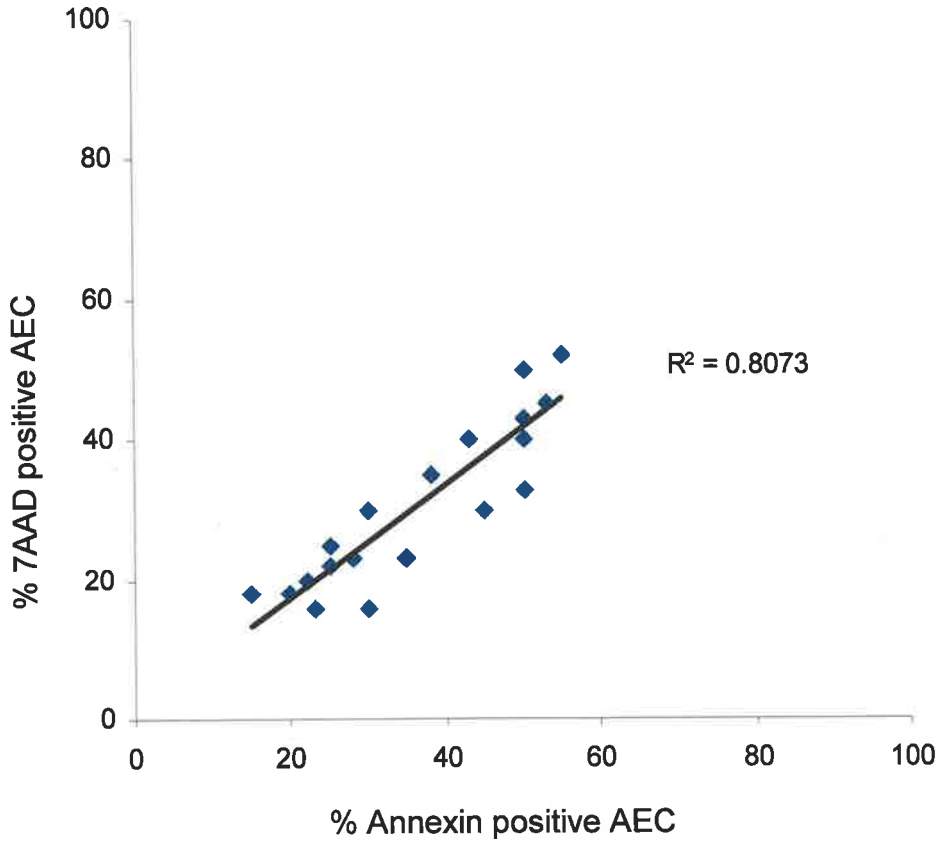


Figure 4-2

Correlation between Annexin + staining and 7AAD + staining for 16HBE AEC

Scatter Plot showing % Annexin positive AEC (x axis) vs % 7AAD positive staining (y axis)

(R^2=0.8; good correlation) (Pearson correlation analysis).

4-3-2 Apoptosis: BAL– derived cells

Based on several validation studies, 7AAD was used to investigate apoptosis of BAL- derived cells. Typical scatter patterns of FSC vs 7AAD are presented in **Figure 4-3**. The percentage of apoptotic neutrophils or AM in COPD patients and control subjects was not significantly different. The percentage of apoptotic lymphocytes was significantly higher for patients with COPD than control subjects (p=0.015) (**Table 4-1**).

4-3-3 Apoptosis: brushing- derived AEC

Brushing- derived AEC were identified by positive staining with epithelial antigen FITC. Leucocytes were excluded by CD45 PE positive staining. Apoptotic AEC were identified by FSC (x axis) vs 7AAD (y axis) staining to distinguish viable (7AAD negative staining) and apoptotic (7AAD bright staining) AEC (**Figure 4-4**).
The percentage of apoptotic AEC was significantly higher for patients with COPD (p=0.004) than control subjects (the difference was significant for total, mild (p=0.027) and moderate-severe (p=0.003) COPD groups) (**Table 4-1**).

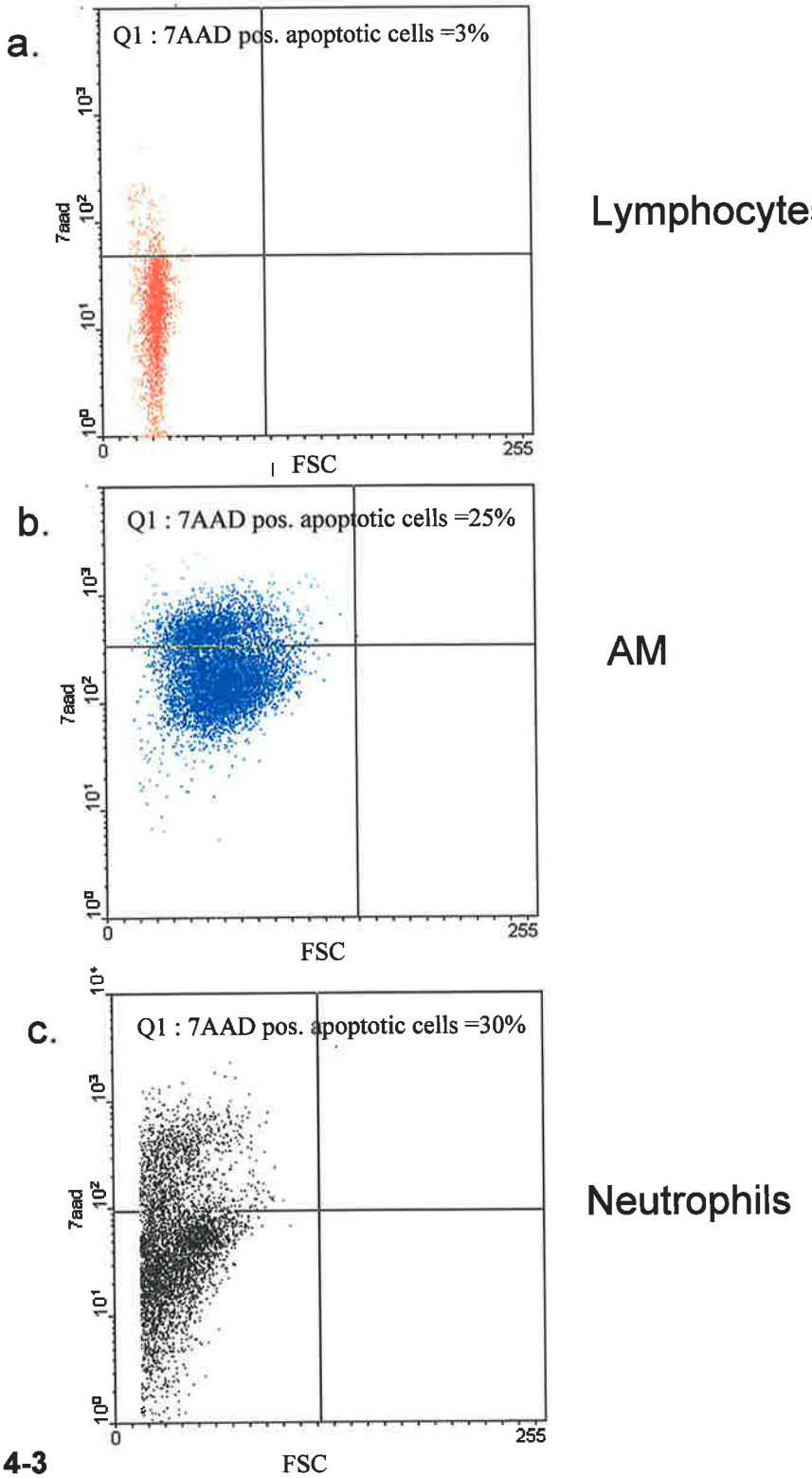


Figure 4-3

7AAD staining of apoptotic BAL- derived cells from control subject

Representative dot plots showing:

(a) Lymphocytes, (b) AM and (c) Neutrophils. The percentages of apoptotic cells were determined by bright staining with 7AAD (Quadrant 1 (Q 1)). Cells were initially gated in R1 on the basis of FSC and SSC characteristics to exclude debris, red blood cells and fragmented cells and cell types identified as outlined in Chapter 3.

% Apo. Cells	Control Subjects	COPD Total	COPD Mild	COPD Mod-severe	p value C/COPD
	N=11	N=16	N=9	N=7	
Lymphocytes	5.1 ± 1.2	19.8 ± 5.2 *	17.1 ± 7.3 *	22.6 ±7.5 *	0.015
AM	19.4 ± 2.6	25.8 ± 3.3	27.5 ± 5.1	22.3 ± 4.3	ns
Neutrophils	44.1 ± 7.9	35.8 ± 5.7	41.5 ± 9.2	30.3 ±5.1	ns
AEC	17.2 ± 4.1	36.4 ± 5.4 * (p=.004)	33.5 ± 7.6 *(p=.027)	36.0 ± 7.9 *(p=.003)	0.004

Table 4-1

Apoptosis of BAL- derived leucocytes and brushing- derived AEC in COPD.

Apoptosis from COPD patients (COPD) and control subjects (C) was measured by 7AAD staining. Results are expressed as mean values ± SEM. * denotes significant p value of ≤ 0.05.

Note significantly increased percentages of apoptotic AEC and lymphocytes in mild and moderate-severe COPD.

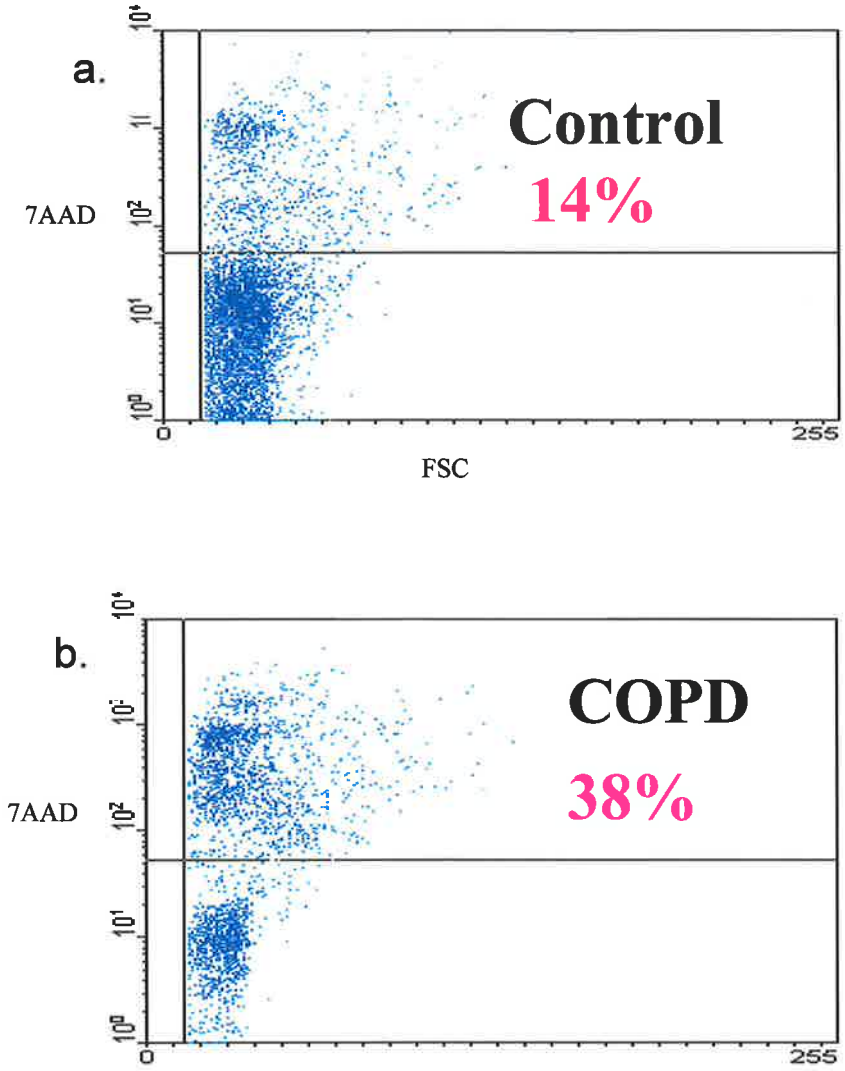


Figure 4-4

7AAD staining of apoptotic brushing- derived AEC

Representative dot plots showing

FSC (x axis) vs 7AAD (y axis) staining to distinguish viable (7AAD negative staining) and apoptotic (7AAD bright staining) AEC

(a) Control subject :14% apoptotic (b) COPD subject : 49% apoptotic.

4-3-4 Investigation of membrane damage during bronchoscopy

To validate results presented in this chapter, apoptosis of brushing-derived AEC from four healthy control subjects was measured by four separate techniques. Results are presented in **Figures 4-5, 4-6**. Higher rates of apoptosis were detected using methods that detect caspase activation and single stranded DNA (SSDNA) than methods that detect cell membrane changes (Annexin V and 7AAD). As COPD AEC would be substantially more "fragile" than AEC from normal subjects, similar testing was carried out on AEC from a group of six COPD subjects. The results were consistent with those obtained using AEC from control subjects: Positive staining with Annexin V 26.4% ± SD 7.2; 7AAD staining 32.0% ± SD 14.7; ssDNA 27.0% ± SD 15.6; Caspace 44.8% ± SD 16.6). These results indicate that artifactual membrane damage during the bronchoscopy procedure did not influence the rates of apoptosis presented in this chapter.

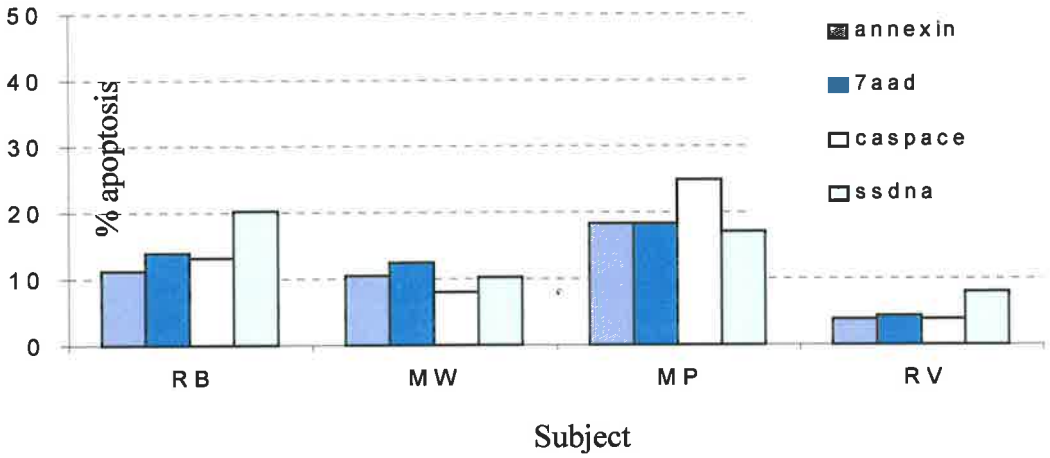


Figure 4-5 Apoptosis of brushing-derived AEC measured by different techniques

Apoptosis of brushing-derived AEC from four healthy control subjects, measured by:

(a) annexin V staining of PTS on the cell membrane

(b) 7AAD staining due to non-functional membrane pump action

(c) staining of i/cellular caspase activation in early apoptosis

(d) staining of i/nuclear SSDNA in early apoptosis

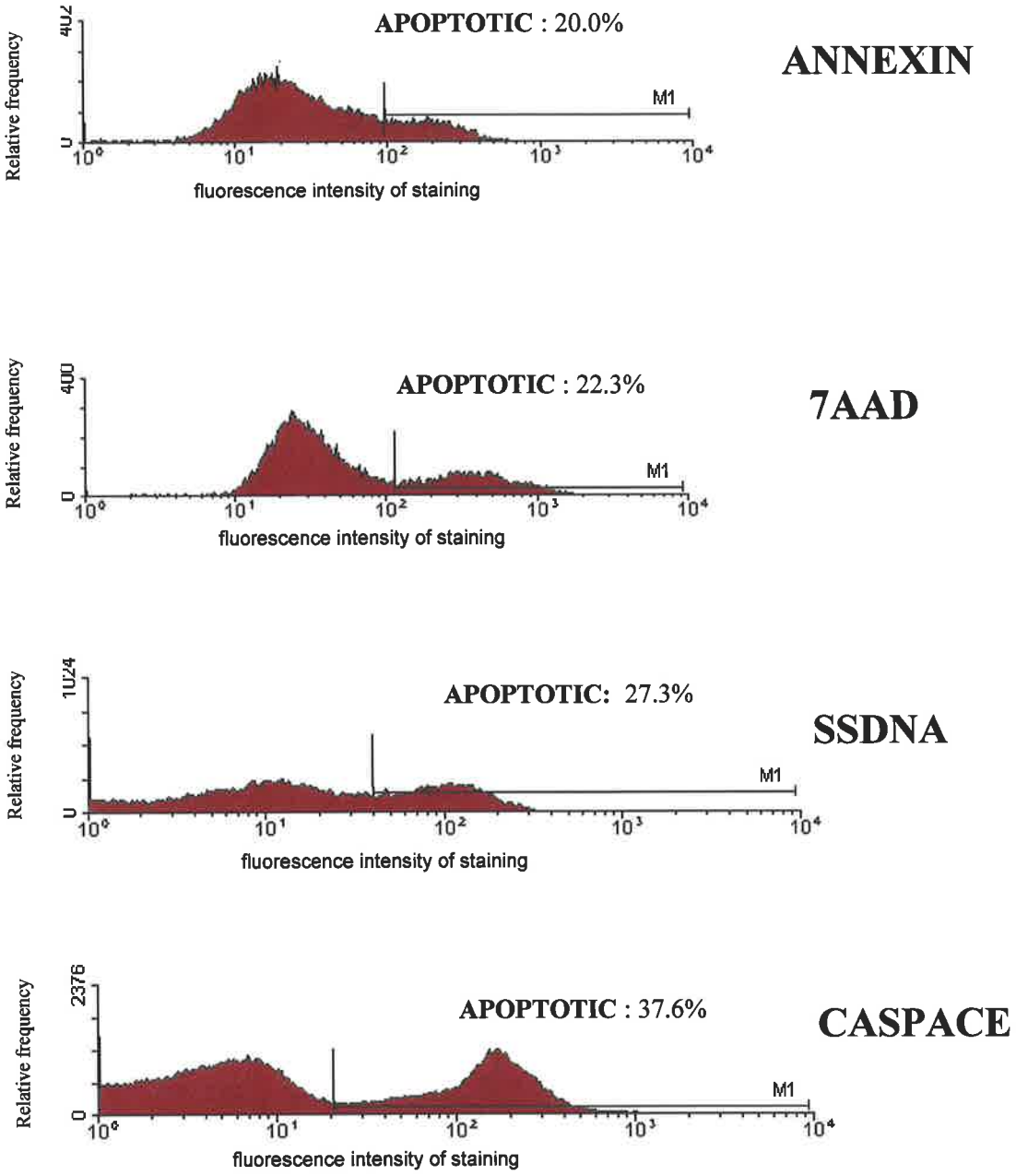


Figure 4-6

Apoptosis of brushing-derived AEC measured by different techniques

Representative histograms showing lower percentage staining for methods that detect cell membrane changes (Annexin V and 7AAD) and higher percentage staining for methods that detect single stranded DNA (SSDNA) and caspace activation (Caspace), indicating actual apoptosis rather than artifactual membrane damage.

4-4 Discussion

The simultaneous identification of cell types and apoptosis in BAL and bronchial brushing enabled investigation of apoptosis in COPD patients, and control subjects. To my knowledge, apoptosis has not previously been quantified in discrete leucocyte subsets from heterogenous cell populations obtained from the lungs of patients with COPD. Flow cytometry allowed easier quantitation of apoptosis in these discrete cell types than previous studies using immunohistochemical staining of tissue sections.

Apoptosis of BAL-derived lymphocytes and brushing- derived AEC was increased in COPD subjects compared with controls.

Disruption of epithelial cells from the basement membrane has been reported to induce apoptosis (Frisch and Francis, 1994). In addition, the local anaesthetic, lignocane, has been reported to diminish epithelial cell viability, *in vitro* (Kelsen et al. 1992). The two techniques originally applied to measure apoptosis in this study (Annexin V and 7AAD) relied on changes to the cell membrane. To investigate whether cell removal or use of lignocaine (used at a minimum dose, 100mg) caused artifactual membrane damage during the bronchoscopy procedure, I further applied techniques to specifically detect apoptosis based on changes that did not involve the cell membrane (binding to single stranded DNA and activation of caspases). The results obtained validated the results obtained using Annexin V and 7AAD, and show that AEC from both normal subjects and COPD patients did not suffer overt artifactual damage from the bronchoscopy.

Enhancement of oxidative stress and release of enzymes by neutrophils and AM in COPD, can destroy connective tissue in the airways and also provoke death by apoptosis (Rahman et al. 2002). TGF-β induces apoptosis in AEC (Hagimoto et al. 2002) and lymphocytes (Andjelic et al. 1997). TGF-β also has the ability to auto-induce its own production (Kim et al. 1989). It is therefore possible that increased production of TGF-β in COPD (by these autocrine and paracrine mechanisms) (refer to next chapter) may contribute to the increased apoptosis of AEC and result in cell lysis and tissue destruction in COPD.

There was a trend for reduced apoptosis of BAL- derived neutrophils in the COPD group (although this difference was not statistically significant). This difference may be due to the effects of TGF-β, which augments survival of neutrophils (Lagraoui and Gagnon, 1997). Increased survival of neutrophils may be a problem in COPD, as release of neutrophil contents has the potential to cause tissue injury and amplify inflammation by degradation of matrix proteins into chemotactic fragments (Haslett et al. 1989). COPD is associated with an increase in neutrophil numbers, a finding that was supported in this study. Neutrophil presence has also been reported to induce apoptotic cell death in AEC after ozone exposure *in vitro* (McDonald and Usachencko, 1999), and may contribute to the increased apoptosis of these cells in COPD.

Apoptosis of BAL-derived lymphocytes was increased in the COPD group compared to controls. TGF-β has been shown to induce apoptosis of T-cells, and its increased production in COPD (investigated in the following chapter) may contribute to the increased rates of apoptosis of T-cells observed in this study.

Several mechanisms may contribute to the failure to resolve epithelial damage in COPD. I have now shown that the defective repair may result from increased rates of apoptosis of AEC and lymphocytes in the airway. Failure to clear these cells rapidly may result in secondary necrosis with release of toxic cell contents, tissue damage and chronic inflammation (Hagimoto et al. 1997; Kuwano et al, 1999). To verify that secondary necrosis is present in the airways in COPD, future studies will investigate the release of lactate dehydrogenase (LDH) in BAL samples. When the cells of a tissue become damaged or necrotic, their contents leak out into the extracellular fluid. Thus the existance of secondary necrosis in the airways can be inferred by the presence of LDH in the BAL. Although apoptosis of AEC plays an active role in reducing inflammation and repair of acute epithelial injury in the normal lung, increased apoptosis and defective clearance may result in chronic inflammation and defective repair in COPD. To further investigate the findings in this chapter, in Chapter Five I describe studies which were undertaken to investigate the production of cytokines and surface molecules, known to be involved in the apoptotic process. In Chapter Six, I clarify the role of AM in the clearance of apoptotic cells in COPD.

Chapter Five

Cytokines and mediators in the airways in COPD

5-1 Introduction

Destruction of the extracellular matrix and loss of cells critical to tissue architecture, such as AEC, are prevalent in COPD (Nakamura et al. 1995; Carnevali et al. 1998). In the previous chapter, increased apoptosis of AEC in COPD was investigated. This chapter describes studies to further characterise the cytokines and surface molecules involved in signalling and modulation of increased rates of apoptosis and in defective repair processes in the airway epithelium in COPD. The hypothesis that increased activation of apoptotic pathways can be detected in AEC and in AM from heterogenous cell populations derived from BAL and bronchial brushings from patients with COPD was investigated. Flow cytometry was applied to measure synthesis of cytokines and evaluate the potential of cells to produce cytokines. Anti-cytokine Mabs provide a tool for multi-parametric flow cytometric analysis of individual cytokine-producing cells within unseparated cell populations in samples such as whole blood or BAL. Some form of stimulation is genererally required for detection of cytokine synthesis by flow cytometry. In this study, stimulation with a polyclonal activator (PMA), which directly activates protein-kinase C, and *E-coli* LPS, a patho-physiological stimuli for monocytes/macrophages were applied.

As described in Chapter One, apoptosis can be induced by extrinsic mechanisms such as binding of cytokines (eg, TNF-α and TGF-β) to their cognate receptors on

the cell surface, or binding of 'death ligands' to the corresponding receptor eg, Fas ligand (Fas L) to Fas (Blobe et al. 2000; Hagimoto et al. 2002). All of these pathways activate intrinsic cell pathways that result in a cascade of proteolytic caspases and the other changes leading to apoptotic cell death. Although increased production of TNF-α in the airways in COPD has been established (Keatings et al. 1996; Eid et al. 2001; Aaron et al. 2001; Qvarfordt et al. 1998; Takabatake et al. 2000), reports of changes in TGF-β production have been variable (Aubert et al. 1994; Vignola et al. 1997; de Boer et al. 1998, Takizawa et al. 2001, Buhling et al, 1999).

IL-8 production by AEC and AM and IL4 production by AEC in COPD was also investigated. Increased production of IL-8 and IL-4 has been reported in COPD (Jeffery, 1999) and lung injury (Jain-Vora et al. 1997). It is possible that IL-4 contributes to increased AM and T-cell numbers in COPD as IL-4 increases AEC expression of chemokines including IL-8 (Striz et al. 1999), and adhesion molecules such as ICAM-1 (Tosi et al. 1992) which are increased in COPD (Riise et al. 1994 (1)). These adhesion molecules are thought to contribute to increased AM numbers in the airway wall. In addition, IL-4 increases recruitment and proliferation of T-cells to the site of inflammation (Thornhill et al. 1990; Thornhill et al. 1991). In a baboon model, IL-4 increased T-cell infiltration into the skin when combined with an otherwise ineffective dose of TNF-α (Briscoe et al. 1992). IL-4 production has also been reported to be upregulated during lung injury and inflammation and to cause progressive pulmonary infiltration with AM, lymphocytes and neutrophils in transgenic mice (Jain-Vora et al. 1997).

To investigate changes in the production of cytokines and expression of cytokine receptors and surface molecules, I used flow cytometric techniques and ELISA to

analyse samples of AM obtained from BAL and AEC obtained from bronchial brushings from patients with COPD.

5-2 Methods

5-2-1 Study population

Patients undergoing clinically indicated fibreoptic bronchoscopy were invited to participate in the study, and informed consent obtained. The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital following the guidelines of the Declaration of Helsinki. BAL and bronchial brushings were collected from 16 patients with COPD (mean age 67). 11 control subjects with no evidence of COPD, asthma or allergy were used as controls (mean age 64) (**Table 5-1**). (There was some overlap of subjects enrolled in this study and those described in Table 3-1). The diagnosis of COPD was established using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (US Department of Health and Human Services, 2001) (studies described in the preceding chapters used the ERS criteria for diagnosis of COPD. In 2001, the Global Strategy for the Diagnosis, Management and Prevention of Chronic Obstructive Pulmonary Disease (**G**lobal Initiative for Chronic **O**bstructive **L**ung **D**isease: GOLD criteria) workshop report was published. The criteria for diagnosis of COPD contained in this report was thereafter employed in my studies).

Nine COPD patients were categorised as mild COPD ($FEV_1 \geq 70\%$, with clinical correlation) and 7 as moderate–severe COPD ($FEV_1 < 70\%$) (**Table 5-1**).

Table 5-1

Demographic characteristics of the population studied

Subjects	Control group	COPD (total group)	Mild COPD (FEV1≥70%)	Moderate-severe COPD (FEV1<70%)
No. of subjects	11	16	9	7
Age (y)	64 ± 16	66 ± 9	69 ± 8	60 ± 9
Smoking, pack yr	2.5 ± 9	67 ± 26	61 ± 24	83 ± 31
FEV1, % pred	91.9 ± 12.5	68.1 ± 21.1	82.6 ± 6.7	54.0 ±18.5
FVC, % pred	96.9 ± 9.4	82.5 ± 18.7	94.0 ± 17.0	75.6 ±13.7
FEV1 % FVC	79.4 ± 18.0	70.8 ± 13.7	73.9 ± 5.0	64.3 ±19.3
DLCO	96.0 ± 4.4	66.4 ± 27.4	69.0 ± 38.3	63.7 ±4.3

Results are expressed as mean values.

5-2-2 Bronchoscopy sampling

Brushings and BAL were obtained from COPD control subjects as outlined in the previous chapters. Patients underwent pulmonary function tests as part of their routine clinical assessment.

5-2-3 Preparation of samples

The two BAL specimens were pooled. The BAL-derived cells were pelleted by centrifuging at 200 x g for 10 min. The supernatant was discarded and cells re-suspended to 4 x 10^5 cells per ml with RPMI 1640 media (Gibco), supplemented with 10% fetal calf serum (Gibco) and 1% weight per volume penicillin/ streptomycin (Gibco) (hereinafter referred to as 'culture medium'), in 10 ml culture tubes. Cellular morphology was assessed as described in Chapter Three. Bronchial brushings were centrifuged at 200 x g for 10 min, supernatant was discarded and the cells re-

experiments to investigate intracellular cytokine production, brefeldin A (1μg/ml)(Sigma) was added as a 'Golgi block' to inhibit intracellular transport and thus retain cytokines produced during activation inside the cell. After 24h incubation in the presence of *E. coli* lipopolysaccharide (LPS) (Sigma) (1μg/ml), the supernatant was decanted and the cells resuspended in 2 ml RPMI 1640 medium. To investigate production of soluble TGF-β using ELISA, another batch was cultured without FCS or 'Golgi block', and 24h culture supernatant from bronchial brushing decanted and stored at -70^0C.

5-2-4 Cytokines and Mabs

Mabs used in the ex vivo investigation of cytokines and surface molecules are detailed in the Appendix. PE-CY5-conjugated Mab to the monocyte/macrophage marker CD14 (Pharmingen) was used to identify AM. This was supported by the addition of FITC-labelled CD45 (BD) in some tubes. PE-CY5-conjugated Mab to the leucocyte marker CD45 (Pharmingen) was used to identify leucocyte contamination in bronchial brushings. Anti-human TGF-β antibody (unconjugated) (R&D, Min, USA) and rat anti-mouse antibody, conjugated to PE (BD), was used for an indirect staining technique for detection of TGF-β. PE conjugated IgG1 Mab (BD) and unconjugated IgG1 (Dako, Denmark) were used as negative controls. All other Mabs to cytokines used in the study were directly conjugated to PE. Expression of IL-4R's α (CD124; Coulter Immunotech) and γ (CD132; Pharmingen), TNFRI (R&D) and Fas (Pharmingen) were investigated using PE-conjugated Mabs. TNFRII was measured using an FITC-conjugated Mab (R&D).

suspended to 4 x 10^5 cells per ml with culture medium in culture tubes. In

5-2-5 Cell type identification and analysis of surface markers

For cell type identification and analysis of surface marker expression, 200μl aliquots of washed, unstimulated BAL and 100μl aliquots of washed, unstimulated, bronchial brushings were added to labeled 5ml FACS tubes. Surface staining with Mabs was performed as described in Chapter Three (**Figure 3-1**). For brushing-derived AEC, 10,000 events were collected and analysed by flow cytometry. For BAL, 50,000 events were analysed.

The following panels of Mabs were used in the study.

Table 5-2

Mabs used for surface marker analysis of BAL-derived AM

	FITC	PE	PE-Cy5
1)	CD45	CD124 (IL-4R)	CD14
2)	CD45	CD132 (IL-4R)	CD14
3)	TNF-RI	TNF-RII	CD14
4)	CD36*	CD95 (Fas)	CD14
5)	IgG1	IgG1	CD14

*Results discussed in Chapter 5

(b) Mabs used for surface marker analysis of brushing-derived AEC

	FITC	PE	PC-5
1)	ECA	CD124 (IL-4R)	CD45
2)	ECA	CD132 (IL-4R)	CD45
3)	TNF-RI	TNF-RII	CD45
4)	CD36*	CD95 (Fas)	CD45
5)	IgG1	IgG1	CD45

*Results discussed in Chapter 5.

5-2-6 TGF-β receptor expression

TGF-β receptor expression was investigated in cultured AEC lines and patient samples, using a commercial FLUOROKINE^{TM} kit (R&D), following the manufacturer's instructions, with minor modifications. Biotinylated recombinant (rh) TGF-β was supplied as 1.25 μg of lyophilised material to which 1ml of sterile distilled water was added and mixed gently until completely dissolved. As a negative control reagent, a protein soybean trypsin inhibitor was biotinylated to the same degree as the cytokine (5μg/ml). To measure TGF-β receptor expression, 200 μl cell suspension was added to labeled FACS tubes. Cell surface staining (ie, with CD14 to identify AM in BAL) was carried out as described above. Cells were re-suspended in 25 μl wash buffer. Biotintylated cytokine reagent (7 μl) was added to the cell suspension for a total reaction volume of 35 μl. As a negative staining control, an identical sample of cells was stained with 7 μl of biotinylated negative control reagent. Cells were incubated overnight at 4^0 C, after which time 7 μl of avidin-FITC reagent was added to each tube (Cells were not washed prior to adding avidin-FITC reagent). Cells were incubated for a further 30 minutes at 4^0 C in the dark, then washed twice with 2 ml of wash buffer to remove unreacted avidin-fluorescein, and resuspended in 50 μl of wash buffer for flow cytometric analysis. Avidin-FITC reagent and biotintylated cytokine reagent were titrated for optimal staining. Comparison of testing using wash buffer and commercial buffer (supplied with the kit) confirmed that identical results were obtained using either buffer (data not shown).

5-2-7 Cytokine production: analysis by flow cytometry

(a) Intracellular TNF-α, IL-4 and IL-8

Directly conjugated Mabs were used for measurement of intracellular cytokines by flow cytometry. Stimulated BAL that had been cultured for 24h were treated for 10 min with 20mM EDTA (Sigma), at 4^0C, to remove adherent monocytes from the polypropylene tubes. Brushing and BAL-derived cells were removed from culture tubes by gentle pipetting with an 'Ultrafine' 29-gauge needle (BD) (refer Appendix for validation of this method). Cell surface staining to identify cell types was carried out as described in Chapter Three (**Figure 3-1**). For intracellular cytokine staining, cells were permeabilised with 500 μl of FACS permeabilising Solution (BD) for 10 min at room temperature, then washed with wash buffer, centrifuged, and supernatant discarded. To block Fc receptors and reduce non-specific binding, 20 μl normal human immunoglobulin was again added to each tube for 30 min at room temp. Three microlitres of fluorescent-conjugated antibodies to intracellular cytokines TNF-α, IL-8 and IL-4 were added and the cells incubated again at room temperature for 30 min. After a further wash, acquisition was carried immediately by flow cytometry.

(b) Intracellular TGF-β

An indirect flow-cytometric staining technique was applied to measure intracellular TGF-β production by AEC from bronchial brushing and AM from BAL. Stimulated cells were treated with EDTA and removed from tubes as described above. Washed cells (200μl) were permeabilised with 500 μl of FACSperm (BD) for 10 min. Cells were washed with wash buffer, centrifuged and the supernatant discarded. Fc receptors were blocked and non-specific binding reduced by the addition of normal

human immunoglobulin as described above. After a further incubation for 30 min with unconjugated Mab to TGF-β (R&D), cells were washed with wash buffer and the blocking step repeated. Cells were incubated with PE-conjugated rabbit-anti-mouse Mab (Dako) for a further 30 min the washed with wash buffer. Surface staining for cell type identification using CD14 PE-CY-5 or CD45PE-Cy-5 was carried out as described above, cell washed then reconstituted to a total volume of 20 μl in wash buffer and events acquired immediately by flow cytometry.

5-2-9 Soluble TGF-β: analysis by ELISA

Culture supernatants were decanted and stored at -20^0 prior to testing. 'Active' TGF-β (ie, non-polymerised) was quantified using a Quantikine immunoassay kit (R & D) following instructions supplied by the manufacturer.

5-2-10 Flow cytometric analysis

Red blood cells and debris were excluded from Region 1 (R1) on the basis of FSC and SSC characteristics. All subsequent analysis was carried out on cells from R1. BAL-derived AM were identified by bright staining with CD14 PE-CY5 in R2 (**Figure 5-1**). Leucocyte contamination was excluded from the AEC gate (R2) by bright staining with CD45, as described in the previous chapter and shown in **Figure 5-2**. All subsequent analysis was carried out on cells from R1 and R2. The experiments were processed under identical settings and results expressed as a percentage of cells exhibiting positive fluorescence (eg, for PE-conjugated IL-4 in FL2). Analysis quadrants were based on less than 2% staining for the negative control (an irrelevant antibody of the same isotype). **Figures 5-1; 5-2** show details of gating procedures.

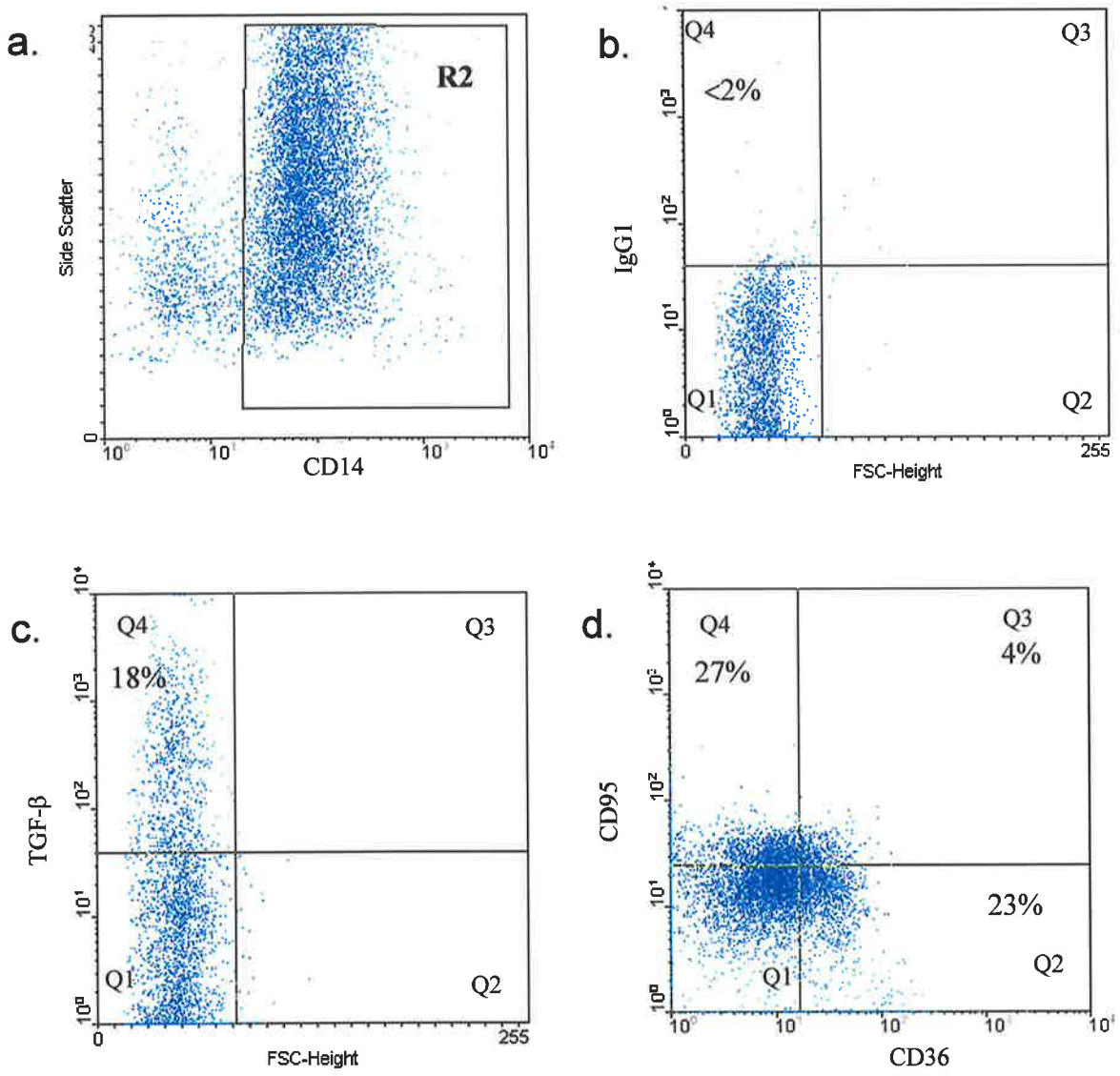


Figure 5-1

Flow cytometry gating strategies for BAL-derived AM

(a) AM were identified by bright staining with CD14 in Region 2 (R2) All subsequent analysis carried out on cells from this region.

(b) Analysis quadrants set on less than 2% staining for the negative control (an irrelevant antibody of the same isotype).

(c) Intracellular cytokine production:18% of cells produced TGF-β (Quadrant 4 (Q4))

(d) Surface marker expression: CD36 = Q2 + Q3 = 27%, CD95 (Fas) = Q4 + Q3 = 31%.

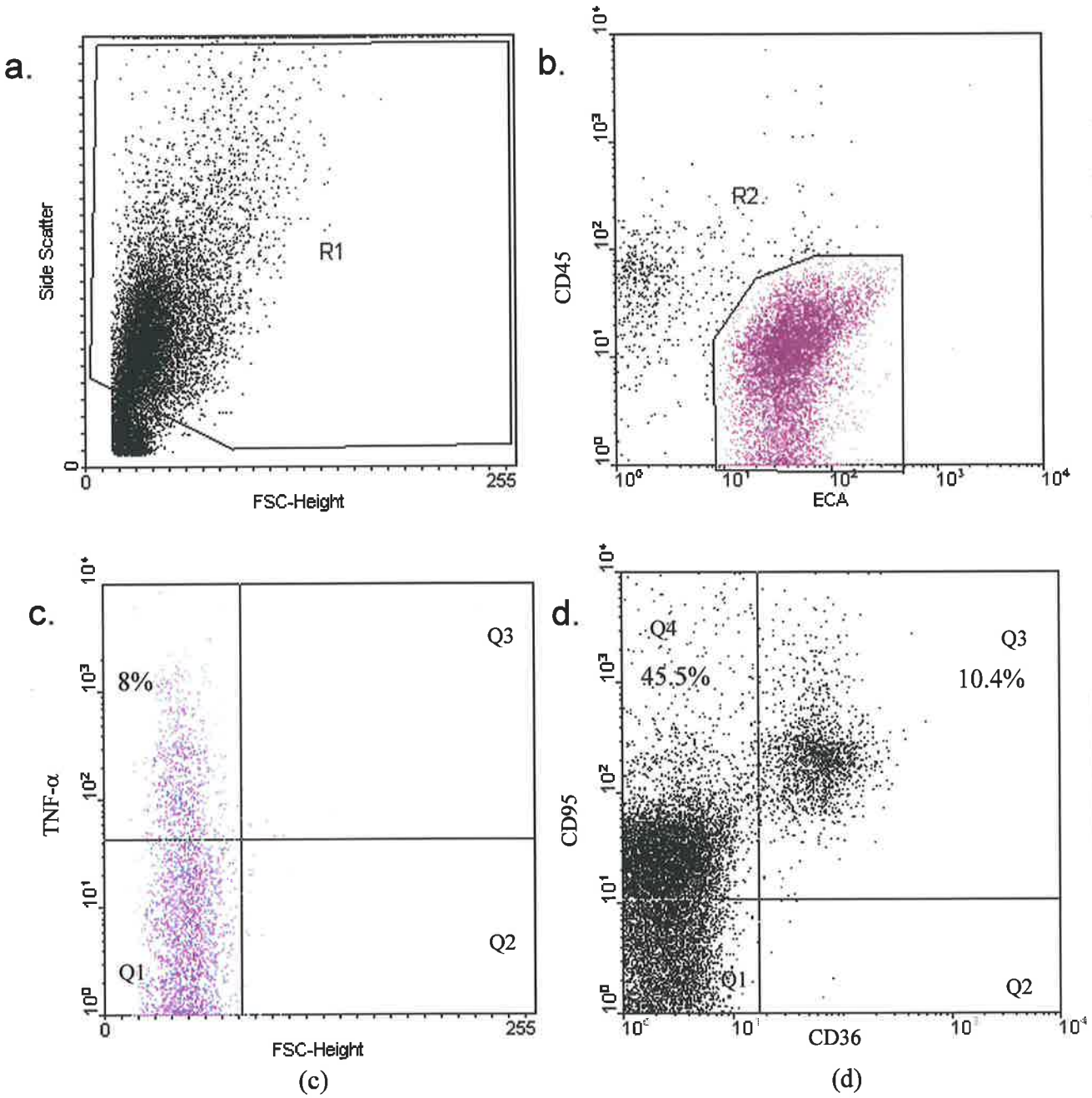


Figure 5-2 Flow cytometry gating strategies for bronchial brushing-derived AEC

(a) Red blood cells and debris were excluded from Region 1 (R1) based on FSC and SSC characteristics. All subsequent analysis carried out on cells from this region.

(b) Contaminating leucocytes identified by bright staining with CD45 and negative staining with epithelial cell antigen (ECA) and excluded from AEC in R2

(c) Intracellular cytokine: Quadrant 4 (Q4): 8% of cells produced TNF-α

(d) Surface marker expression: CD36 (Q2 + Q3) = 10.4%, CD95 (Fas) (Q4 + Q3) = 55.9% (Quadrants set on <2% staining with appropriate isotype controls).

5-3 Results

5-3-1 Intracellular cytokine production

A significantly greater percentage of AEC from COPD patients produced TGF-β (Total: active + latent), IL-4, IL-8 and TNF-α when compared with control subjects (**Table 5-3**). TNF-α, IL-8 and TGF-β production by AM was also significantly increased (**Table 5-3**).

Table 5-3

Cytokine production and Fas expression by AM and AEC in COPD

Cytokine	Cell type	Control n=11	COPD n=16	P value C/COPD
TNF-α	AM	10.3 ± 9.4	21.1 ± 14.5	.023 *
TGF-β		12.3 ± 9.9	28. 0 ± 15.7	.021 *
IL-8		28.3 ± 15.8	43.5 ± 22.5	.002*
TNF-α	AEC	2.1 ± 1.8	11.4 ± 10.5	.001 *
TGF-β		8.4 ± 6.8	14.6 ± 9.5	.027 *
IL-4		9.9 ± 6.0	16.7 ± 9.8	.010 *
IL-8		28.0 ± 23.5	52.0 ± 25.9	.035 *

Results (% positive cells) expressed as mean ± SD * significant difference from control group (Wilcoxon Test)

5-3-2 Soluble 'active' TGF-β release

Soluble active TGF-β secretion in COPD measured by ELISA was significantly higher for AEC from COPD patients when compared with control subjects (**Table 5-4**).

Table 5-4

Soluble active TGF-β secretion by AEC in COPD

Control (n=7)	COPD (n=6)	P value
812.6 ± 126.6	1701.6 ± 1318.4	.013 *

Results (pg/ml) expressed as mean ± SD

*significant difference from control group (Mann-Whitney Test)

5-3-3 Surface marker expression

Brushing-derived AEC from COPD patients expressed Fas (CD95) at a higher frequency than control subjects (86.6% vs 74.7 %, **Figure 5-3**). Fas expression by AM was not significantly different for COPD and control groups. There was no difference in expression of TNF-R's, TGF-R, IL-4R (CD124 and CD132) for AEC or AM in COPD (**Table 5-5**).

Table 5-5

Cytokine receptor expression by AM and AEC in COPD

Receptor	Cell type	Control n=11	COPD n=16	P value C/COPD
TNF-RI	AM	5.5 ± 1.7	5.7 ± 6.6	Ns
TNF-RII		9.7 ± 7.7	8.4 ± 4.6	Ns
TGF-R		4.9 ± 4.4	2.8 ± 1.5	Ns
IL-4Rα (CD124)		3.3 ± 4.1	5.8 ± 8.8	Ns
IL-4Rγ (CD132)		4.6 ± 2.7	5.0 ± 6.9	Ns
TNF-RI	AEC	5.3 ±3.4	4.1 ±3.4	Ns
TNF-RII		6.2 ± 4.6	8.3 ± 9.4	Ns
TGF-R		12.2 ± 8.0	18.4 ± 16.9	Ns
IL-4Rα (CD124)		9.3 ± 6.1	7.5 ± 6.2	Ns
IL-4Rγ (CD132)		2.8 ±3.3	4.6 ±3.5	Ns

Results (%) expressed as mean ± SD * significant difference from control group

(Mann-WhitneyTest)

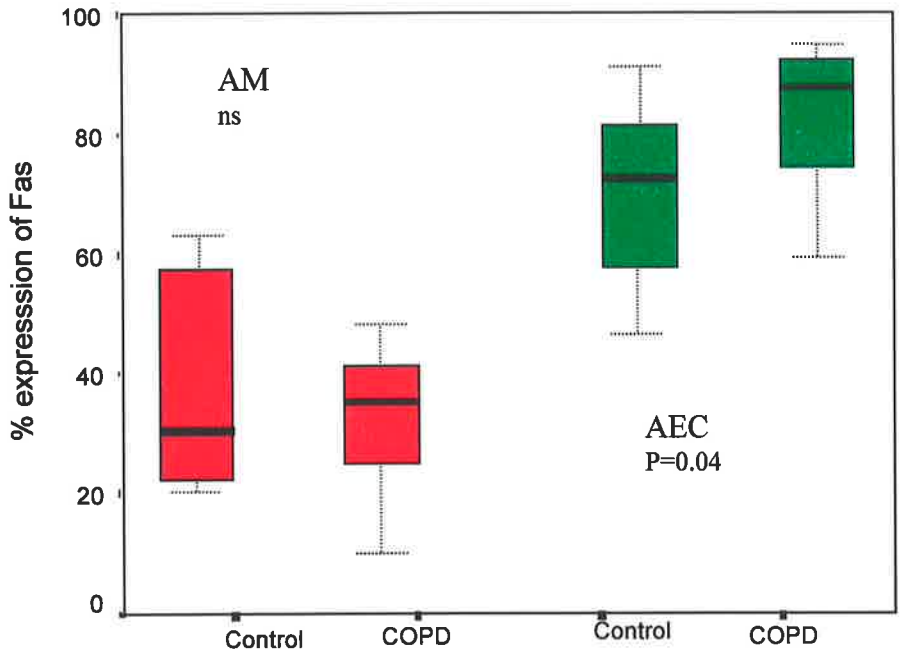


Figure 5-3

Fas expression by AEC and AM in COPD. Note significantly greater expression of Fas by AEC but no significant increase in expression by AM (Mann-Whitney Test; chart shows median ± 2SD; broken lines show data spread)

5-4 Discussion

Cytokine production, cytokine receptor and Fas expression have not previously been quantified in discrete leucocyte subsets from heterogenous cell populations obtained from the airways of patients with COPD.

Increased production of cytokines IL-4, IL-8, TGF-β and TNF-α by AEC and increased production of IL-8, TGF-β and TNF-α by AM were detected in COPD. There have been a number of reports of increased TNF-α production in COPD (Keatings et al. 1996; Soler et al. 1999; Eid et al. 2001; Aaron et al. 2001; Takabatake et al. 2000). TNF-α is widely thought to contribute to the pathogenesis of COPD. My studies show increased rates of apoptosis of AEC in COPD (Chapter Four), findings consistent with reports of others (Kasahara et al. 2001; Segura-Valdez et al. 2000). TNFα exerts pro-apoptotic effects by binding to its type I receptor on AEC (Krunkosky et al. 1996). TNFα also induces cell activation and anti-apoptotic effects by binding to TNF-R II. Using flow cytometry I did not observe any significant changes in expression of TNF-Rs in this study. However, detectable levels of these receptors were very low. Alternative methods of measuring expression (eg, enzyme amplification techniques or indirect biotin-streptavidin staining which increase sensitivity of detection by flow cytometry (Zola et al. 1992)) may be warranted for further studies.

Reports of TGF-β production in the airways in COPD have been variable (Aubert et al. 1994; Vignola et al. 1997; de Boer et al. 1998, Takizawa et al. 2001; Buhling et al, 1999). My studies show increased production of total (active and latent) TGF-β by AEC and AM in COPD as well as an increase in active secreted TGF-β. As

discussed in Chapter 1, TGF-β is known to induce apoptosis in AEC (Hagimoto et al. 2002) and its increased production may contribute to the high rates of apoptosis of these cells in COPD.

The increased IL-8 production noted in the airways of COPD subjects in this study is consistent with numerous previous reports (Jeffery, 1994; Hill et al. 1999; Keatings et al. 1996; Pesci et al. 1998; Yamamoto et al. 1997). However, IL-8 production has not previously been quantified in discrete leucocyte subsets from heterogenous cell populations obtained from the airways of patients with COPD. I have now shown that the increased IL-8 in the airways in COPD is a result of its increased production by both airway epithelial cells and AM. IL-8 can damage airway structure and potentiate the inflammatory response by stimulating the release of chemotactic factors (including MCP-1 and IL-8) (Senior et al. 1980; Kwon et al. 1994), and therefore may a significant role in the chronic inflammation that is characteristic of COPD.

CD95 (APO-1; Fas) is activated to induce apoptosis following interaction with its natural ligand (Fas L) (Kuwano et al. 2000). The regulatory role of Fas-initiated apoptosis in T-cells is well recognised. Consistent with previous reports (Hamann et al. 1998; Amsellem et al. 2002) I observed high Fas expression by AEC, suggesting that the Fas pathway may be involved in apoptosis of AEC cells in COPD. These observations are also consistent with a report of a link between upregulation of Fas and apoptosis of alveolar epithelial cells and lymphocytes in bleomycin-induced pulmonary fibrosis in mice (Hagimoto et al. 1997). Furthermore, a recent report has shown that hydrogen peroxide induces Fas upregulation in AEC (Fujita et al. 2002). As increased levels of exhaled hydrogen peroxide have been reported in COPD (Dekhuijzen et al. 1996) there may be a link between this reactive

oxygen species and increased Fas in AEC, leading to increased apoptosis of AEC. In addition, activation of the Fas/FasL pathway induces apoptosis of mature CD4+ T-cells after repeated antigenic stimulation (Singer et al. 1994), thus the raised levels of Fas I report here may be one cause of the increased lymphocyte apoptosis in the airway in COPD, reported in Chapter 4.

Under normal conditions, apoptosis is followed by rapid phagocytosis of apoptotic cells by macrophages/monocytes. We can speculate that an increased rate of apoptosis of AEC in the airways in COPD may outweigh the phagocytic capacity of AM and result in defective clearance of apoptotic cells. Impaired capacity for phagocytic clearance may lead to accumulation of apoptotic cells, disintegration and tissue damage (Ogasawara et al. 1993).

IL-4 production by AEC from COPD subjects was increased relative to controls in the present study. IL-4 plays an important role in promotion of tissue adhesion and mediating inflammation. IL-4 upregulates production of the chemokine IL-8, increases expression of the adhesion molecule ICAM-1 and acts with TNF-α to induce expression of VCAM-1 on vascular endothelial cells, thus facilitating the recruitment of T-cells to the inflamed lung (Ying et al. 1997; Thornhill et al. 1991). IL-4 can also prolong the survival of cultured T-cells (Hu-Li et al. 1987). It is therefore possible that this cytokine plays a role in COPD by increasing AM and T-cells numbers in the airway wall.

As presented in the previous chapter, COPD is associated with an increase in neutrophil numbers in the airway lumen. The presence of neutrophils has been reported to induce apoptotic cell death in AEC after ozone exposure *in vitro* (McDonald and Usachencko, 1999), and may contribute to increased apoptosis of these cells in COPD. Furthermore, increased TGF-β release in the airways, as found

in the present study, may augment the already prolonged survival of neutrophils in COPD (Lagraoui and Gagnon, 1997).

In summary up-regulation of Fas, TNF-α and TGF-β in the airways in COPD suggested that these apoptotic pathways are active in COPD. The defects in repair noted in this debilitating disease may result from an imbalance between tissue defense mechanisms, such as apoptosis and phagocytosis, and inflammatory mediators.

In Chapter Six I report an investigation of the phagocytic clearance of apoptotic AEC by AM in COPD.

Chapter Six

Phagocytosis of apoptotic AEC by AM in COPD

6-1 Introduction

Cell turnover, repair of injured epithelium and resolution of inflammation in the normal lung are highly regulated processes whereby unwanted AEC and inflammatory leucocytes are removed once the repair process is complete. Although the cellular mechanisms for resolution of excess AEC (as well as fibroblasts and endotheial cells) have not been elucidated, the process most likely involves apoptosis followed by phagocytosis without disruption of neighbouring tissue (Bitterman et al. 1994; Polunovsky et al. 1993).

Excessive apoptosis and/or reduced clearance of apoptotic cells is associated with tissue damage and inflammation in the airways (Kasahara et al. 2001; Hagimoto et al. 1997 (1); Vandivier et al. 2002(1); Hansen et al. 2000), liver (Ogasawara et al. 1993; Hiramatsu et al. 1994), skin (Trautmann et al. 2000) and heart (Felzen et al. 1998). While a single episode of inflammation may resolve quickly, a second episode may result in progressive tissue damage, possible due to a reduced capacity to remove apoptotic cells (Johnson R. 1994)

There have been few studies on phagocytic ability of AM or peripheral monocytes in COPD (Ferrara et al. 1996; Meloni et al. 1996; Prieto et al. 2001). These studies have used opsonised yeast, *E. coli* or *Candida albicans* as the targets for

phagocytosis, mostly assessed by subjective manual counting techniques. Flow cytometry has been applied to assess AM phagocytic function in asthma, again using opsonized yeast as targets (Alexis et al. 2001).

To assess the role of AM in airway repair, the use of apoptotic AEC as phagocytic targets would be more physiologically relevant for the study than previous methods. A comparison of phagocytosis of non-physiological targets, such as polystyrene microbeads, would enable detection of specific defective AM phagocytosis of apoptotic AEC in COPD. Furthermore, flow cytometry may allow more accurate quantitation of the percentage of AM which have ingested apoptotic cells whem compared to manual counting methods.

I hypothesised that the accumulation of apoptotic material in COPD (described in Chapter Four) may result from defects in AM function, AM recognition of apoptotic cells or a combination of these processes.

To investigate this hypothesis, I developed an *ex vivo* flow cytometric assay to quantify phagocytosis of apoptotic AEC by AM. This novel technique was applied to study the phagocytic capacity of AM from BAL obtained from COPD patients and control subjects.

6-2 Methods

6-2-1 Study Population

Patients undergoing fibre-optic bronchoscopy for clinically indicated reasons were invited to participate in the study and informed consent obtained as described in Chapter Two. BAL was collected from 6 current non-smokers with COPD (mean age 68) (**Table 6-1**). For 8 never-smokers, with no history of COPD, asthma or allergy and undergoing bronchoscopy for other clinically indicated reasons, specimens were obtained and used to establish optimum techniques and as controls (mean age 69) (**Table 6-1**). A further group of five recruited healthy never-smokers were also tested. The diagnosis of COPD was established using the GOLD criteria (US Department of Health and Human Services, 2001). BAL was obtained in accordance with international published guidelines as described in Chapter Three. Aspirated BAL was immediately transferred to 50ml polypropylene tubes (to avoid attachment of cells to the polystyrene plastic trap) and kept on ice. Cells were processed within one hour of collection.

Table 6-1

Demographic characteristics of the population studied

Subjects	Control	'Recruited normals'	COPD
No. of subjects	8	5	6
Age (y)	69.3 ± 7.5	40 ± 12.8	68.0 ± 11.4
Smoking, pack yr	0	0	36.0 ± 21.0
FEV1, % pred	90.7 ± 21.5	107.0 ± 15.0	63.6 ± 9.1
FVC, % pred	94.7 ± 23.7	93.3 ± 9.7	83.9 ± 15.9
FEV1, % FVC	80.3 ± 10.3	115.8 ± 5.0	55.7 ± 11.7

Results are expressed as mean values ± SD.

6-2-2 Cell culture and preparation of cells

A human transformed normal bronchial epithelial cell line (16HBE14$_o$-[160], a gift from Dr. D. Greunert, University of California at San Francisco) (Cozens et al. 1994) was used for the phagocytosis assay. The cell line was maintained in culture medium. Cell cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were plated in 25cm^2 tissue culture plates (Corning - Costar, New York, USA) and experiments were carried out between passages 20 and 45. Cells were passaged by treatment with 2 ml .05% trypsin and re-seeded when 80% confluent.

6-2-3 Optimisation of methods

6-2-3(a) Optimal methods for inducing apoptosis

For use as targets in the phagocytosis assay, 16HBE AEC were induced to undergo apoptosis. Preliminary experiments were carried out by subjecting cells to UV radiation for 5min, 10min, 15min and 20min using a 305nm transilluminator UV source (UVP, Ca, USA), as well as aFAS (500ng/ml) (Sigma) for 48h and 10^{-5} dexamethasone (David Bull Laboratories, Australia) for 20h. Staining was also carried out with PI to detect 'necrosis' (nuclei that have passed through necrosis and lost their cytoplasm), evident by PI positive and Annexin negative staining (Vermes et al. 2000) as described in Chapter Four.

6-2-3(b) Reproducibility of apoptosis induction by UV radiation

To investigate reproducibility, AEC were exposed to UV radiation for 20min on nine separate occasions. The percentage of apoptotic cells was assessed by staining with Annexin V and flow cytometry as described in Chapter Two.

6-2-3(c) Reproducibility of Phagocytosis Assay

To assess the variability, the assay was carried out in quadruplicate on five separate occasions. Standard Error was calculated as SD/ $\sqrt{}$ N.

6-2-3(d) Time frame experiments for phagocytosis

To establish the optimum time frame for maximum detection of phagocytosed AEC (before collapse and digestion of the apoptotic cells), the assay was carried on two separate occasions for 1h, 1½h and 2h, in triplicate.

6-2-4 Phagocytosis Assay

6-2-4(a) Induction of apoptosis and staining of AEC targets

For use as targets in the phagocytosis assay, 16HBE AEC were induced to undergo apoptosis by exposure to UV radiation for 20 min. Cells were removed from the plates by treatment with 2 ml of 0.05% trypsin for 5 min at 37^0C until they had rounded and loosened. Cells were released from the flask with a sharp tap, then trypsin was inhibited by adding 20 ml culture medium. Cells were centrifuged at 500 x g for 5 min then re-suspended in 10 ml culture medium. The AEC were stained

with 50 μl mitotracker green (MTG) (Molecular Probes, OR, USA) (25μg/ml) for 15 min then washed twice in wash buffer. For some experiments, cells were stained with 5 μl anti-epithelial cell antigen Mab (ECA, Dako). Stained cells were re-suspended in culture medium to 10x the AM cell count, then 2 ml aliquots added to the AM monolayers for quantitation of phagocytosis as described below.

6-2-4(b) Confirmation of apoptosis with Annexin V and PI

To confirm that >80% of AEC were apoptotic before use as targets in the phagocytosis assay, aliquots were stained with FITC labelled Annexin V (Pharmingen) and Propidium iodide (PI, Sigma) and analysed using flow cytometry as described in Chapter Four.

Briefly, three ml of HEPES buffer were added to 200 μl aliquots of UV treated AEC. Specimens were centrifuged at 500 × g for 5 min. Supernatant was discarded and 2 μl FITC-conjugated Annexin V added to the cell pellet and mixed. After 15 min at room temperature in the dark, cells were washed again in 3ml HEPES buffer and re-suspended in 100 μl 5 μg/ml PI in HEPES buffer prior to acquisition and analysis by flow cytometry.

6-2-4(c) Preparation of BAL

The two BAL specimens (samples 2 and 3) were pooled and cells pelleted by centrifuging at 200g for 10 min. The supernatant was discarded and cells re-suspended to half the original volume with culture medium. This yielded an average cell count of 7.4×10^6 (± SD 13×10^6) AM. Cellular morphology was assessed by

sedimenting a 100 μl aliquot of cells onto a microscope slide using a Shandon Cytospin 2 (Shandon Southern Products Ltd, Astmore UK) for 5 min at 500 rpm. Slides were alcohol fixed and stained with a standard May Grunwald Giemsa stain prior to manual cell counting of BAL using light microscopy to assess the morphological features of the cells. All BAL samples contained less than 2% AEC contamination.

Apoptosis of AM was quantified using CD14 to identify AM and 7AAD staining as described in Chapter Two.

6-2-4(d) Phagocytosis assay

Phagocytosis of apoptotic AEC by AM was quantified using a flow-cytometric assay. 2 ml aliquots of BAL (cell count adjusted to $2x10^5$ cells / ml) were added to 10 ml culture tubes, on a slope, and incubated for 2h at 37C/5% CO_2. The fluid was removed by suction and the adherent AM monolayer washed with culture medium then re-incubated for 18h at 37C/5% CO_2.

At this time point, the apoptotic AEC were prepared and stained with MTG, as described above. The fluid was gently removed from the adherent AM monolayer. Stained, washed, epithelial cells (2 ml) were adjusted to a cell concentration of 10× the AM cell count and added to the AM monolayer. After 1 ½ h incubation on a slope at 37C/5% CO_2, the fluid was removed by gently pipetting, to minimise agitation of the sedimented cells. The adhered AM were exposed to 2 ml ice cold 0.5 mM EDTA for 15 min, removed from tubes by vigorourous pipetting, transferred to flow tubes and pelleted by centrifugation without further washing. AM were then stained with 5μl PE-CY5-conjugated Mab to CD33 (Immunotech) for 15 min. Addition of crystal violet (Gurrs) (0.8 mg/ml for 30sec) was carried out to quench autofluorescence of

AM and fluorescence of adherent but not phagocytosed cells (Hed et al. 1987; Bjerknes and Bassoe, 1984; Pruzanski and Saito, 1988). Unfixed cells were washed then acquired immediately by flow cytometry.

For analysis by flow cytometry, debris and red blood cells were excluded from region 1 (R1) based on forward (FSC) vs side (SSC) scatter characteristics (**Figure 6-1 (a)**). All subsequent analysis carried out on cells from R1. A further region (R2) defined cells that exhibited positive staining for CD33 (AEC that had not been phagocytosed did not fall in this region) (**Figure 6-1 (b)**). Using boolean logic, all subsequent analysis of AM that had ingested apoptotic AEC was carried out on cells from R1 and R2. Control tubes (**Figure 6-1 (c)-(e)**) of AM only, labeled AEC only, and AM + un-labeled AEC were included and their staining patterns used to set quadrant markers for flow cytometric analysis of the percentage of AM that had ingested apoptotic AEC (**Figure 6-1(f)**).

To assess the ability of AM to ingest particles, FITC-labeled carboxylate-modified polystyrene microbeads (1.7 μm mean diameter) (Polysciences, Warr, USA), adjusted to 40× the AM cell count, were added to the AM monolayer in one tube and assayed in parallel with tubes containing the AEC targets (**Figure 6-1 (g)**).

6-2-5 Confirmation of purity of test quadrant (AM + ingested AEC)

Cells that had been gated as described in **Figure 6-1**, and that were considered to be AM that had ingested apoptotic AEC, were sorted using a FACScalibur flow cytometer. The resulting cell suspension was deposited onto a microscope slide using a Shandon Cytospin 2 as described above, alcohol fixed and stained with H&E.

Figure 6-1

Analysis of phagocytosis by flow cytometry

6-1 (a) For analysis by flow cytometry, debris and red blood cells were excluded from region 1 (R1) based on forward (FSC) vs side (SSC) scatter characteristics. All subsequent analysis carried out on cells from R1.

6-1 (b) A further region (R2) defined cells that exhibited positive staining for CD33 (AEC that had not been phagocytosed did not fall in this region). All subsequent analysis of AM that had ingested AEC was carried out on cells from R1 and R2.

Figre 6-1 (c) Control tubes: unstained AEC and AM (<2% staining in FL-1 and FL-2).

Figure 6-1 (d) Control tube of MTG labeled AEC (<2% positive staining with CD33-PE-CY5).

Figure 6-1 (e) Control tube of CD33 PE-Cy5 labeled AM (<2% positive staining with MTG-FITC).

Staining patterns from control tubes were used to set quadrant markers for flow cytometric analysis of the percentage of:
Figure 6-1 (f) AM that had ingested apoptotic cells (20% of AM ingested apoptotic cells ie, positive staining with CD33 and MTG).

6-1 (g) AM that had ingested beads (27% of AM ingested beads ie, positive staining with CD33 and FITC).

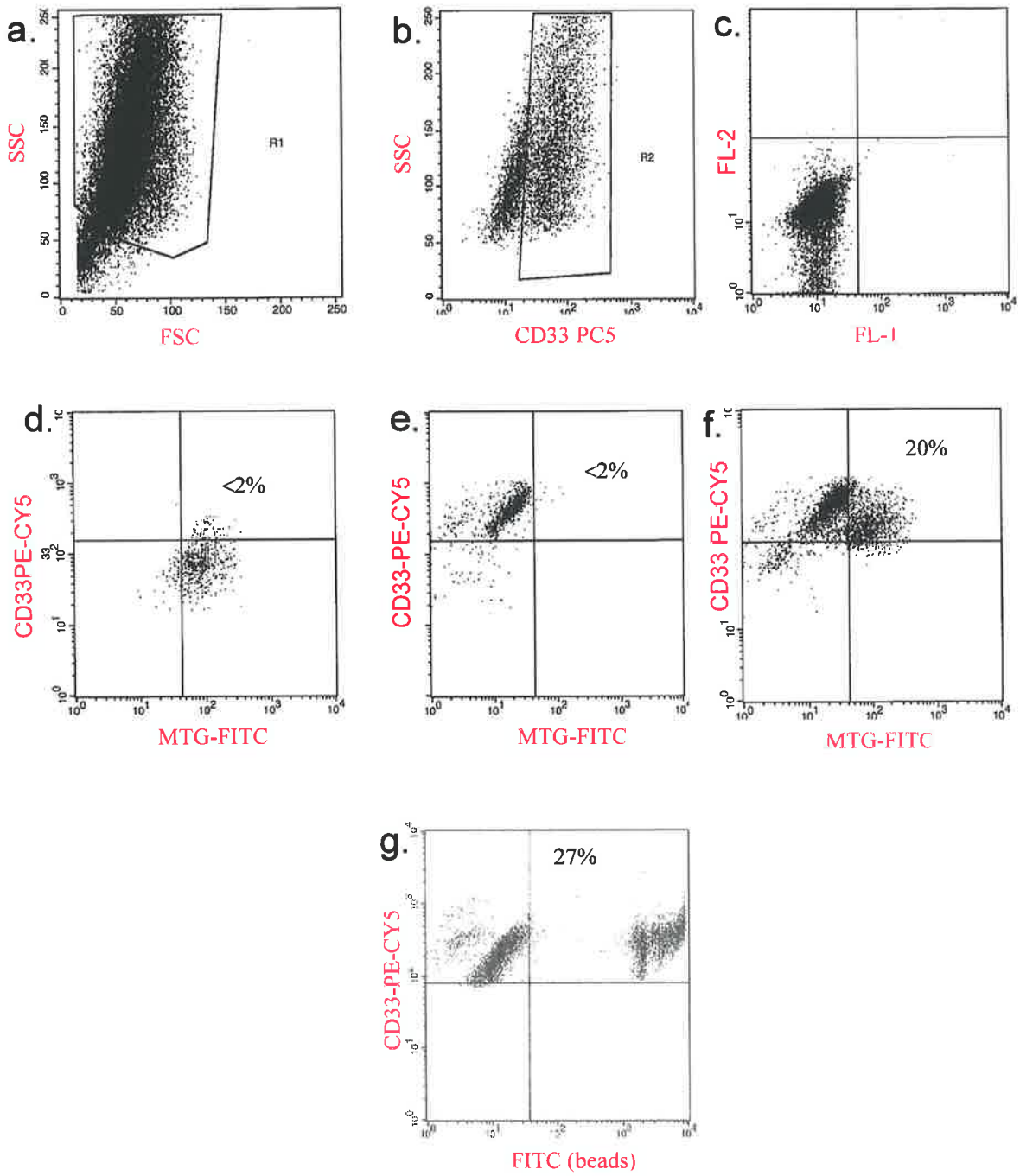


Figure 6-1 Analysis of phagocytosis by flow cytometry

6-2-6 Investigation of thrombospondin receptor and αVβ3 integrin by AM

For cell type identification and analysis of surface marker expression, 200 μl aliquots of washed, unstimulated, BAL were added to labeled FACS tubes. Cell surface staining with Mabs (CD33 PC-5 (for identification of AM as described above), CD36 (thrombospondin receptor), CD51 (αV integrin) and CD61 (β3 integrin)) was carried out as described in Chapter Three.

6-3 Results

6-3-1 Optimisation of methods

6-3-1(a) Optimal methods for inducing apoptosis in AEC

Preliminary experiments were carried out by subjecting AEC to UV radiation for 5 min, 10 min, 15 min and 20 min as well as aFAS for 48h and 10^{-5} dexamethasone for 20h.

Results presented in **Figure 6-2** show that UV radiation for 20min yielded the highest rate of apoptosis, assessed by positive staining with Annexin V.
When apoptosis was induced with Dexamethasone or aFas in five separate experiments, there was significant variation in rates of apoptosis (**Figure 6-3**).
When apoptosis was induced with Dexamethasone or aFAS, the rate of 'necrosis' (nuclei that have passed through necrosis and lost their cytoplasm) was increased compared with UV radiation for 20 min (UV 20 min 4.3% ± SD 1.4% necrotic vs

aFAS 26.5% ± 3.1%, **Figure 6-3**). Based on these results, subjection of 16HBE AEC to UV radiation for the optimal time, 20 min, was employed to induce apoptosis immediately prior to commencing the phagocytosis assay.

6-3-1(b) Reproducibility of apoptosis induction by UV radiation

To ensure reproducibility the AEC were exposed to UV radiation for 20 min on nine separate occasions. In every experiment, apoptosis was detectable in over 80% of treated cells (**Figure 6-4**). Staining with FITC-labeled Annexin V and PI (as described in Chapter Four) was used to determine the mean percentage of cells in early apoptosis (Annexin V+/PI-) was 35.1% with 52.2% of cells in late apoptosis (Annexin V+/PI+).

6-3-1(c) Reproducibility of Phagocytosis Assay

For analysis of the percentage of AM that had ingested apoptotic AEC, variability ranged from 1.85-2.85.

6-3-1(d) Time frame experiments for maximum detection of phagocytosis

The mean percentage phagocytosis of apoptotic AEC increased from 30.7% ± SD 12.5% at 1h to 34.3% ± SD 2.8% at 1 ½ h. At 2h, the percentage of detectable phagocytosis had decreased to 24.9% ± SD 3.9% ($p=0.002$, compared with 1 ½ h, Non parametric Wicoxon Test). Based on these results, all subsequent assays were performed for 1 ½ h.

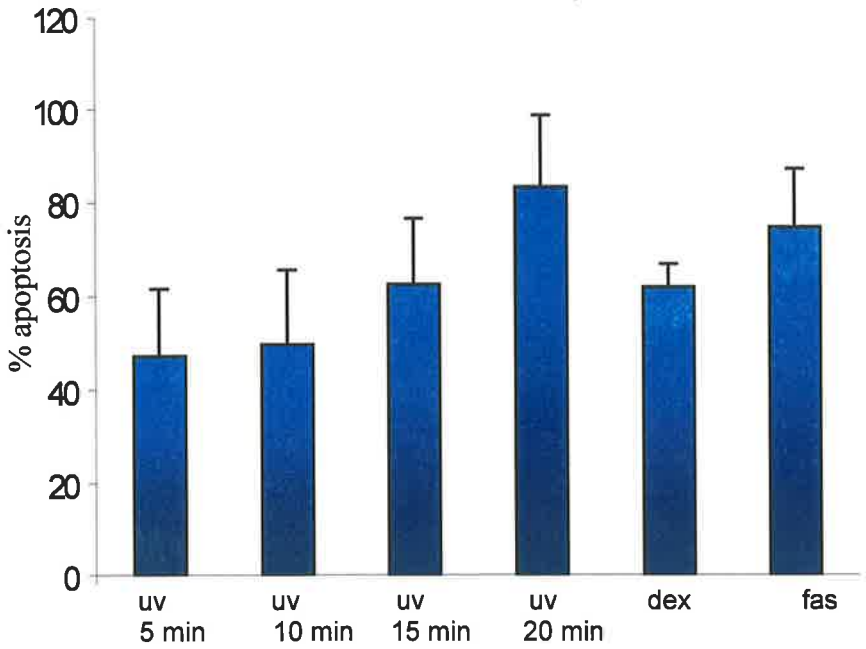


Figure 6-2

Induction of apoptosis in 16HBE AEC

Representative experiment showing variable percentages of apoptosis (mean ± SD) using a range of apoptosis-inducing agents (UV for 5, 10, 15, 20 min; 10^{-5} M Dexamethasone, 500ng/ml aFas 48h). UV radiation for 20min yielded the highest rate of apoptosis, assessed by positive staining with Annexin V.

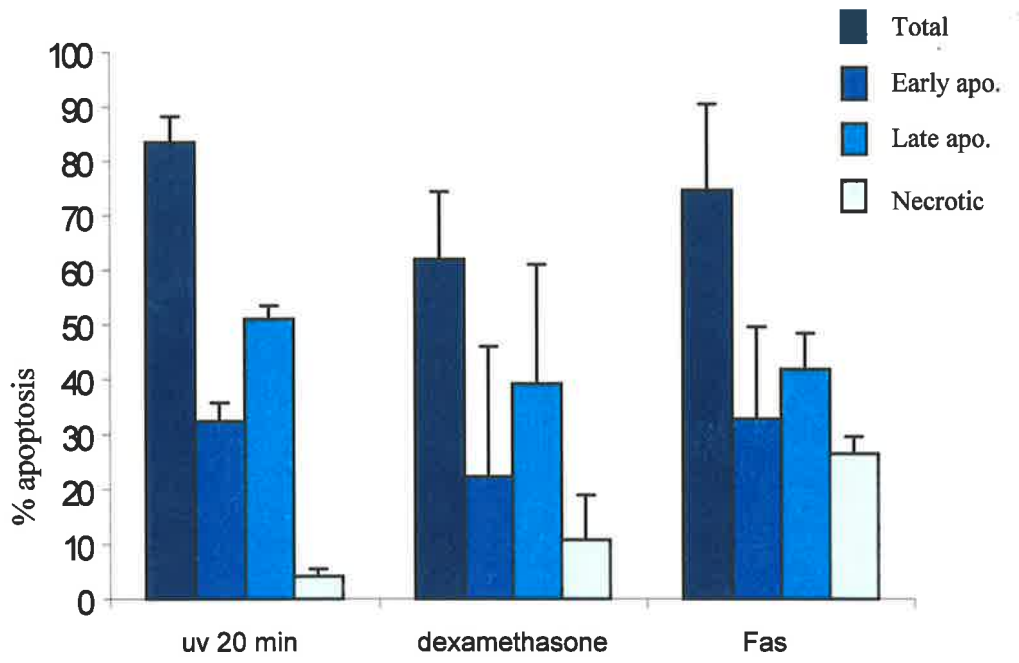


Figure 6-3

Various stages of apoptosis using a range of apoptosis-inducing agents

Various stages of apoptosis were quantified using flow cytometry and dual staining with Annexin V-FITC and PI. Early apoptosis was assessed as negative staining with PI and positive staining with Annexin V. Late apoptosis was assessed as positive staining with both Annexin V and PI. Necrosis was assessed as positive staining with PI and negative staining with Annexin V. UV radiation for 20 min produced the maximum, most consistent rates total apoptosis with minimal necrosis. Results represent mean values of 5 experiments ± SD.

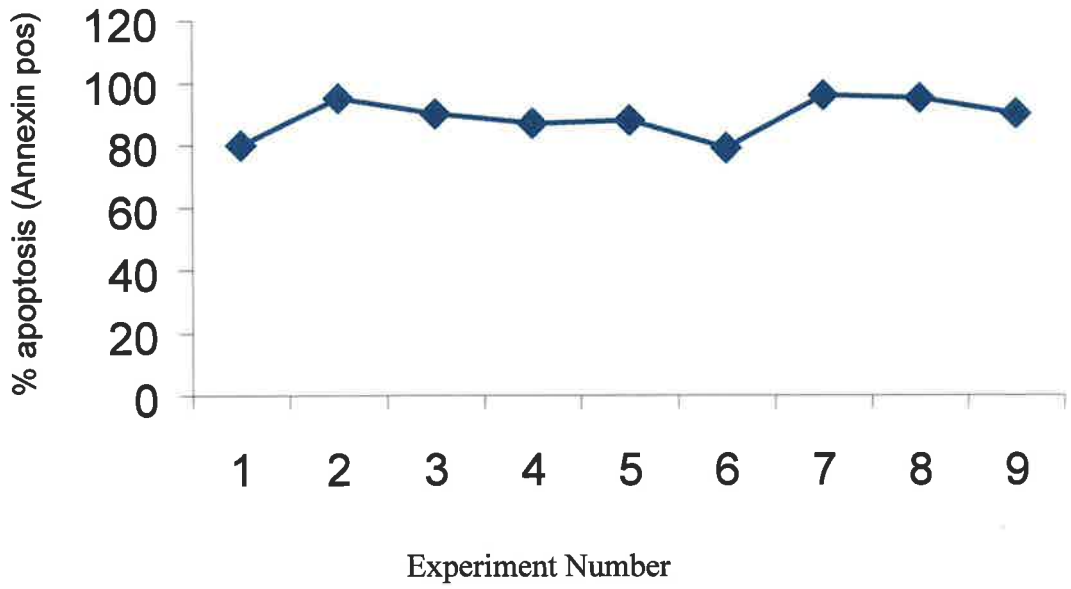


Figure 6-4

Reproducibility of induction of apoptosis with UV

To ensure reproducibility, apoptosis was induced on nine separate occasions. In every experiment, apoptosis was detectable in over 80% of treated AEC.

6-3-2 Phagocytosis Assay

6-3-2(a) Induction of apoptosis and staining of AEC targets

Some early experiments were carried out using ECA staining of apoptotic AEC. Comparison between the two stains showed no significant difference in the levels of detectable phagocytosis (data not shown). MTG exhibited brighter staining for apototic AEC than ECA FITC and was thereafter routinely applied for the phagocytosis assay.

6-3-2(b) Crystal violet quenching of AM autofuorescence

Crystal violet quenching (0.8 mg/ml for 30sec) successfully quenched autofluorescence of AM and prevented fluorescence signal crossover into fluorescence channel 1 (FL-1).

6-3-2(c) Confirmation of purity of test quadrant (AM + ingested apoptotic AEC)

H&E staining confirmed that the gated population contained only AM that had ingested apoptotic AEC, thus validating the flow-cytometric analysis and quenching procedure.

6-3-2(d) Morphology of ingested apoptotic AEC: H&E staining

On microscopic analysis, the morphology of ingested AEC showed features of apoptosis, including nuclear condensation (**Figure 6-5**). The ingested cells appeared

to be intact, which suggested that the prior cell surface staining with MTG would still be detectable by flow cytometry.

6-3-2(e) Ingestion of apoptotic AEC: fluorescence microscopy

On fluorescent microscopic analysis, ingestion of intact apoptotic AEC by AM were evident (**Figure 6-6**), consistent with the findings from light microscopy and flow cytometry.

6-3-3 Phagocytic ability of AM from COPD and control subjects

6-3-3(a) Viability of AM

7AAD staining was used to investigate whether increased apoptosis of AM from COPD compared to control subjects could account for the defective phagocytic capacity. At harvest, there was no significant difference in 7AAD staining for AM from COPD compared to control subjects (control 13.4% ± 8.8%; COPD 18.8 ± 12.9).

6-3-3(b) Phagocytosis of apoptotic AEC

AM from COPD patients showed reduced ability to phagocytose apoptotic AEC when compared with control subjects (12.4% ± SD 4.1% of AM from COPD ingested apoptotic AEC vs 25.6 % ± SD 9.2% for control group, p=0.004) (**Figure 6-7 (a)**). Phagocytic ability of AM (to ingest apoptotic AEC) from a small (n=4) group of recruited volunteers with no history of chronic lung disease (subject details: refer 4-

2-3) was also measured. The values from this group were within the mean ± SD obtained from the control group, validating the study results (**Figure 6-7 (a)**).

(c) Phagocytosis of carboxy-modified polystyrene beads

To assess the specific ability of AM to ingest apoptotic AEC, I compared the capacity of AM from COPD and control groups to ingest FITC-labelled carboxy-modified polystyrene beads. There was no significant difference in the ability of either group to ingest beads (31.8% ± SD 13.7% of AM from COPD ingested beads vs 33.3% ±SD 9.2% for control group) (**Figure 6-7 (b)**).

Phagocytic ability of AM (to ingest FITC-labelled carboxy-modified polystyrene beads) from a small (n=4) group of recruited volunteers with no history of chronic lung disease (subject details: refer 4-2-3) was also measured. The values from this group were within the mean ± SD obtained from the control group, validating the study results (**Figure 6-7 (b)**).

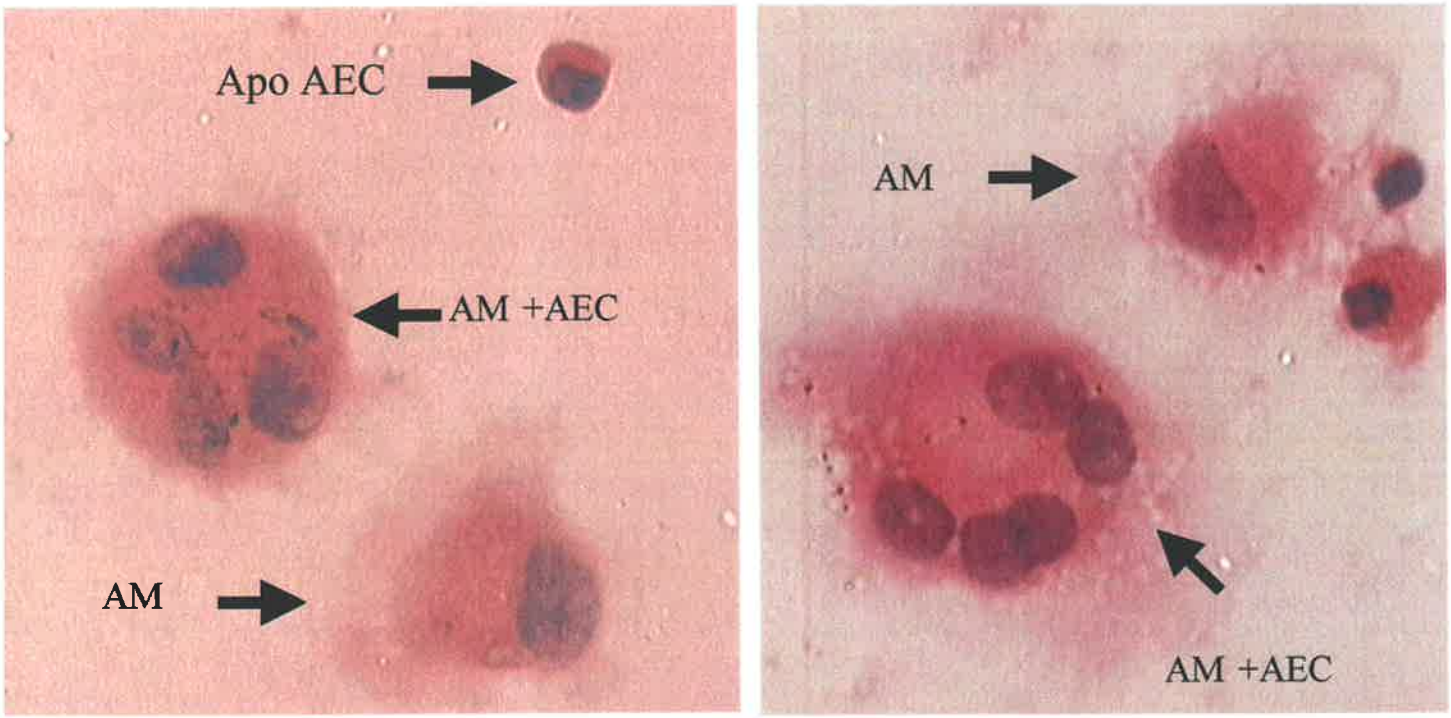


Figure 6-5

Morphological analysis of ingested AEC

Features of apoptosis are present, including nuclear condensation (400 x magnification; light microscopy). The ingested cells appear to be intact, suggesting that the prior cell surface staining with MTG would still be detectable by flow cytometry.

Figure 6-6

Fluorescence microscopy showing AM and AM that has ingested apoptotic AEC Nuclei were stained with Dapi; AEC were stained with MTG.

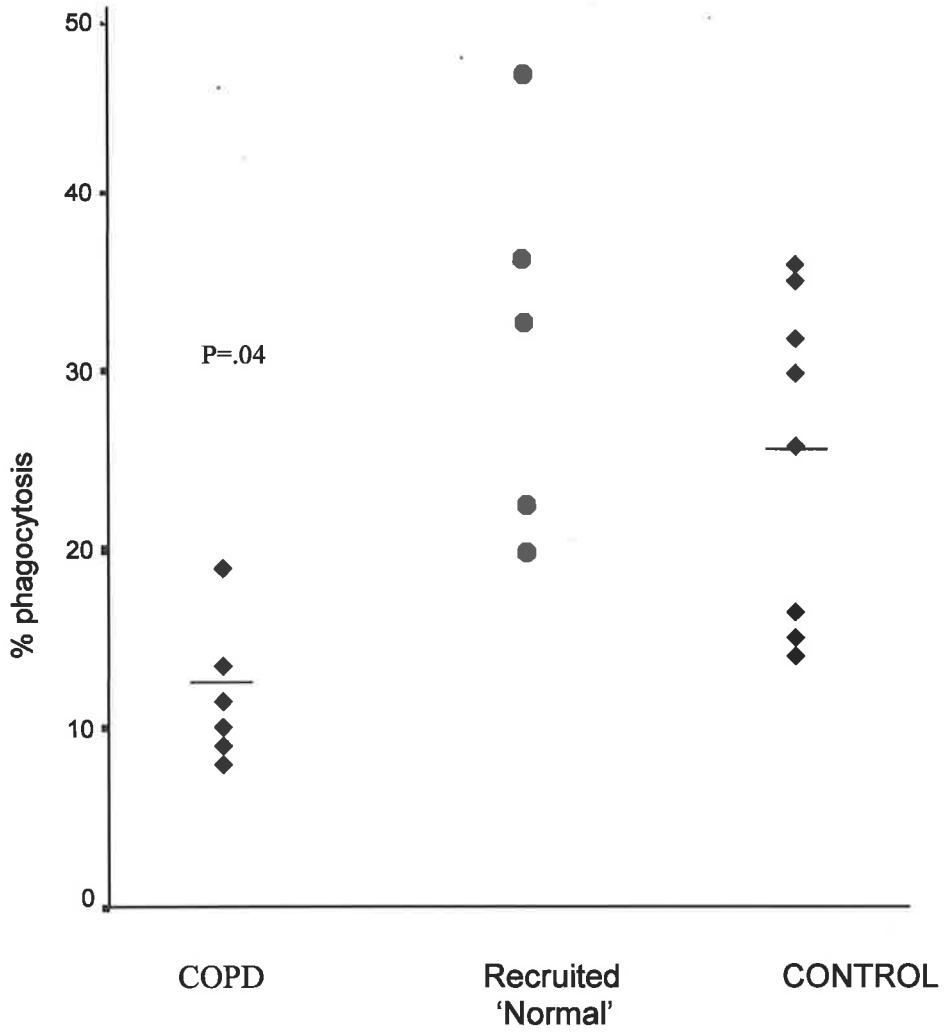


Figure 6-7 (a)

Phagocytosis of apoptotic AEC

AM from COPD subjects were significantly deficient in their ability to phagocytose apoptotic AEC when compared with control subjects (p=0.04, Mann Whitney test).

Red circles denote results from five recruited non-smoking volunteers with no history of chronic lung disease.

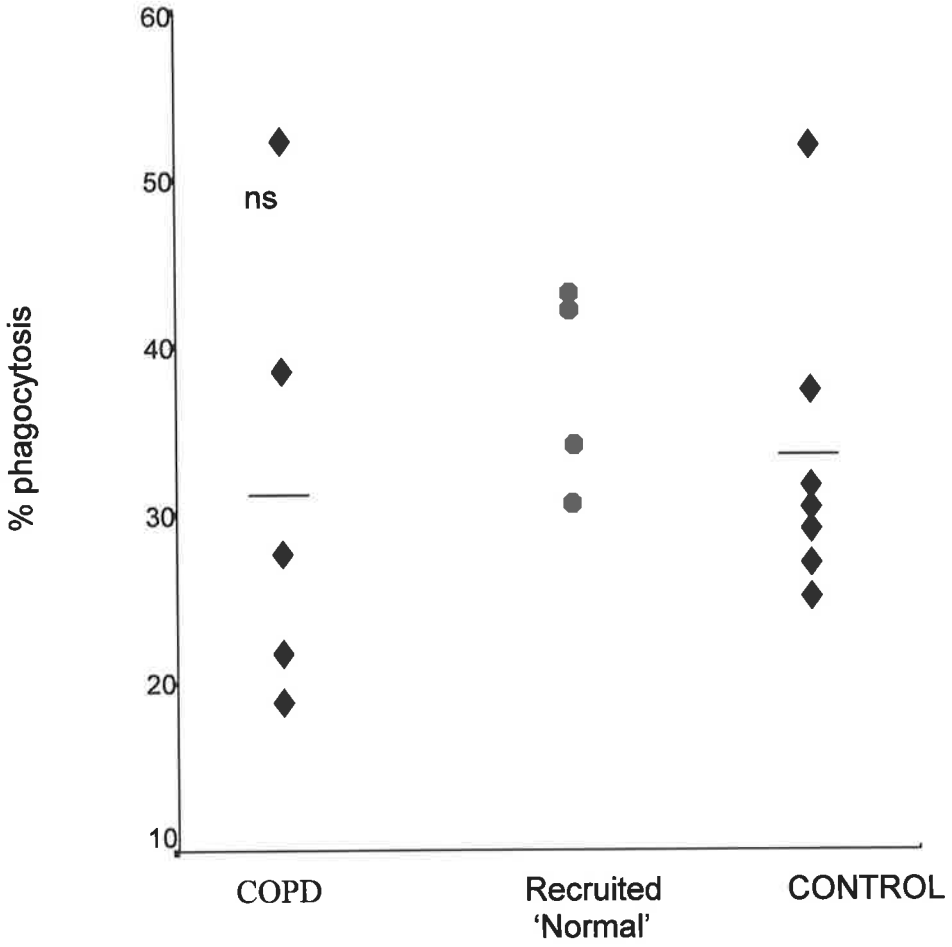


Figure 6-7 (b)

Phagocytosis of polystyrene beads

AM from COPD subjects: No significant deficiency in their ability to phagocytose apoptotic AEC when compared with control subjects (p=0.04, Mann Whitney test).

Red circles denote results from four recruited non-smoking volunteers with no history of chronic lung disease.

6-3-4 No changes in expression of CD36 or αVβ3 integrin in COPD

There was no significant difference in expression of thrombospondin receptor (CD36) by AM from COPD and control subjects (COPD: 18.5 ± 10.3% of AM expressed CD36 vs control: 18.7 ± 8.3%, ns). There was no difference in expression of CD51 (αV integrin) (COPD: 31.3 ± 7.3% of AM expressed CD51 vs control: 23.1 ± 13.2%, ns). CD61 (β3 integrin) was expressed at very low levels on AEC (<3% for COPD and controls). For the five recruited 'normals', 16.0 ± 3.6% of AM expressed CD36, 34.3 ± 20.1% expressed CD51 and <3% expressing CD61. These results were consistent with those obtained from the control group.

6-4 Discussion

Many factors associated with COPD have the potential to cause the increased rates of apoptosis of AEC described in Chapter Four. These include cigarette smoke, LPS (expressed as a contaminant on cigarette smoke), increased production of cytokines TGF-β, IL-8 and TNF-α (shown in Chapter Five), neutrophil activity and disruptions of epithelial cell/ matrix interactions (Gadek et al. 1984; D'Agostino et al. 2001; Frisch and Francis, 1994; McDonald and Usachencko, 1999). I have now shown that, in addition to an absolute increase in pro-apoptotic mechanisms, impaired capacity for phagocytic clearance of apoptotic material may be a further factor contributing to COPD pathogenesis. Uncleared apoptotic cells may undergo secondary necrosis with discharge of injurious cell contents (Hagimoto et al. 1997; Kuwano et al, 1999), resulting in tissue destruction and further inflammation (Ogasawara et al. 1993).

Importantly, I developed a novel *ex vivo* flow-cytometric technique for quantitation of the phagocytic ability of AM. In this assay I induced apoptosis in cultured AEC which were then exposed to AM obtained from BAL. This method has several advantages over conventional assays, which have utilised slide or plate-based techniques. Firstly, flow cytometry enables accurate quantitation of large numbers of AM that have engulfed apoptotic cells, based on staining properties of AM and AEC. This overcomes the need for manual counting of AM that have engulfed apoptotic cells, a time consuming technique that may be open to observer error. Furthermore, the use of exposure to UV radiation for 20 min to induce apoptosis of target cells was shown to overcome potential problems of overnight incubation of cells with commonly used apoptotic mediators such as Dexamethasone and aFas. The data suggests that these long incubations may cause inconsistent rates of apoptosis with up to 20% of cells progressing to necrosis. In nine separate experiments, I exposed AEC to UV radiation for 20 min and consistently achieved greater than 80% apoptosis. Minimal necrosis as assessed by Annexin V negative / PI positive staining was also observed following UV treatment. In general these are nuclei that have passed through necrosis and lost their cytoplasm (and hence their phosphotidylserine residues) (personal communication: M Ormerod, Purdue Cytometry Mailing List Feb., 2000). These changes would render the cells unsuitable as phagocytic targets for the annexin-based assay.

Additionally, the use of AEC as targets for phagocytosis enabled a more physiologically relevant appraisal of phagocytosis in the airways than previously possible. This was clearly demonstrated when I applied the technique to assess phagocytic capacity of AM from patients with COPD and control subjects. I found that AM from COPD patients had significantly reduced phagocytic capacity for apoptotic AEC. Interestingly, this deficiency appeared to be specific for apoptotic

cells as tests carried out in parallel using carboxylate-modified polystyrene microbeads revealed no significant difference between COPD patients and control subjects. This finding not only stresses the importance of using physiologically relevant apoptotic targets but also suggests that there may be a specific deficiency in the phagocytosis of apoptotic AEC by AM in COPD.

There are several possible reasons for this deficiency. The mechanisms by which apoptotic cells are recognised and phagocytosed have been extensively studied over the last few years. For fibroblasts at least, the apoptotic cell can actively recruit phagocytes, through secretion of TSP (Moodley et al. 2003). Phagocytosis of apoptotic neutrophils by AM has been shown to be mediated by a pathway in which the integrin $\alpha V\beta 3$ (CD51/CD61) cooperates with the thrombospondin receptor, CD36 (Gibson et al. 1997). This complex then binds to secreted thrombospondin, which binds to an incompletely characterised ligand on the apoptotic cell (Savill et al. 1992) (also refer 1-7 and **Figure 1-11**). The phagocytic process is complex and incorporates chemotaxis, actin polymerisation (to surround and internalise the target), migration, adhesion, aggregation, phagocytosis, degranulation, and reactive oxygen species production. I investigated expression of recognition/adhesion molecules CD36, CD51 and CD61 on both BAL-derived AM and AEC from COPD and control subjects but no significant differences were noted, indicating that expression of these molecules does not play a role in the deficient phagocytosis noted. Whilst there are obvious differences between the process of phagocytosis of apoptotic cells and polystyrene beads (such as beads not expressing molecules such as CD36, CD51 and CD61), the actin polymerisation is required for phagocytosis of apoptotic cells and beads over 0.9 microns in size (the beads employed in my study were 1.7 microns diameter) (Toyohara et al. 1989). As no defect of phagocytosis of beads was noted by AM from COPD subjects in my study,

it may be that the defect lies in the apoptotic AEC rather than the AM. If this is the case, then future studies using the AEC derived from bronchial brushings would be important additions to these experimentsA second reason may be that previous ingestion of digestible particles or even apoptotic AEC (increased in number in COPD) may downregulate the subsequent ability of AM to ingest these cells. Previous uptake of apoptotic neutrophils has been shown to downregulate the ability of macrophages to ingest apoptotic neutrophils. The study showed that an initial round of phagocytosis led subsequently to decreased phagocytosis of apoptotic neutrophils, but not of opsonised erythrocytes, which would be recognised via Fc receptors (Erwig et al. 1999).

The role of pulmonary collogenous C-type lectins (collectins) in regard to these findings needs consideration. Collectins are surfactant proteins, synthesised by AEC, that interact with pathogens and are involved in host defense and clearance of pathogens by phagocytosis. The interaction involves binding of the collectin to the bacterial cell wall (glycoconjugates) as well as to the LPS receptor (CD14) and toll like receptors (TLR) 2 and 4 (Palaniyar et al. 2002), resulting in enhanced killing and clearance by phagocytes (Haagsman HP. 2002). In addition, the collectin surfactant protein A (CSA) has been reported to enhance apoptotic neutrophil uptake by AM (Reidy et al. 2003; Vandivier et al. 2002; Schagat et al. 2001), and may play a similar role with apoptotic AEC. Interestingly, SPA deficient mice exhibited defective phagocytosis, delayed microbial clearcnce, increased neutrophil influx and increased TNF-α and IL-6 levels in BAL (Levine et al, 1997; Levine et al, 2000; Levine et al, 1999). It is therefore possible that there are reductions in levels of SPA in the airways in COPD that contribute to the deficient phagocytic capacity of AM and this hypothesis requires further investigation.

There was an unexpectedly low rate of phagocytosis in the assay described in this Chapter. As collectins would be present in the airways when phagocytosis of

apoptotic AEC occurs it is possible that reduced levels in the culture conditions may affect the rate of phagocytosis in the assay, although this would not invalidate the COPD vs control comparison.

In conclusion, I showed that AM from subjects with COPD are deficient in their ability to phagocytose apoptotic AEC. A flow cytometric assay enabled me to use physiologically relevant apoptotic AEC as phagocytic targets. Further studies are thus warranted to more clearly identify the reasons for the deficient phagocytosis of AEC in the airways in COPD.

Chapter Seven

Apoptosis and cytokine production in the peripheral blood in COPD

7-1 Introduction

Apoptosis is important for the regulation of normal cell turnover in the lung and is a key mechanism in the control of the repair process. I have demonstrated in previous chapters that COPD is associated with increased apoptosis of AEC and lymphocytes, increased production of apoptotic mediators that AMs from COPD subjects have a reduced capacity to ingest apoptotic AECs.

Activated T-cells, which are increased in the airways in COPD, are thought to play an important role in the pathogenesis of the disease, with their numbers in the alveolar wall correlating with the extent of emphysema (Saetta et al. 1993; Finklestein et al.1995). These T-cells must be removed by apoptosis at the end of an inflammatory response in order to maintain cellular homeostasis (Lenardo et al. 1999). As previously discussed, under normal conditions, apoptosis is followed by rapid phagocytosis of apoptotic cells with minimal inflammatory response. However, increased rates of apoptosis may result in unbalanced homeostasis leading to an overloading of the local capacity for phagocytosis and defective clearance (Ogasawara et al. 1993). This could potentially lead to retention of apoptotic material, secondary necrosis and perpetuation of the inflammatory response.

Increased apoptosis of lymphocytes obtained from the airways by bronchoalveolar lavage (BAL) in COPD was described in Chapter Four. Lymphocytes are known to traffic from the bloodstream to the bronchoalveaolar space and then may later re-join the peripheral circulation (Schuster et al. 2000; Lehmann et al. 2001). Based on this known trafficking of lymphocytes between the airways and the peripheral blood, and the increased rates of apoptosis in airway derived T cells, I hypothesised that I could detect an increase in the propensity of peripherally derived T cells from COPD patients to undergo apoptosis compared to cells from subjects without this disease.

Several pathways have been reported to be involved in inducing apoptosis of T-cells. These include TGF -β/TGF-R1, TNF-α/TNF-R1 and Fas/Fas ligand (Lenardo et al. 1999; Sillett et al. 2001; Siegmund et al. 2001). In Chapter Five I described increased production of TGF-β and TNF-α in the airways in COPD. In the present study, I investigated production of these apoptotic mediators and expression of their receptors in the peripheral blood from COPD subjects. These factors may contribute not only to T-cell apoptosis, but apoptosis of alveolar wall cells thereby contributing to the development of emphysema (Majo et al. 2001; Kasahara et al. 2001).

Alterations in lymphocyte subsets in the peripheral blood and airways of patients with COPD have also been reported (Majo et al. 2001; Saetta et al. 1998). The CD4:CD8 ratio is significantly decreased with the percentage of CD8 lymphocytes increased. These ratio changes in COPD could be due to a number of factors, including relatively increased rates of apoptosis of CD4 compared with CD8 T-cells. Alternatively, the changed ratio could primarily be due to an absolute increase in CD8 T-cell numbers. I therefore specifically investigated apoptosis of the CD4 and CD8 subsets of T-cells in the COPD patients and control subjects.

I used multiparameter flow cytometry to determine cytokine, cytokine receptor and apoptosis levels as previously reported (Hodge et al..2000; Hodge et al..2001). It was important to use whole blood as we have previously shown that puification of peripheral blood mononuclear cells results in increased levels of apoptosis (Hodge et al. 2000), which would confound the analysis.

7-2 Methods

7-2-1 Study population

Following informed consent, peripheral blood was collected into tubes containing sodium heparin (50 IU/ml) as anticoagulant from 18 patients with COPD (mean age 66, 3 females, 15 males). All COPD subjects were reformed smokers with the exception of one current smoker. For 16 non-smoking volunteers, with no history of respiratory diseases, specimens were obtained and used as controls (mean age 41, 8 females, 8 males) (**Table 7-1**). The diagnosis of COPD was established using the 'GOLD' criteria as outlined in Chapter Six. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee.

Table 7-1 Demographic characteristics of the population studied

Subjects	Control group	COPD
No. of subjects	16	18
Age (y)	41 ± 13	66 ± 8
Smoking, pack yr	0.5	69 ± 39
GCS treatment	0	14
BD treatment	0	4
FEV1, % pred	96.1 ± 15.5	47.5 ± 18.0
FVC, % pred	98.7 ± 9.7	88.8 ± 18.5
FEV1,% FVC	81.8 ± 16.2	53.9 ± 20.1

Results are expressed as mean values. GCS: corticosteroid, BD: bronchodilator only

7-2-2 Reagents

PE-conjugated monoclonal antibodies (Mabs) against TGF-β (IQ products) and Fas (CD95), TNF-α (BD), TNF-RI (BD), CD45 (BD), and CD8 (BD) were used for flow cytometry. FITC-conjugated MoAbs to TNF-α (BD), TNF-RII (BD) were also employed. PE-CY5- MoAbs to the monocyte/macrophage marker CD14 and the T-cell marker, CD3 (Immunotech) were also included. FITC conjugated Annexin V (Pharmingen) and 7 aminoactinomycin D (7AAD, Sigma) were used for investigation of apoptosis for stimulated T-cells by flow cytometry.

7-2-3 Stimulation of peripheral blood

Aliquots of 500 μl of blood were added to 500μl culture medium in 10ml culture tubes, and incubated for 24 h, at 37^{O}C in 5%CO_2 in air. The samples were stimulated with 25ng/ml PMA (Sigma) plus 1μg/ml ionomycin (Calbiochem, CA, USA) for T-cell cytokine analysis, or 10μg/ml PHA for T-cell surface marker and apoptosis analysis or 100ng/ml *E. coli* LPS, serotype 0111:B4 (Sigma) for monocyte stimulation. For intracellular cytokine investigation, cells were cultured in the presence of brefeldin A (1μg/ml) (Sigma) as a 'Golgi block' to inhibit intracellular transport and thus retain cytokines produced intracellularly during activation.

7-2-4 Staining with Mabs to surface markers

Mabs to identify cell types (T-cell markers CD3 (Pharmingen), CD4 (BD) and CD8 (BD) and monocyte marker CD14 (Pharmingen)), cytokine receptors (TNF-RI (R&D), TNF-RII (R&D) and CD132 (Pharmingen)) and Fas (CD95) (Pharmingen) were used in the study. Heparinised whole blood (50 IU/ml heparin final concentration) was

stained with Mabs within 6h of collection using the whole blood lysis method (Caldwell and Taylor, 1986). Briefly, 200 μl aliquots of blood were stained with 5 μl of the appropriate Mab for 20 min on ice in the dark and then 2 ml of FACSlyse (BD) was added to lyse the red blood cells in a further 10 min incubation at room temperature in the dark. After centrifugation at 300 x g for 5 min the tubes were inverted and drained on filter paper for 30 sec, cells were washed twice with 1% BSA Isoton II (Coulter) and events acquired immediately by flow cytometry.

7-2-5 Staining for apoptosis with Annexin V

Staining with Annexin V was used for evaluation of translocation of PTS to the outer cell membrane in early apoptosis. Staining for cell type identification was carried out as described above. Cells were further washed and stained with Annexin V as described in Chapter Four.

7-2-6 Intracellular cytokine production

Surface staining to enable cell subset identification, as described above, was carried out immediately prior to intracellular cytokine staining. Intracellular staining with Mabs to intracellular cytokines and isotype-matched controls was performed as described in Chapter Five. Cells were washed, and events acquired immediately by flow cytometry with collection gates set on the cells of interest.

As TNF-α and Fas have been reported to induce apoptosis of CD8+ T-cells (Kaser et al. 1999) production of these mediators was investigated for CD3+, CD4+ and CD8+ T-cells.

Analysis of intracellular cytokine production was carried out on T-cells and monocytes, identified by staining characteristics with Mabs (**Figure 7-1**). T-lymphocytes were gated based on known staining characteristics with CD3 PC5 versus side scatter (**Figure 7-1(a)**). CD8+ events were identified then CD4+ events gated by CD3+CD8- staining characteristics. Monocytes were gated based on known staining characteristics with CD14 PC-5 (**Figure 7-1(c)**).

Figure 7-1

Intracellular cytokine production in peripheral blood

Representative dot plots showing

(a) Debris excluded from region 1 (R1) based on forward (FSC) vs side (SSC) scatter characteristics. All subsequent analysis carried out on cells from R1.

(b) Identification of cell types. T-cells were gated in R2 based on positive staining with CD3 and low SSC characteristics. Monocytes were gated based on positive staining with CD14 and high SSC characteristics.

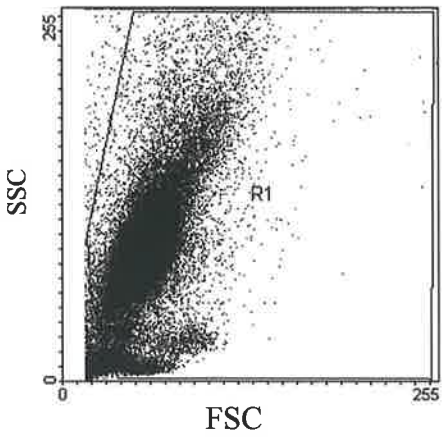
(c) T-cells gated in R2 were further analysed for production of TNF-α (COPD: total TNF 23%; CD4 negative (CD8 T-cells) 9%; CD4 positive T-cells 14%).

(d) CONTROL: total TNF 6%; CD4 negative (CD8 T-cells) 2%; CD4 positive T-cells 4%).

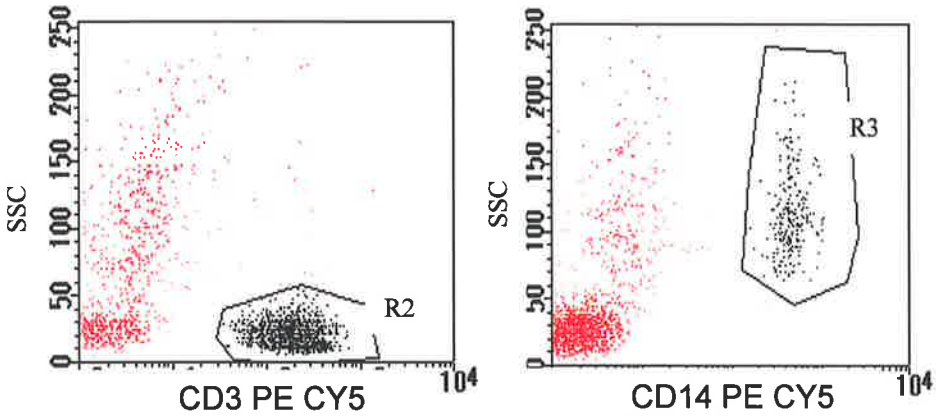
(e) Monocytes gated in R3 were further analysed for production of TNF-α (COPD 65%).

(f) CONTROL: Monocyte production of TNF 51%.

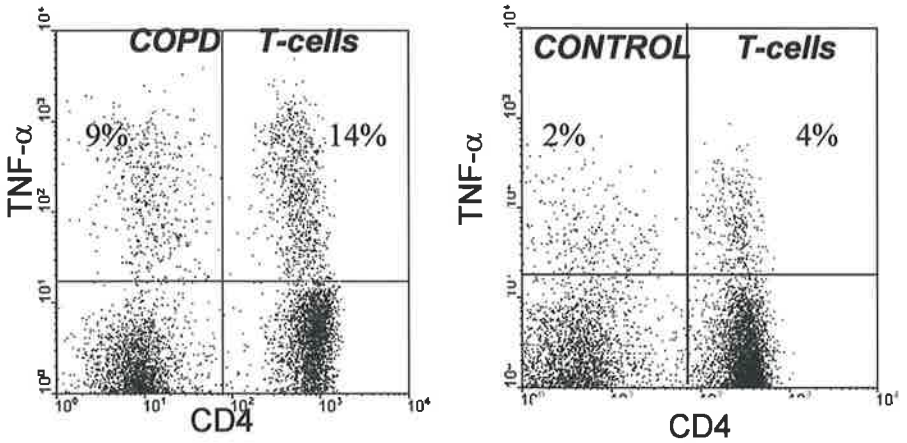
a.


SSC
R1
FSC
0
255

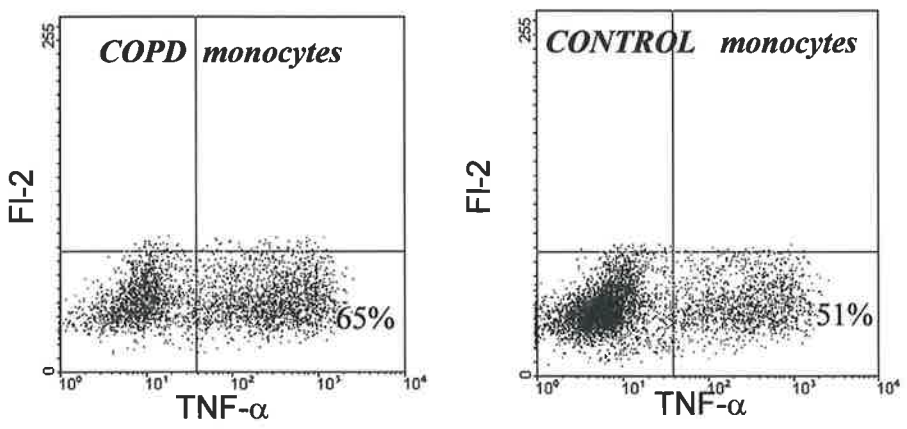
b.


SSC
R2
CD3 PE CY5
R3
CD14 PE CY5
0
50
100
150
200
250
10⁴

c.


COPD T-cells
9%
14%
TNF-α
CD4
CONTROL T-cells
2%
4%

d.


COPD monocytes
65%
Fl-2
TNF-α
CONTROL monocytes
51%

7-2-7 Soluble TGF-β release

Plasma from 24h-LPS-stimulated blood was removed after centrifugation at 1800g for 5 minutes. Plasma was stored at -20^{0}C prior to batch testing. 'Active' TGF-β (ie, non-polymerised) was quantified with a Quantikine immunoassay kit (R & D), following instructions supplied by the manufacturer.

7-2-8 TGF-β receptor expression

PBMCs were obtained by diluting peripheral blood with an equal volume of RPMI, layering 7 ml over 3 ml Lymphoprep (Nycomed, Norway) and centrifuging for 15 min at 500g. The monolayer was removed and washed twice with 10 ml RPMI containing 10% FCS (Gibco). The cells were resuspended in RPMI at 5 x 10^6 cells / ml, and incubated for 24 h, at 37^{O}C in 5%CO_2 in air. TGF-R was quantified using a sensitive indirect kit (FLUOROKINE^{TM} , R&D) following the manufacturer's instructions as described in 5-2-6.

7-2-9 Influence of age on apoptosis and cytokine production

There was a difference in the mean age of control subjects (mean age 41) tested in parallel with the COPD subjects (mean age 66) in this study. To assess the influence of age, levels of apoptosis and production of cytokines were investigated in peripheral blood from five normal, non-smoking volunteers aged 35-45, seven aged 45-55 and seven aged 55-65 as described above.

7-2-10 Influence of sex on apoptosis and cytokine production

There was a difference in the ratio of male: female control subjects (male 8: female 8) tested in parallel with the COPD subjects (male 15: female 3) in this study. To investigate the influence of sex, levels of apoptosis and production of cytokines were investigated in peripheral blood from eight normal, non-smoking volunteers and eleven males as described above.

7-2-11 Statistical analysis

The Wilcoxon non-parametric test for related samples was used to analyze the data. P values ≤ 0.05 were considered significant.

7-3 Results

7-3-1 CD4+ and CD8+ T-cells in peripheral blood in COPD

The percentage of CD8 T-cells was slightly but significantly increased in the peripheral blood of COPD subjects compared with control subjects (31.7% ± SD 14.3% versus 26.6% ± SD 11.2%, p=0.049). The percentage of CD4 T-cells was not significantly different between the groups (COPD 68.2% ± SD 14.3% versus 73.1% ± SD 11.2%). The ratio of CD4:CD8 T-cells was decreased in the peripheral blood of COPD subjects compared with control subjects (3.1 ± SD 2.4 versus 4.3 ± SD 4.1, p=0.041).

7-3-2 Apoptosis in peripheral blood in COPD

Apoptosis, identified by staining with Annexin V, was higher for PHA-stimulated T-cells from patients with COPD than those from the control group (p=0.006, **Table 7-2**). Apoptosis of stimulated T-cells was significantly increased in both CD4 and CD8 T-cells from COPD subjects (p=0.043 for both subsets). There was no significant difference in levels of apoptosis between CD4 and CD8 peripheral blood-derived T-cell subsets in COPD patients. To confirm these findings, 7AAD staining was carried out in parallel. As I found in earlier preliminary studies of AEC (Chapter Four), there was good correlation between the two methods (**Figure 7-2**). Apoptosis, identified by staining with 7AAD, was higher for PHA-stimulated T-cells from COPD subjects than from control subjects (COPD 70.0% ± SD23.1% vs control 58.2% ± SD15.9%, p=0.043).

7-3-3 Fas expression in peripheral blood in COPD

Having found an increase in the propensity of T cells from COPD subjects to undergo apoptosis, I next investigated a number of potential pathways, which may explain this. To investigate the role of the Fas pathway in apoptosis of T-cells in peripheral blood, I measured Fas (CD95) expression for CD3+, CD4+ and CD8+ T-cells, and CD14+ monocytes. T-cells from COPD patients expressed Fas (CD95) at a higher frequency than control subjects (**Table 7-2**). This difference was significant for both CD4 and CD8 T-cells (Table 2). However, Fas expression by monocytes was not significantly different for COPD and control groups.

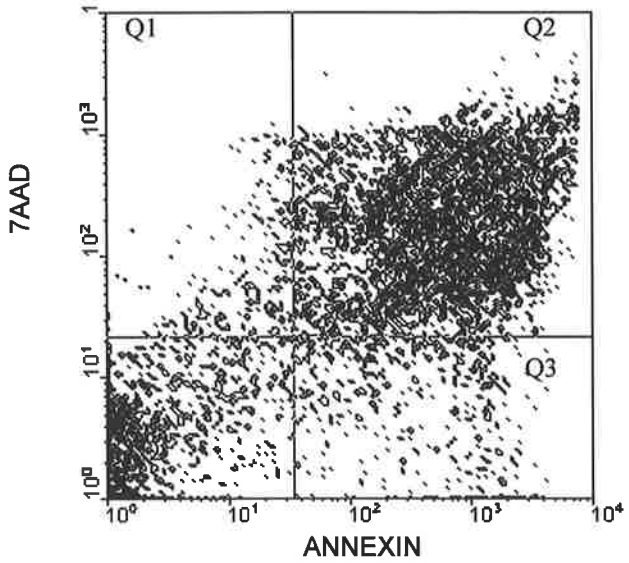


Figure 7-2

Correlation between Annexin V and 7AAD staining for peripheral blood derived T-cells

Representative contour plot showing Annexin V and 7AAD staining (apoptosis) of PHA-stimulated peripheral blood – derived T-cells (Control subject, stimulated with PHA for 24h). Annexin V positive staining (Quadrants 2 and 3) 50.9%; 7AAD staining (Quadrants 1 and 2) 50.5%

Table 7-2

Apoptosis and Fas expression in PHA stimulated blood in COPD

Cell type	Marker	Control	COPD	P value	
T-cell	Annexin V	50.2 ± 21.8	75.0 ± 14.7	.006	*
CD4+		54.8 ± 27.7	68.5 ± 20.7	.043	*
CD8+		43.1 ± 25.9	52.0 ± 20.7	.043	*
T-cell	Fas (CD95)	35.5 ± 12.5	44.8 ± 16.3	.013	*
CD4+		41.1 ± 10.4	53.6 ± 16.0	.002	*
CD8+		24.2 ± 20.4	36.6 ± 28.0	.004	*
Monocyte		89.9 ± 17.4	86.0 ± 18.6	Ns	

Results (% of cells exhibiting positive staining measured by flow cytometry) are expressed as mean values ± SD *denotes significant difference from control group

7-3-4 TNF-α and TGF-β and receptors in peripheral blood in COPD

In addition to Fas, TNF-α has also been reported to induce apoptosis of T-cells (Zheng et al. 1995), thus, I hypothesised that changes in the levels of this mediator in COPD might be involved in the alteration of T-cell apoptosis rates and/or the CD4:CD8 ratio. Using flow cytometry, I found significantly increased production of TNF-α by peripheral blood CD3+ T-cells in COPD (p=0.008, **Figure 7-3**). In a group of 10 Control subjects and 10 COPD patients, there was no significant difference noted for CD4+ or CD8+ T-cells (CD4: COPD 17.8% ± 20.1% vs control 3.6% ± 2.9%; CD8: COPD 15.2% ± 24.0% vs control 2.0% ± 15.2%, ns).

The effect of TNF-α is dependent upon which TNF-α receptor is activated, with TNF-RI and TNF-RII having pro- and anti-apoptotic roles respectively. Increased expression of TNF-RI was observed for CD3+ T-cells from COPD subjects compared with controls (p=0.003, **Table 7-3**). There was no significant difference in expression of TNF-RII between COPD and control groups.

I also investigated the role of TGF-β and its receptor in induction of apoptosis of peripheral blood T-cells in COPD. Flow cytometric analysis showed low production of TGF-β by activated T-cells and no significant difference between COPD and control groups (**Figure 7-3**). However, monocyte production of TGF-β was significantly increased in COPD (**Figure 7-3**). In contrast to TGF-β, TGF-β receptor expression was significantly increased for T-cells from COPD subjects compared with controls (p=0.018, **Table 7-3**).

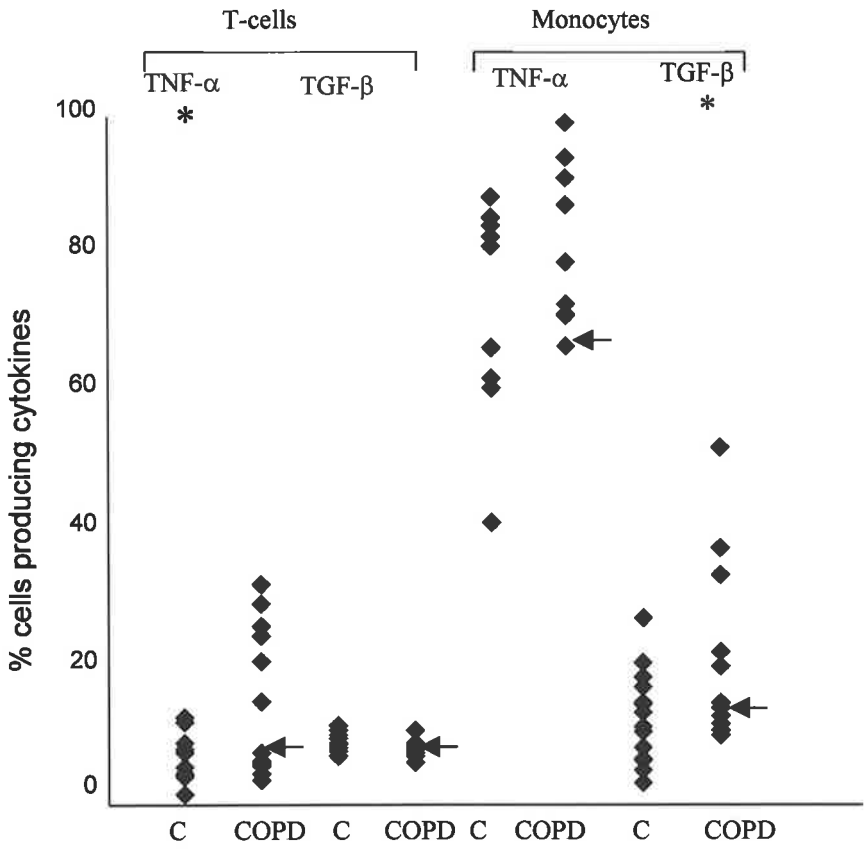


Figure 7-3.

Intracellular cytokine production in peripheral blood in COPD and control subjects (C). Note significantly increased production of TNF-α by T-cells and monocytes and increased TGF-β production by monocytes. * denotes significant difference between COPD and controls. Arrows denotes values from the COPD subject who was currently smoking.

Table 7-3

Cytokine receptor expression by T-cells in COPD

Receptor	Control	COPD	p value C/COPD
TNF-RI	0.6 ± 0.8	1.2 ± 1.3	0.003 *
TNF-RII	3.8 ± 2.6	3.7 ± 3.2	Ns
TGF-βR	5.5 ± 2.8	15.8 ± 16.1	0.018 *

Results (% of cells exhibiting positive staining measured by flow cytometry) are expressed as mean values ± SD *denotes significant difference from control group

7-3-5 Secreted, active TGF-β in peripheral blood in COPD

Although I found increased cellular TGF-β levels, TGF-β is produced in a latent form and is biologically inactive and unable to bind to its receptors until it has been activated (Blobe et al. 2000). The flow cytometric method utilised a Mab to both active and latent TGF-β and hence measured total TGF-β. To measure secreted, active TGF-β in the peripheral blood I applied ELISA techniques to measure plasma levels. Release of active TGF-β in the peripheral blood in COPD was significantly increased compared with the control group (p=0.015) (**Figure 7-4**).

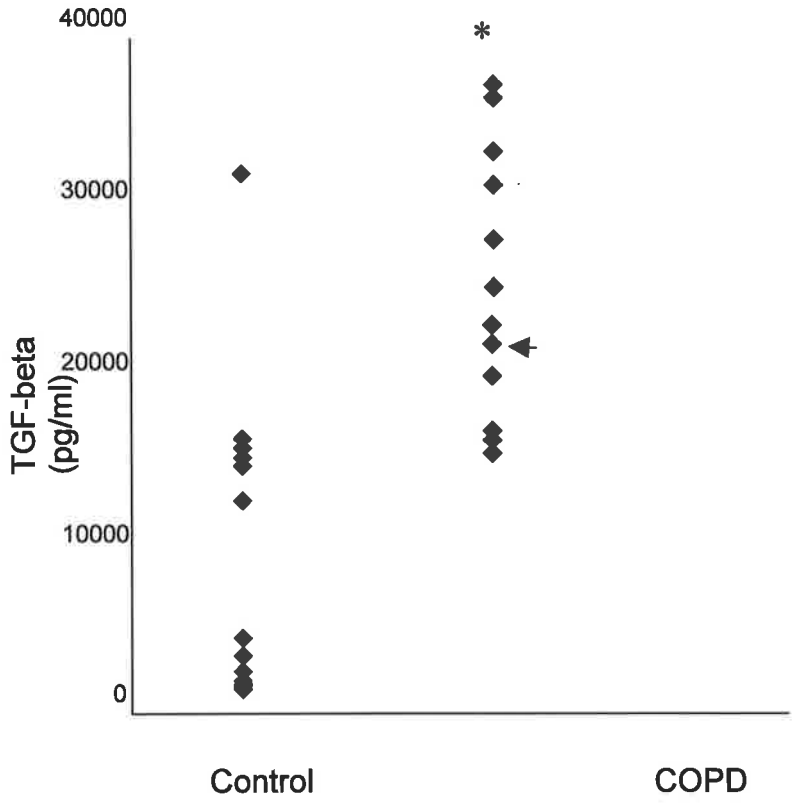


Figure 7-4

Soluble active TGF-β release in peripheral blood in COPD

ELISA was applied to show significant increase in active TGF-β in COPD * denotes significant difference [p=0.015] between COPD and controls. Note. Arrow denotes value from the COPD subject who was currently smoking.

7-3-6 Influence of age on apoptosis and cytokine production

There was no significant difference in the levels of apoptosis and production of TGF-β for the various age groups (Table 7-4). TNF-α production for peripheral blood-derived T-cells increased with age, but the increase did not reach statistical significance (p=0.087, 55-65 year old age group when compared to the 35-45 year old group) (Table 7-4). Monocyte TNF-α production was significantly lower for the 55-65 year old age group compared with the 35-45 year old age group (Table 7-4).

7-3-7 Influence of sex on apoptosis and cytokine production

There was no significant difference in levels of apoptosis or cytokine production between males and females (Table 7-4).

Table 7-4 Effects of age and sex on apoptosis and cytokine production

	Age 35-45	Age 45-55	Age 55-65	Males	Females
N	5	7	7	11	8
T-cell apoptosis	55.6 ± 13.9	59.4 ± 19.8	48.8 ± 11.8	50.9 ± 16.2	55.8 ± 11.7
T-cell TNF-α	13.0 ± 8.5	21.3 ± 5.8	22.4 ± 13.2	23.8 ± 11.8	17.7 ± 11.0
T-cell TGF-β	9.7 ± 8.8	11.0 ± 9.8	13.6 ± 5.5	9.46 ± 6.9	12.5 ± 7.4
Monocyte TNF-α	60.3 ± 5.9	66.4 ± 7.4	47.2 ± 7.9* (p=.01)	56.7 ± 8.8	48.5 ± 10.6
Monocyte TGF-β	8.4 ± 7.7	7.9 ± 5.6	8.80 ± 4.4	12.1 ± 8.5	6.6 ± 3.4

Results (% of cells exhibiting positive staining measured by flow cytometry) are expressed as mean values ± SD *denotes significant difference from 35-45 year old age group, otherwise no significant difference between 45-55 age group and 35-45 age group; 55-65 age group and 35-45 age group; and males and females.

7-4 Discussion

In Chapter Four I reported increased rates of apoptosis of T-cells in the airways in COPD, consistent with reports by others (Majo et al. 2001; Segura-Valdez et al. 2000). I report here the novel finding of increased apoptosis of T-cells from the peripheral blood in COPD. The increase was not age or sex related.

A study in our laboratory reported that the detectable level of apoptosis of T-cells (identified by Annexin V staining) in peripheral blood samples is less than 1% (Hodge G. and Han. P. 2001). Direct comparisons of unstimulated blood samples between groups of subjects has not previously been reported due to the low levels of apoptosis detectable, which is likely a reflection of the rapid removal of apoptotic cells from the circulation. Such comparisons are thus likely to miss important differences between groups.

The present study utilised PHA stimulation of peripheral blood T-cells from COPD and control subjects, using flow cytometry and Annexin V and 7AAD staining methods. PHA been widely reported for studies of the potential of T-cells to undergo apoptosis when stimulated (Frassanito et al. 1998; Kaser et al. 1999; Alcouffe et al. 1999; Chen et al. 1998; McLeod et al. 1998; Novelli et al. 1997; Ito et al. 1997).

Common contaminants of cigarette smoke, such as LPS, induce an increase in T-cells in the airways in COPD (Sandstrom et al. 1994). This increase may result from local proliferation of T-cells in the lung or enhanced trafficking from the bloodstream (Tschernig et al. 2001; Schuster et al. 2000), although the rapidity of the increase following LPS instillation (within a few hours) suggests that enhanced entry from the

bloodstream is more likely (Pabst and Tschernig, 1997). This trafficking of T-cells also occurs from the airways to the bloodstream. Lymphocytes from the bronchoalveolar space have been reported to re-enter the lung tissue, migrate to regional lymph nodes and re-join the systemic immune system (Lehmann et al. 2001). Therefore, it is possible that increased apoptosis of T-cells in the peripheral blood in COPD may result from either local apoptotic stimuli in the airways or alternatively from apoptotic stimuli in the peripheral blood.

Activation of the Fas/FasL pathway induces apoptosis of mature CD4+ T-cells after repeated antigenic stimulation (Singer et al. 1994), thus the raised levels of Fas I report here may be significant for apoptosis induction in the peripheral blood in COPD (Van Parijs and Abbas, 1996).

Apoptosis of CD8+ T-cells can be mediated by either TNF-α or Fas pathways (Zheng et al. 1995). The effect of TNF-α is dependent upon which TNF receptor is activated, with TNF-RI and TNF-RII having pro and anti-apoptotic roles respectively. In the present study, production of TNF-α and expression of TNF-RI by peripheral blood T-cells was increased in COPD. These results are consistent with previous reports of increased circulating TNF-α in the peripheral blood in COPD (Takabatake et al. 2000). As there was a trend for increased increased T-cell production of TNF-α with age, further studies using age-matched controls are warranted.

Although in absolute terms the level of TNF receptors seen was low, the difference between the COPD and control groups was clear. The low levels may be a reflection of the sensitivity of the flow cytometry technique and does not discount the potential significance of the findings (Zola et al. 1990; Zola et al. 1992). Cytokine binding has

been shown to occur through receptors that need only to be expressed at low concentrations (around 100 molecules per cell) to transmit activation signals (Zola et al. 1992), which is well below the level of sensitivity of flow cytometry.

The TGF-β/ TGF-β receptor pathway enhances apoptosis of peripheral blood T-cells by inhibiting proliferation at G1 to S phase transition (Bright et al. 1997), inhibiting IL-2 induced expression of alpha and beta chains of IL-2R, and IL-2 induced activation of signal transduction molecules Jak-1 and Stat 5 (Bright et al. 1997). The findings of increased production of TGF-β by monocytes, increased release of active TGF-β and upregulated expression of TGF-β receptor by T-cells suggest that this cytokine may contribute to excess apoptosis of T-cells in the peripheral blood in COPD. This could be tested using blocking antibodies to TGF-β, and forms the basis for future studies. Interestingly, our laboratory has previously reported that plasma-derived factor VIII concentrate has apoptosis- promoting effects on T-cells (Hodge and Han, 2001). The presence of TGF-β was shown to be a major component responsible for the apoptotic effects seen in PHA-stimulated T-cells from haemophilia patients receiving factor VIII prophylaxis therapy (Hodge and Han, 2001).

Recent reports have shown that phagocytosis of apoptotic cells by macrophages leads to release of TGF-β (Fadok et al. 1998). Monocytes may therefore increase their secretion of TGF-β in the peripheral blood, as a result of ingestion of increased numbers of apoptotic cells, potentially explaining our findings of increased intracellular production of TGF-β by monocytes and secreted TGF-β in COPD.

Fourteen of eighteen COPD patients in this study were treated with inhaled corticosteroids (GCS). GCS-induced apoptosis is a well- recognised regulator of T-cell apoptosis and function. Dexamethasone, for example, has been shown to induce upregulation of Bax with mitochondrial membrane disruption, release of cytochrome c and caspase-3 mediated apoptosis in thymocytes (Yoshino et al. 2001). It is therefore possible that treatment of COPD patients with GCS may have contributed to the increased apoptosis in peripheral blood-derived T-cells. Consistent with this concept was the observation that three of the four COPD patients treated with bronchodilators alone showed no evidence of increased apoptosis of peripheral blood-derived T-cells

These inhibitory effects of GCS on T-cell proliferation and function are similar to those induced by TGF-β. It is possible that the effects of GCS are at least partially achieved through modulation of the expression of TGF-β. This is supported by reports that GCS, dexamethasone and cycloheximide, increase TGF-β mRNA in normal T-cells (Ayanlar Batuman et al. 1991).

The ratio of CD4:CD8 cells has important implications for the host response to infective and inflammatory stimuli. COPD is associated with a relative increase in CD8+ T-cells (Saetta et al. 1998), a finding that was confirmed in the present study. Reasons for this have not been determined. TNF-α and Fas have been shown to induce apoptosis of CD8+ T-cells (Zheng et al. 1995). These mediators were therefore investigated for CD4 and CD8 lymphocyte subsets. The increase in CD8+ T-cells could not be explained by a relative increase in apoptosis of CD4+ T-cells nor by relative changes in TNF-α and Fas. TGF-β, although well-recognised as a growth inhibitor of T-cells, has been demonstrated to be costimulatory for naïve CD8+ T-cells (Lee and Rich, 1993). Thus, increased TGF-β in the peripheral blood in COPD

as well as increased expression of TGF-R by CD8+ T-cells may partially explain the increase of CD8+ T-cells in COPD'

These raised levels of TGF-β may be relevent to the increased susceptibility to infection seen in COPD (Sethi, 2000). TGF-β has broad inhibitory effects on immune function and may increase susceptibility to opportunistic infections and malignancies (Letterio and Roberts, 1998). We can speculate that elevated TGF-β and TGF-β receptor expression in COPD may result in an increase in T-cell apoptosis following an infection. This would lead to a diminished immune response to the infective organism and contribute to the increased frequencies of infection, which are associated with the disease (Sethi, 2000).

In conclusion, I have demonstrated the novel finding of increased propensity of peripheral blood T-cells in COPD to undergo apoptosis. Whether this finding represents a systemic effect of COPD on peripheral cells, or whether these cells have re-entered the circulation after passing through the airway epithelium requires further study.

Chapter Eight

Apoptotic pathways in peripheral blood in COPD

8-1 Introduction

In the previous chapter I demonstrated that stimulated peripheral blood T-cells from patients with COPD have increased propensity to undergo apoptosis. These results were extended to examine the mechanisms by which T-cells in the peripheral blood undergo apoptosis.

The intracellular pathway to apoptosis may involve pro-apoptotic genes (eg, p53 and target genes situated on the downstream pathway of p53 transcription, such as BAX) as well as some genes (eg, Bcl2 and NFκB) and cytokines (eg, IL-2 and IL-7) that can inhibit apoptosis (refer Chapter One, Figure 1-11).

P53 is a nuclear protein that is upregulated in response to DNA damage (Hall et al. 1993; Di Leonardo et al. 1994). The damage may occur in response to cytotoxic drugs, viruses or oxidative stress associated with the generation of free radicals. The latter is particularly relevant to COPD, as increased oxidative stress (assessed by decreased Trolox equivalent antioxidant capacity (TEAC) and increased levels of products of lipid peroxidation) has been reported in the peripheral blood in COPD (Rahman et al. 2000; Rahman et al. 1996). These mediators induce phosphorylation of p53 leading to increased expression of the protein BAX. A BAX-generated reduction in mitochondrial membrane potential and subsequent permeabilisation, leads to activation of the caspase proteolytic cascade and

apoptosis (Kastan et al. 1991; Lowe et al. 1993). Alternatively, p53 may function by inhibiting cell division by arresting the cell cycle at the G1 stage (Di Leonardo et al. 1994).

The protein product of the proto-oncogene, Bcl-2, negatively regulates apoptosis by binding to BAX, and its expression is required for the continued survival of lymphocytes in vivo (Cory et al. 1995).

I hypothesised that alterations in expression of p53 and Bcl-2 may be important in determining the susceptibility of T-cells to undergo apoptosis in COPD. The regulation of T-cell apoptosis is complex and involves several cytokines, which may act via BAX and other pathways. IL-7, a product of stromal cells, plays a role in the continued survival of lymphocytes by upregulating Bcl-2 and inhibiting BAX-generated apoptosis (Hofmeister et al. 1999; Kim et al. 1998). IL-2 promotes the proliferation of T-cells following primary activation with antigen by binding to its high affinity receptor (Nelson et al, 1998). The common gamma chain of the high affinity IL-2 receptor complex is shared by other cytokines, including IL-4 and IL-7, so the collective ability of these cytokines to protect activated T-cells from death may be mediated by signals which include the common γ chain (Vella et al. 1998). Paradoxically, IL-2 also programs T-cells for activation-induced apoptosis following repeated antigenic stimulation (Lenardo, 1991). The mechanism has been shown to involve IL-2 upregulation of FasL expression on activated CD4+ and CD8+ T-cells and downregulation of IL-2Rγ on activated CD8+ T-cells (Dai et al. 1999; Refaeli et al, 1998).

Based on these reports, I further hypothesised that changes in T-cell expression of cytokines IL-2 and IL-4 or receptors, IL-7R IL-4R and IL-2Rγ may be important in

determining the susceptibility of peripheral blood-derived T-cells (CD4+, CD8+ or both) to undergo apoptosis in COPD.

To test these hypotheses I investigated expression of p53, Bcl-2, IL-2, IL-4, IL-2Rγ, IL-4R and IL-7R for peripheral blood-derived T-cells from COPD and control subjects.

8-2 Methods

8-2-1 Study population

Following informed consent, peripheral blood was collected into tubes containing sodium heparin (50 IU/ml) as anticoagulant from 11 patients with COPD (mean age 68). For 11 non-smoking volunteers, with no history of allergies or asthma, specimens were obtained and used as controls (mean age 42) (**Table 8-1**) There was overlap between these subjects and those characterised in Table 7-1. The diagnosis of COPD was established using the GOLD criteria (US Department of Health and Human Services, 2001) and ethics approval obtained as described in the previous chapter.

Table 8-1

Demographic characteristics of the population studied

Subjects	Control group	COPD
No. of subjects	11	11
Age (y)	42 ± 13	68 ± 9
Smoking, pack yr	0	75 ± 49
FEV1, % pred	117.3 ± 3.3	41.3 ± 17.9
FVC, % pred	107.0 ± 8.5	80.1 ± 18.5
FEV1,% FVC	93.5 ± 10.6	51.0 ± 18.8

Results are expressed as mean values.

8-2-2 Reagents

PE-conjugated monoclonal antibodies (Mabs) against IL-2Rγ (CD132, Pharmingen), IL-4 (BD), IL-4R (CD124) (Immunotech), IL-7R (Immunotech), p53 (Novo Castra Labs) and CD45 (BD) were used for flow cytometry. FITC-conjugated Mabs to IL-2 (BD), CD8 (BD) and Bcl-2(Dako) were also employed. PC-5-conjugated Mabs to the T-cell receptor, CD3, (Immunotech) were also included.

8-2-3 Stimulation of peripheral blood

Aliquots of 500 μl of blood were added to 500μl RPMI 1640 culture medium (Gibco), containing 1% weight per volume penicillin/ streptomycin (Gibco) in 10ml culture tubes, and incubated for 24 h, at 37^{O}C in 5%CO_2 in air. The samples were stimulated with 10μg/ml PHA for cytokine receptor, Bcl-2 and p53 analysis or 25ng/ml PMA (Sigma) plus 1μg/ml ionomycin (Calbiochem) for intracellular T-cell cytokine analysis. For cytokine investigation, cells were cultured in the presence of brefeldin A (1μg/ml) (Sigma) as a 'Golgi block' to inhibit intracellular transport and thus retain cytokines produced during activation inside the cell.

8-2-4 Staining with Mabs to surface markers

Staining and flow cytometric analysis was performed as described in the previous chapter. Briefly, 200 μl aliquots of blood were stained with 3 μl directly conjugated Mabs to surface markers of interest, lysed with FACSlyse (BD), washed, then acquired immediately by flow cytometry.

8-2-5 Staining for intracellular markers

Staining for cell type identification was carried out as described above. Nuclear antigens Bcl-2 and p53 were stained using a modification of a direct staining technique for a suspension of unfixed nuclei (Jinquan et al. 1999). This method avoids centrifugation and washing so nuclei are less likely to adhere to each other. Aliquots of 1 x 10^6 cells were added to FACS tubes, washed with calcium and magnesium- free PBS, and centrifuged at 300 x g for 5 minutes. Supernatant was discarded and 200 μl of Nonidet-based membrane lysing solution (995 ml calcium and magnesium free PBS, 5.0 ml 9.5% v/v NP-40 (Sigma), 0.186 g Na2 EDTA* 2H2O (0.5 nM), 5.0 g bovine serum albumin (0.5% W/V) added to the cell pellet. Tubes were mixed and left at room temperature, in the dark, for 15 min. Permeability of the cell membrane was checked with Trypan Blue using light microscopy. FITC-conjugated Mab to Bcl-2 or PE-conjugated Mab to p53 (10 μl) was added for 15 min at room temperature, in the dark. Irrelevant, conjugated antibodies of the same isotypes were used a negative controls. Analysis of unwashed, unfixed cells was carried out immediately by flow cytometry. T-cells were gated based on known staining characteristics with CD3 PC5 versus side scatter. CD8+ events were identified and CD4+ events identified by CD3+CD8- staining characteristics as described in the previous chapter.

8-2-6 Statistical analysis

The Wilcoxon non-parametric test for related samples was used to analyze the data, using SPSS software. p values ≤ 0.05 were considered significant.

8-3 Results

8-3-1 Cytokines and receptors in blood-derived T-cells in COPD

To investigate the role of IL-2 and IL-4 in apoptosis of peripheral blood T-cells in COPD, I measured production of IL-2 and IL-4 and expression of IL-4R (CD124) and IL-2Rγ (CD132, common γ receptor that is shared by other cytokines including IL-4 and IL-7) by flow cytometry. I found no significant difference in production of IL-2 or IL-4 or expression of IL-2Rγ or IL-4R for T-cells from COPD and control subjects (**Tables 8-2, 8-3**). IL-7R (CD127) expression by peripheral blood T-cells from subjects with COPD was significantly decreased compared to controls **(Table 8-3, Figures 8-1 (c))**. Based on this significant difference for CD127 expression by CD3+ T-cells, further analysis of CD127 expression by CD4+ and CD8+ T-cells was performed (**Figure 8-2**). CD127 expression was significantly decreased for all T-cell subsets tested, indicating that reduced expression of the anti-apoptotic IL-7R may play a role in the increased susceptibility of peripheral blood T-cells to undergo apoptosis in COPD.

8-3-2 p53 and Bcl-2 expression by blood-derived T-cells in COPD

To investigate the role of intracellular molecules that are involved in pro- and anti-apoptotic pathways (p53 and Bcl-2 respectively), I analysed expression of these markers by flow cytometry. There was no significant difference in expression of Bcl-2 by CD3+ peripheral blood T-cells from COPD subjects compared to controls (**Table 8-3, Figure 8-1**). P53 expression by CD3+ peripheral blood-derived T-cells from subjects with COPD was significantly increased compared to controls (**Table 8-3,**

Figure 8-1

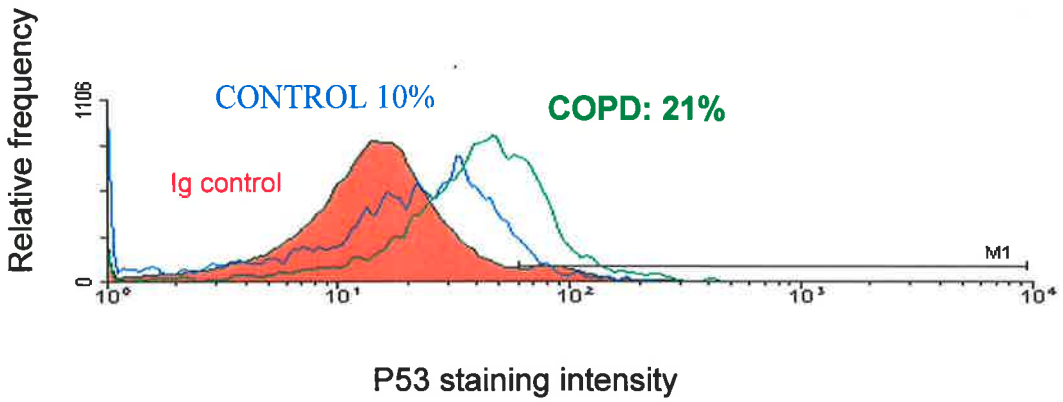
P53, Bcl-2 and IL-7R in blood-derived T-cells in COPD

(a) Representative histograms of p53 staining for CD3+ peripheral blood T-cells from COPD and control subject: significantly increased expression in COPD (p=0.021).

(b) Representaive dot plots of Bcl-2 staining for CD3+ peripheral blood T-cells from COPD and control subject: no significant difference between Bcl-2 expression for COPD and control groups.

(b) Representaive dot plots of CD127 staining for peripheral blood T-cells from COPD and control subjects. T-cells were identified by staining characteristics with CD3, as described in Chapter Seven. Further staining with FITC-conjugated CD8 and PE-conjugated CD127 allowed discrimination of CD4+ T-cells (negative staining with CD8) and CD8+ T-cells. The percentage of events from each subset that exhibited positive staining with CD127 was calculated: significantly decreased expression of IL-7R in COPD (p=0.028).

a.



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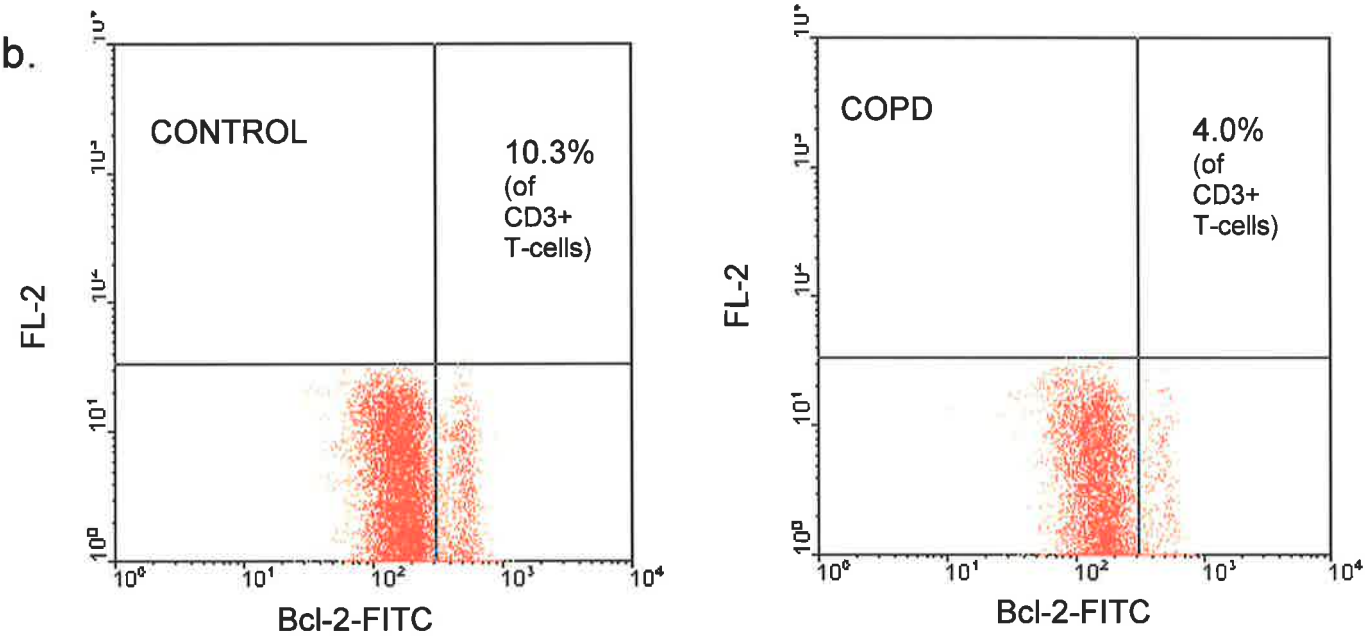


c.

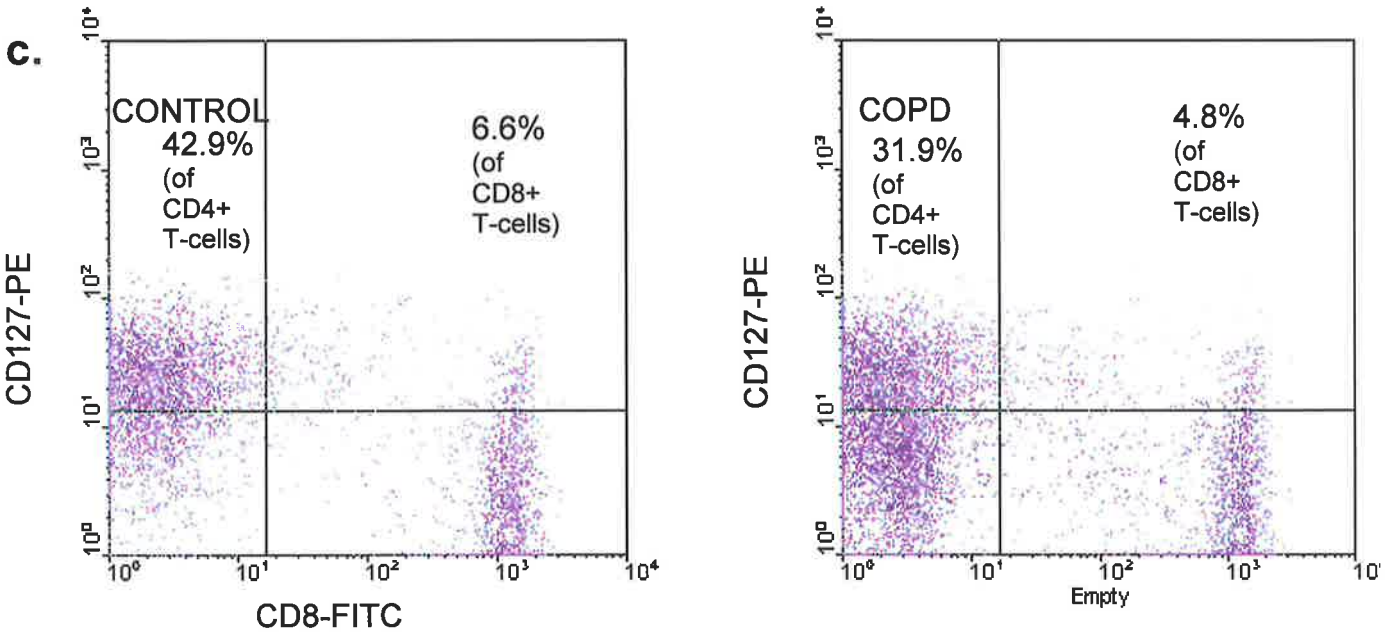


Figure 8-1

Figure 8-2). Based on the significant difference for CD3+ T-cells, further analysis of p53 expression by CD4+ and CD8+ T-cells was performed. P53 expression was significantly increased for all T-cell subsets tested (**Figure 8-2**), indicating that the p53 apoptotic pathway may play a role in the increased propensity of peripheral blood T-cells to undergo apoptosis in COPD.

Table 8-2

Cytokine production by PMA-stimulated T-cells

Cytokine	Control group	COPD	P value
IL-2	20.6 ± 17.1	12.9 ± 9.6	ns
IL-4	7.4 ± 5.1	10.4 ± 6.5	ns

Results (% of cells exhibiting positive staining measured by flow cytometry) are expressed as mean values ± SD.

Table 8-3

Expression of cytokine receptors and nuclear antigens by PHA-stimulated T-cells

Antigen	Control	COPD	p value C/COPD
Bcl-2	11.5 ± 1.8	10.2 ± 1.3	Ns
P53	13.7 ± 4.4	25.7 ± 6.3	.021 *
IL-2Rγ (CD132)	8.8 ± 9.2	12.1 ± 10.3	Ns
IL-7R (CD127)	57.7 ± 6.6	50.3 ± 8.3	.028 *
IL-4R (CD124)	2.4 ± 6.9	2.4 ± 2.5	Ns

Results (% of cells exhibiting positive staining measured by flow cytometry) are expressed as mean values ± SD *denotes significant difference from control group.

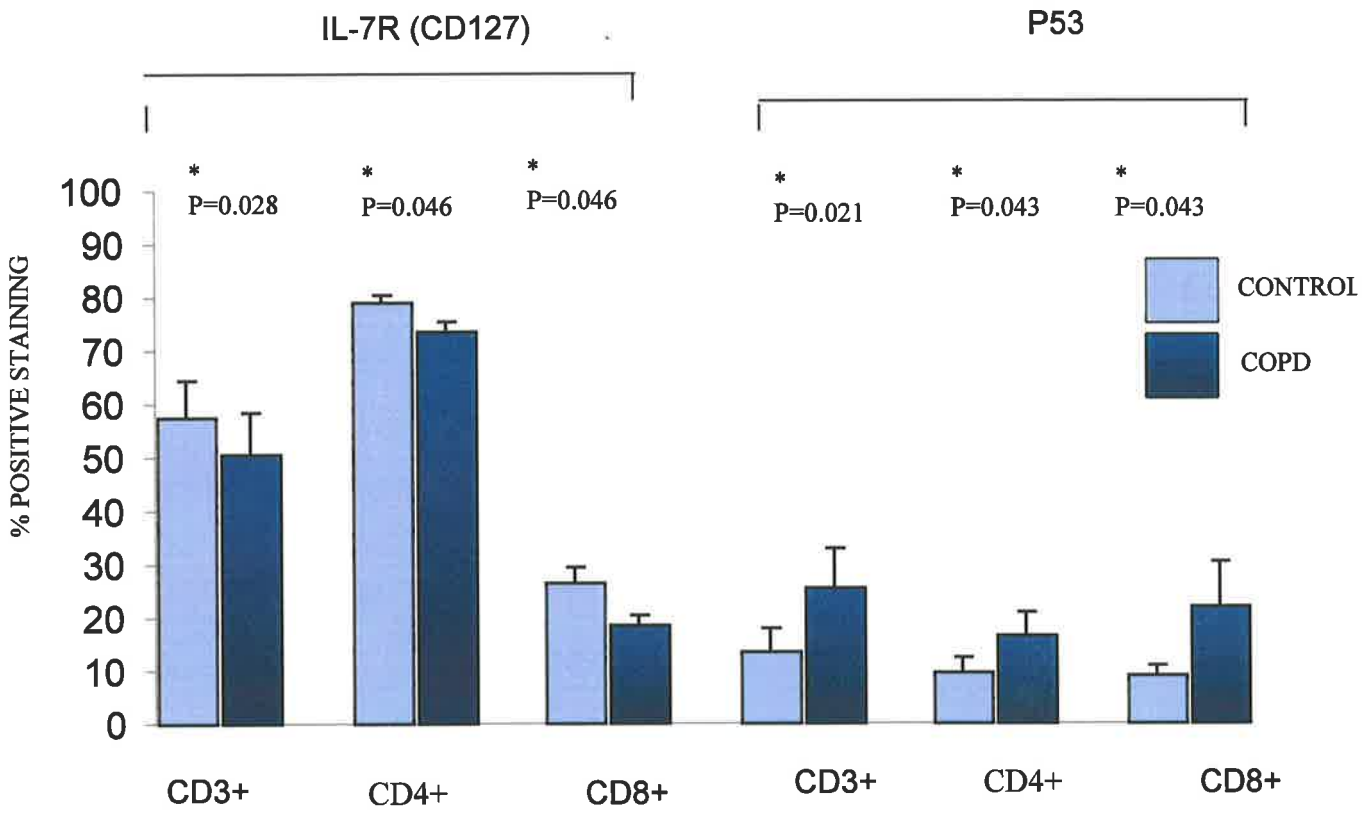


Figure 8-2

IL-7 receptor (CD127) and p53 in blood-derived CD4+ and CD8+ T-cells in COPD

Note significantly decreased expression of CD127 and increased p53 expression by all T-cell subsets. Results are expressed as mean values + SEM

* denotes significant difference from control group.

8-4 Discussion

Increased expression of p53 by peripheral blood T-cells in COPD has important implications in COPD. The p53-induced apoptotic pathway is separate from those induced by TGF-β, Fas and TNF-α, and its upregulation may further explain the increased susceptibility of T-cells to undergo apoptosis in COPD. Consistent with these findings in patients with COPD, increased p53 expression has been reportedly associated with apoptosis in type II pneumocytes in diffuse alveolar damage (Guinee et al. 1996). P53-regulated apoptosis can be induced by oxidative stress associated with the generation of free radicals, that include the superoxide anion (O_2^-, hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen (O_2) (Yin et al. 1998; Ueda et al. 2002). The potential for free radicals to induce apoptosis is thought to involve DNA damage and be regulated by intracellular levels of Bcl-2 and BAX (Korsmeyer et al, 1995). An imbalance between oxidants and antioxidants in the blood, associated with evidence of increased oxidative stress, has been reported in COPD (Rahman et al. 1996). Thus the findings of increased p53, an inducer of BAX, and increased apoptosis in the peripheral blood may at least partially result from the presence of free radicals in COPD.

Other cytokines, including IL-4 and IL-7, promote the survival of resting T-cells (Boise et al. 1995; Rathmell et al. 2001). IL-2 also promotes the proliferation and survival of activated T-cells by binding to its high affinity receptor. The common gamma chain of the IL-2R (IL-2Rγ) is shared by other cytokines including IL-4 and IL-7, and may therefore act as a signalling mechanism for protection of apoptosis in activated T-cells (Vella et al. 1998). However, there was no significant difference in IL-4 or IL-2 production or expression of the IL-4R or the IL-2Rγ for COPD and control

subjects in the present study, indicating that this protective mechanism may not play a role in COPD. IL-7 receptor expression by peripheral blood T-cells was significantly decreased in COPD, possibly indicating diminished IL-7 mediated protection from apoptosis in COPD. However, there was no significant change in expression of the anti-apoptotic protein, Bcl-2. IL-7 has been reported to protect T-cells from apoptotic death by preventing a decline in Bcl-2, suggesting that the Bcl-2/BAX ratio could be important in susceptibility of T-cells to undergo apoptosis (Kim et al. 1998). The findings of decreased IL-7R but no change in Bcl-2 are surprising in the context of the above report, although a recent *in vitro* study reported that Bcl-2 expression for T-cells undergoing apoptosis remained constant, indicating that Bcl-2 expression is insufficient to explain lymphocyte survival *in vivo* (Rathmell et al. 2001). In addition, activation of the TNF and Fas apoptotic pathways can trigger apoptosis independent of Bcl-2 (Strasser et al. 1995; Vanhaesebroeck et al. 1993; Memon et al. 1995). Given the tight relationship between Bcl-2 and Bax, it will be important in future studies to investigate Bax expression. It has been suggested that the ratio of Bax/Bcl-2 may be more important than the individual values for determining apoptosis (Stoetzer et al. 1996). This ratio will therefore be explored in future studies.

In summary, I found increased p53 but decreased IL-7R expression by peripheral blood T-cells in COPD suggesting a possible role for p53 and IL-7 pathways in apoptosis of peripheral blood-derived T-cells in COPD. Given these findings it would be interesting to apply these techniques to brushing-derived AEC in COPD in future studies. Upregulation of p53 (as well as increased apoptosis and upregulation of Fas and Fas ligand) has been reported in bronchial and alveolar epithelial cells in bleomycin-induced pulmonary fibrosis in mice, and intersitial pneumonia and idiopathic pulmonary fibrosis in humans (Kuwano et al. 1996; Kuwano et al. 2000;

Kunitake et al. 1998). One can speculate that similar mechanisms may be involved in the airways in COPD and this forms the basis for future studies.

Chapter Nine

Phase One: Production of cytokines by apoptotic AEC

9-1 Introduction

Mechanisms regulating epithelial repair following airway injury are largely unknown. Response to airway injury includes hyperplasia of AEC, which resolves when repair is complete. Apoptosis is considered to be the most efficient mechanism of removal of unwanted cells and is characterised by cell shrinkage and DNA fragmentation with minimal inflammatory response (Majno and Joris, 1995). The presence of TGF-β increases apoptosis and inhibits proliferation of AEC (Jetten et al. 1986).

Effective clearance of apoptotic cells prevents secondary necrosis and release of tissue damaging enzymes (Savill, 1997(1); Fadok et al. 1998(1)). Thus, apoptosis is followed by rapid phagocytosis of apoptotic fragments by AM (Fadok et al, 1998(1)). IL-4 also may function in an indirect role in the repair of injured epithelium by increasing epithelial cell expression of various chemokines such as IL-8 (Striz et al. 1999), and adhesion molecules such as ICAM-1 (Tosi et al. 1992) and VCAM-1 (Atsuta et al. 1997). Increased expression of adhesion molecules leads to increased AM chemotaxis to the site of injury and increased phagocytosis of apoptotic AEC. In this regard, IL-4 has been shown to increase AM phagocytosis (Capsoni et al. 1995; Raveh et al. 1998).

There have been a limited number of reports of production of cytokines by apoptotic cells. Lymphocytes undergoing FasL induced apoptosis have been shown to release IL-10, an anti-inflammatory cytokine, and TGF-β (Gao et al. 1998; Chen et al.

2001). IL-4 production by apoptotic mononuclear and tumour cells has been reported (Stein et al. 2000). AM which have ingested apoptotic cells produce inhibitory cytokines, such as IL-10 and TGF-β, a further demonstration of the immunoregulatory changes initiated by the apoptotic process (Fadok et al. 1998; Ronchetti et al. 1999).

Based on these reports, I hypothesised that, when AEC enter an apoptotic cycle, cytokines, such as TGF-β and IL-4, would be produced. Elevated local concentrations of these cytokines would act in an autocrine manner to control AEC hyperplasia and resolve inflammation following acute lung injury.

IL-6 was also investigated because, in contrast to IL-4 and TGF-β, it has well documented pro-inflammatory effects and because increased IL-6 levels have been reported in COPD (Hageman et al. 2003; Chung KF. 2001; Cromwell et al. 1992; Bhowmik et al. 2000). In addition, IL-4 has been shown to suppress IL-6 release by LPS stimulated AM (Zissel et al. 1996).

I thus further hypothesised that apoptotic cells would produce less IL-6 than viable cells, thus further explaining the anti-inflammatory nature of the apoptotic process

I further hypothesised that apoptotic cells would produce less IL-6 than viable cells, thus further explaining the anti-inflammatory nature of the apoptotic process.

This hypothesis was investigated using an Annexin V binding assay to distinguish viable and apoptotic cells. Flow cytometric techniques were used to measure cytokine production by AEC undergoing early apoptosis in a bronchial epithelial cell line (16HBE) in an *in vitro* culture model. I reasoned that production of IL-4 and TGF-β by AEC would facilitate control of proliferation, and removal by apoptosis, following acute lung injury. Cytokine production and release would thus play a direct role in resolving inflammation of the lung epithelium.

9-2 Methods

9-2-1 Cytokines and Mabs

Anti-human TGF-β antibody (unconjugated) was obtained from R & D. Rat anti-mouse IgG1 Mab, conjugated to PE, was obtained from BD. PE conjugated IgG1 Mab (BD), and unconjugated IgG1 Mab (Dako) and anti-human cytokeratin 5/6 (Boehringer) were used as negative controls. Anti-human IL-4 and IL-6 Mab (PE-conjugated) were obtained from Pharmingen. Recombinant IL-4 was obtained from Sigma.

9-2-2 Cell culture and preparation of cells

A 16HBE human transformed normal bronchial epithelial cell line was used as an *in vitro* model. The cell line was maintained as described in 6-2-2 and experiments were carried out between passages 20 and 45. Trypsin-treated cells were re-seeded, to a density of 4×10^6 /ml in a final volume of 2ml culture medium, into 10ml culture tubes. One batch was cultured for 24h in the presence of brefeldin A (1μg/ml)(Sigma) as a 'Golgi block' to inhibit intracellular transport and thus retain cytokines produced intracellularly during activation. For experiments investigating the production of soluble active TGF-β and IL-6, another batch was cultured without the 'Golgi block'. Cells were removed from tubes by gentle pipetting with a 29-gauge needle (BD). This method has been shown to result in minimal cell damage (refer Appendix). Sorting was carried out within 1h of labelling. Sorted cells were washed twice in calcium free buffer to remove Annexin V (Annexin V binding is calcium dependent) and then re-suspended in culture medium without foetal calf serum to

equal cell concentrations. Analysis of intracellular cytokine production was carried out within 1h of labelling on the cells cultured in the presence of brefeldin A. After a further 24h culture, the supernatant from tubes which did not contain brefeldin A were collected, frozen at -70^{0}C and thawed immediately prior to assay for secreted TGF-β and IL-6 levels.

9-2-3 Sorting of apoptotic and viable cells

Prior to sorting, cells were stained with Annexin V as described in Chapter Three. Briefly, cells were washed once with HEPES buffer, centrifuged (300 × g for 5 min), and resuspended in 100 μl HEPES buffer. Cells were stained with 1 μl FITC-conjugated Annexin V (Pharmingen) for 15min, washed again and re-suspended in 2 ml HEPES buffer. Apoptotic and viable cells were sorted using a FACstar PLUS on the basis of bright and dim Annexin V staining (**Figure 9-1**), into culture medium (**Figure 9-2**).

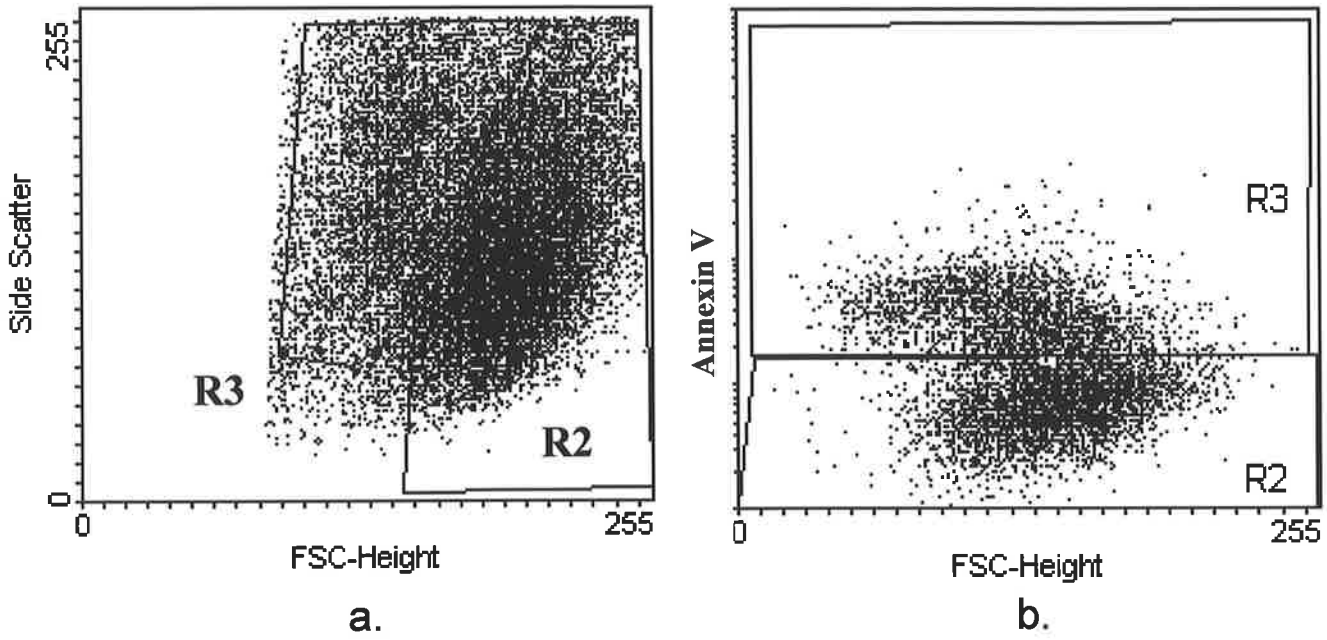


Figure 9-1

Identification of Viable and Apoptotic cell populations for sorting by flow cytometry

a. Representative dot plot showing light scatter characteristics of 16HBE AEC after 24h culture. Apoptotic cells lose water (shrink) and subsequently display decreased FSC. Viable cells are defined in region R2 and smaller, apototic, cells are defined in region R3. (Cells were originally gated in a region (R1), which excluded debris).

b. 16HBE AEC were stained with FITC-conjugated Annexin V staining (shown on the Y axis). Cells that were negative for Annexin V staining were considered Viable (R2) and cells that stained positive with Annexin V Apoptotic (R3). Cells were sorted on the basis of this staining into two tubes, prior to washing with calcium free culture medium and adjusting to equal cell concentrations.

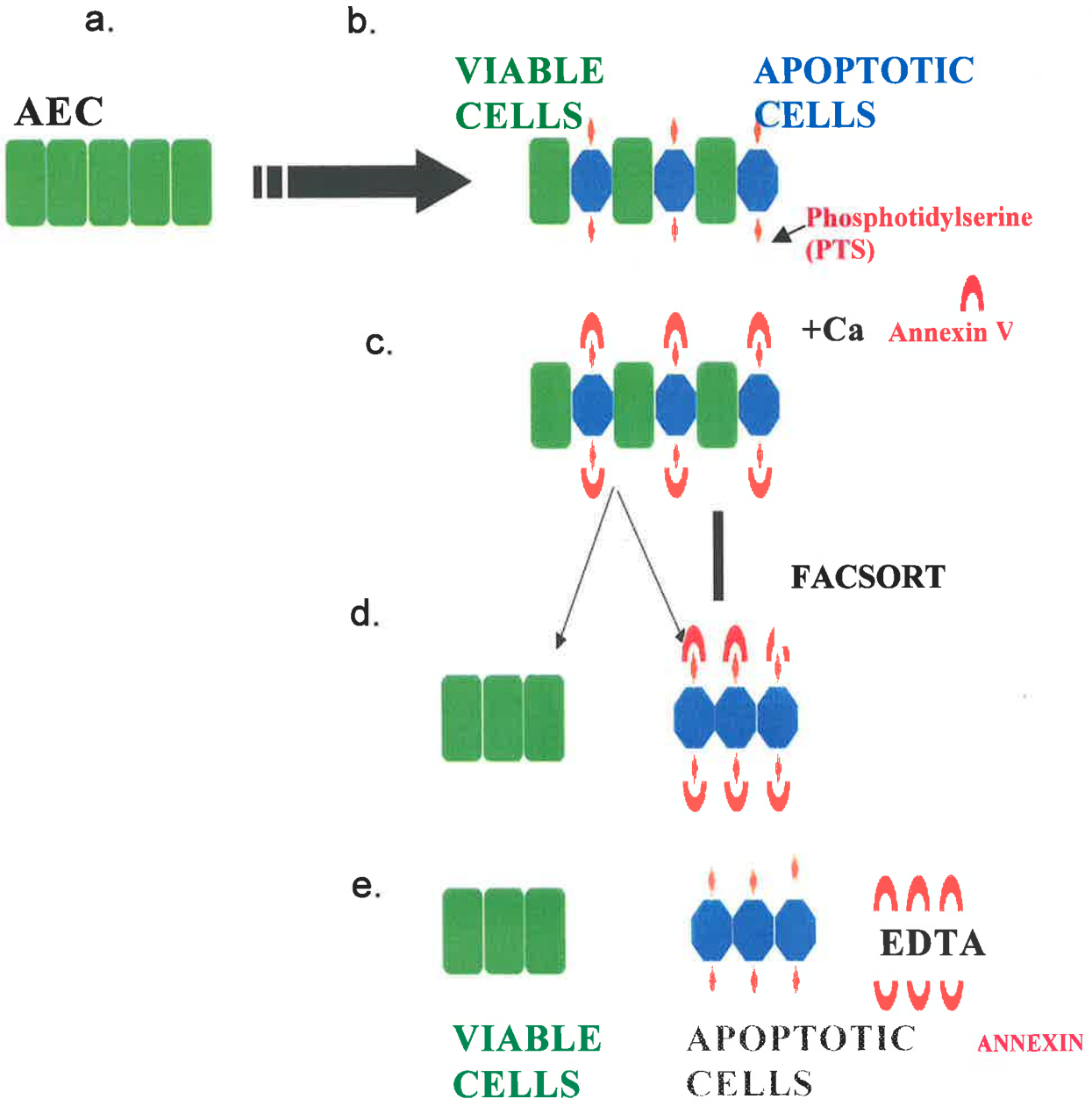


Figure 9-2 Sorting of viable and apoptotic 16HBE AEC by flow cytometry

a. 16HBE AEC cultured alone (for soluble cytokine analysis) or in the presence of brefeldin A (for intracellular cytokine analysis) or aFas (to induce apoptosis).

b. Apoptotic cells express phosphotidylserine (PTS) on the cell surface.

c. PTS was stained with a specific marker, Annexin V, in the presence of calcium.

d. Viable and apoptotic cell populations were sorted based on staining characteristics with Annexin V (as shown in **Figure 9-1**).

e. Annexin V was removed by washing with calcium free buffer.

9-2-4 Confirmation of purity of sorted cell populations

The purity of the sorted cell populations was confirmed by staining with Annexin V. The morphology of sorted cells was examined by cytospin preparations to investigate whether the morphology was consistent with that typical of viable and apoptotic cells. Analysis of necrotic cell contamination was carried out pre- and post-sorting and after further culture, based on bright staining with PI and dim staining with Annexin V (Vermes et al. 2000) Cells were stained with Annexin V as described above. PI (5 μg/ml) was added prior to acquisition by flow cytometry.

9-2-5 Intracellular TGF-β: Flow cytometry

Sorted cells (200 μl) were stained with for TGF-β production using an indirect staining procedure as described in Chapter Five. As negative contols, an isotype matched control and cytokeratin (CK) 5/6 (which was shown immunochemically to be absent from these cells) were included. 10,000 stained events were acquired immediately by flow cytometry.

9-2-6 Intracellular IL-4 and IL-6: Flow cytometry

The percentage of positive events and the median fluorescence intensity (MFI), reflecting intracellular IL-4 and IL-6 production was evaluated using directly conjugated monoclonal antibodies (Pharmingen) as described in Chapter Five.

9-2-7 Flow cytometric analysis

The experiments were processed under identical settings and results expressed as a percentage of cells exhibiting positive fluorescence. Appropriate isotype matched

controls as well as cytokeratin 5/6 were used strictly as the basis for quadrant marker setting for analysis of cytokine production. The quadrant marker settings for live and apoptotic cell populations differed because the apoptotic cells demonstrated more autofluorescence and non-specific staining than their viable counterparts. Median fluorescence intensity (MFI) for test samples was corrected for autofluorescence and non-specific binding of Ig to Fc receptors by subtracting the MFI of the isotype matched negative control.

9-2-8 Blocking of binding sites on antibody with recombinant cytokine

As a single 'proof of principle' that Mabs can be used for specific staining of apoptotic AEC, anti-human PE- conjugated IL-4 Mab (Pharmingen) was pre-incubated with an equal volume of recombinant IL-4 (Sigma) for 30 min to block binding sites on the antibody. The Mab was then used to stain permeabilised apoptotic AEC as described above.

9-2-9 Induction of apoptosis by Fas ligation

To investigate the effect of stimulated apoptosis, trypsin-treated cells were reseeded, to a density of 4×10^6 /ml in a final volume of 2ml culture medium, into 10ml culture tubes in the presence of 500ng/ml aFas (Sigma) and brefeldin A (1μg/ml)(Sigma) for 48h. Control cells were cultured for 24h in the presence of brefeldin A. Apoptosis and intracellular cytokine production were evaluated as described above.

9-2-10 Soluble TGF-β and IL-6: ELISA

Culture supernatants were decanted and stored at -20^{0}C prior to testing. 'Active' TGF-β (ie, non-polymerised) and IL-6 were quantified with Quantikine immunoassay kits (R & D) following instructions supplied by the manufacturer.

9-2-11 Immunohistochemistry (IHC) and electron microscopy (EM)

Cells were cultured in the presence of brefeldin A as described above. One aliquot was prepared for EM. Cytospin cell smears were prepared from the second aliquot for IHC staining for TGF-β. A third aliquot was stained with Annexin V, sorted as described above and cytospin preparations prepared from the sorted cells.

For EM, 8 x 10^6 AEC were fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer at pH 7.2 for a minimum of 30 min. Cells were post-fixed in 2% osmium tetroxide in 0,1M sodium cacodylate buffer with 6% sucrose for 1h. Saturated uranyl acetate in 70% methanol was added for 15 min, followed by dehydration in three changes of 100% methanal for 2h. Cells were left in the presence of propylene oxide for 30 min, followed by 1:1 propylene oxide: resin mixture for 30 min. Infiltration with two changes of resin over 2 h was followed by embedding the cells in capsules filled with fresh resin. Capsules were left in oven at 70^{0}C overnight. The blocks were ultra-thin sectioned and EM photography carried out by the Division of Tissue Pathology, IMVS, Adelaide.

IHC for the presence of TGF-β was carried out as described in the previous chapter. Red staining intensity was assessed semi-quantitatively and scored as follows: 100 cells were counted and the percentage of cells exhibiting positive intracellular staining for TGF-β1 was recorded. In addition, the level of staining for individual cells

was scored as; 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. A scoring index was calculated as: $\sum$ (number of cells $\times$ score).

9-2-12 Statistical analysis

The Wilcoxon Signed Rank test was used to analyze the paired data. This analysis was performed using SPSS software, *P* values ≤ 0.05 were considered significant.

9-3 Results

9-3-1 Confirmation of purity of sorted cell populations

Under the culture conditions described, in the presence of brefeldin A, 45.5% (± SD 13.1) of cells were spontaneously entering apoptosis, confirmed by staining with Annexin V. Necrosis was evident in 7.8% (± SD 3.5) of cells. Viable and apoptotic populations were isolated by flow cytometric sorting, based on Annexin V staining. A combination of Annexin V positive/ PI negative staining, applied post-sorting, confirmed that the 'apoptotic' population contained greater that 90% apoptotic cells and that 'necrotic' cell contamination of both viable and apoptotic cell populations was less than 10%. Cytospin preparations confirmed that the morphology was consistent with that typical of viable and apoptotic cells. Reculture of both viable and apoptotic cell populations for ELISA testing to measure cytokine levels in supernatants resulted in a similar increase in the proportion of necrotic cells (<20%).

9-3-2 Increased TGF-β and IL-4 production by apoptotic cells: Flow cytometry

Flow cytometry was used to evaluate the percentage of cells exhibiting intracellular staining for TGF-β ("total active + latent TGF-β"), IL-4 and IL-6 for viable and apoptotic cells from 16HBE cells, a cultured epithelial cell line. 16HBE epithelial cells expressed TGF-β and IL-4 constitutively. A significantly higher proportion of apoptotic cells exhibited positive intracellular staining for IL-4 (20.0% ± SD 7.5 vs 4.0% ± SD 3.1; p=0.012) and TGF-β (12.9% ± SD 5.2 vs 3.4% ± SD 2.4; p=0.018) compared with viable cells (**Figure 9-3**). The amount of TGF-β and IL-4 produced per cell was also increased as indicated by a significant increase in median

fluorescence intensity (**Figure 9-4**). In contrast, apoptotic AEC exhibited lower percentage staining for IL-6 (18.7% ± SD 15.1 vs 57.6% ± SD 25.4; p=0.018) and lower MFI than viable cells (**Figures 9-3, 9-4**).

9-3-3 Blocking of binding sites on antibody to IL-4 with recombinant cytokine

Specificity of the staining of apoptotic AEC with Mabs was confirmed by blocking of binding sites on the IL-4 Mab with rhIL-4. In three separate experiments, more than 95% antibody neutralisation was achieved, thus proving the principle that flow cytometry and staining with directly-conjugated Mabs can be used for specific staining of apoptotic AEC (**Figure 9-5**).

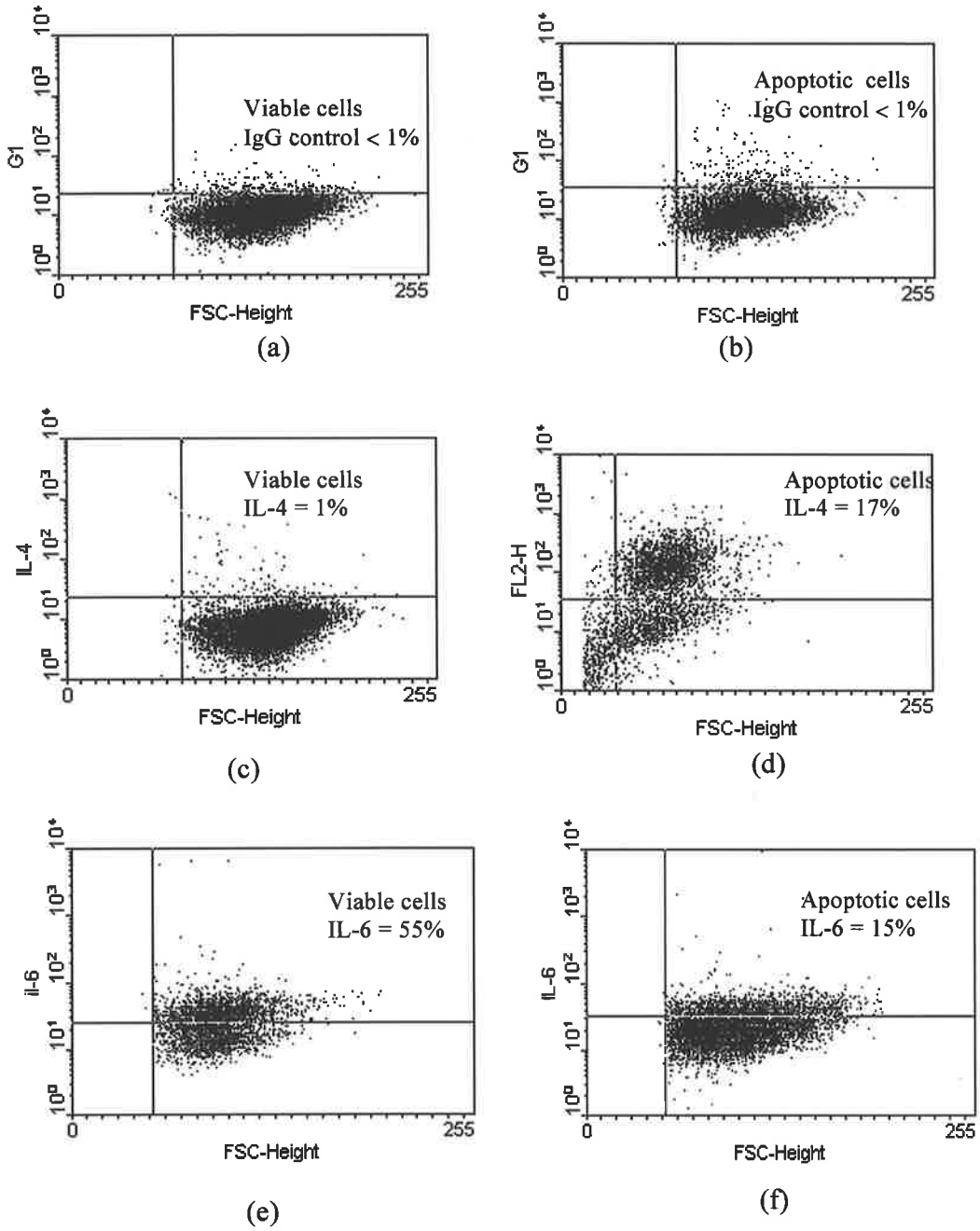


Figure 9-3

Cytokine production by viable and apoptotic AEC: Representative experiment. Note increase in non-specific staining by apoptotic cells and establishment of quadrant markers based on control staining. Cells were sorted on the basis of Annexin V staining, washed with calcium free culture medium and adjusted to equal cell concentrations. Quadrants were based on < 2% staining for viable and apoptotic cells stained with irrelevant IgG control

(a) viable cells: IgG control (b) apoptotic cells: IgG control (c) viable cells: IL-4 = 1% (d) apoptotic cells: IL-4 = 17% (e) viable cells: IL-6 = 55 % (f) apoptotic cells: IL-6 = 15%

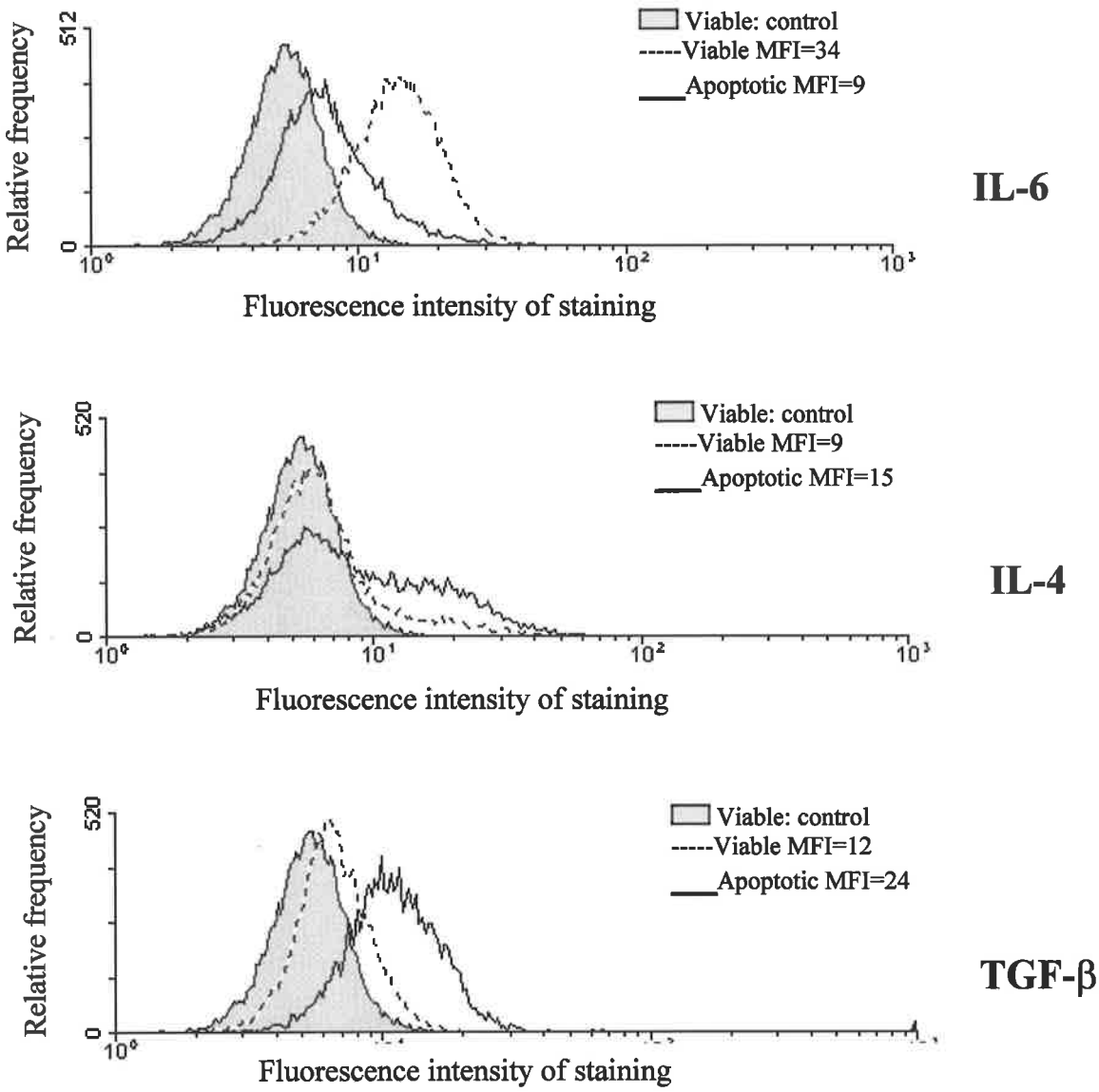


Figure 9-4

Cytokine production by 16HBE AEC

Histograms showing Median Fluorescence Intensity (MFI). Cells were sorted on the basis of positive Annexin V staining (apoptotic cells) and negative Annexin V staining (viable cells). Note increased apoptotic cell MFI for TGF-β and IL-4 but reduced MFI for IL-6.

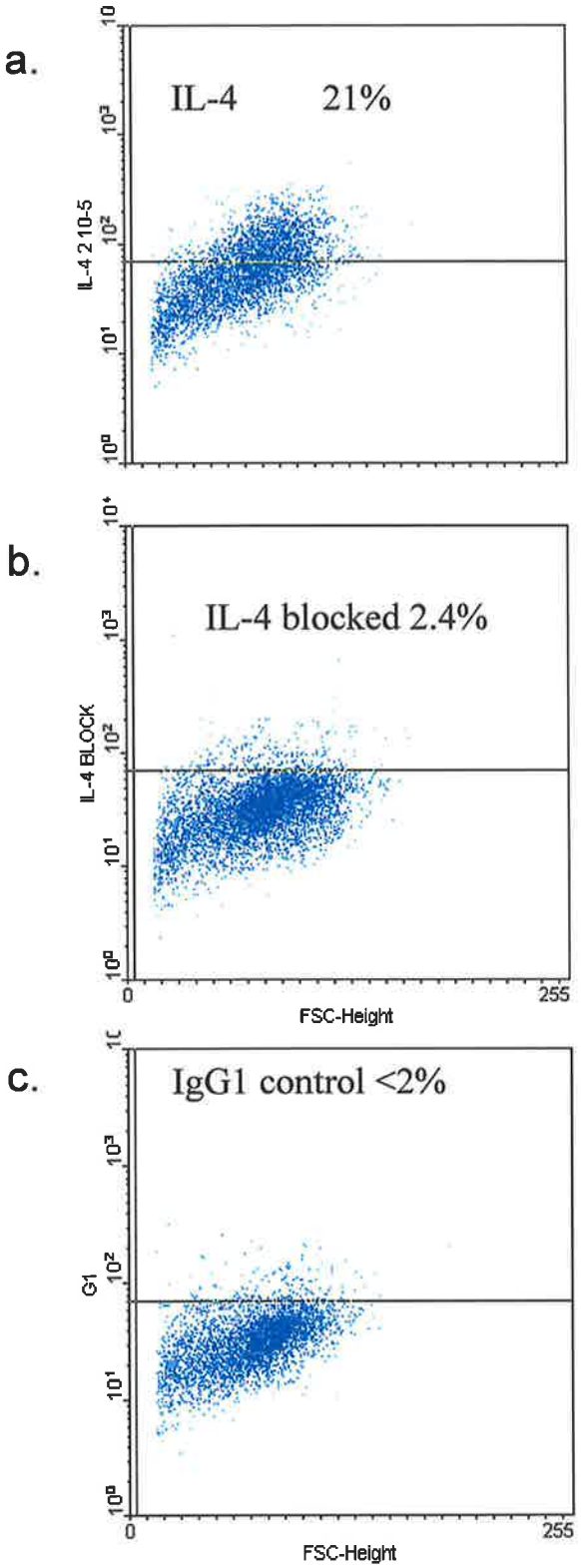


Figure 9-5 Specificity of staining of apoptotic cells with Mabs.

IL-4 Mab was pre-incubated with an equal volume of recombinant (rh) IL-4 for 30 min before staining for IL-4 production. Dot plots from representative experiment show: (a) IL-4 production 21% (b) Blocking of IL-4 antibody with rh IL-4 resulted in almost complete negation of IL-4 staining to similar levels as IgG1 control (<2%;in (c.)).

9-3-4 Increased TGF-β production by apoptotic cells: IHC and EM

To further test my hypothesis, I applied IHC staining for intracellular TGF-β. Small AEC, showing characteristics of apoptotic cells by electron microscopy (**Figures 9-6 (a),(b)**) demonstrated significantly greater intracellular production of TGF-β than viable cells (**Figure 9-7**). Sorted apoptotic cells displayed significantly more intracellular staining for TGF-β than viable cells (mean scoring index 148 ± SD16 for apoptotic vs 84 ± SD 42 for viable, p=0.043). (IHC staining for cytokeratin 5/6 was negative but positive for cytokeratin 19: These were included as specificity controls: data not shown). Staining was not observed when the anti-TGF-β Mab was omitted from the IHC staining protocol or when a monoclonal antibody of the same isotype (which did not react with any of the components of the 16HBE cells) was used in place of anti-TGF-β.

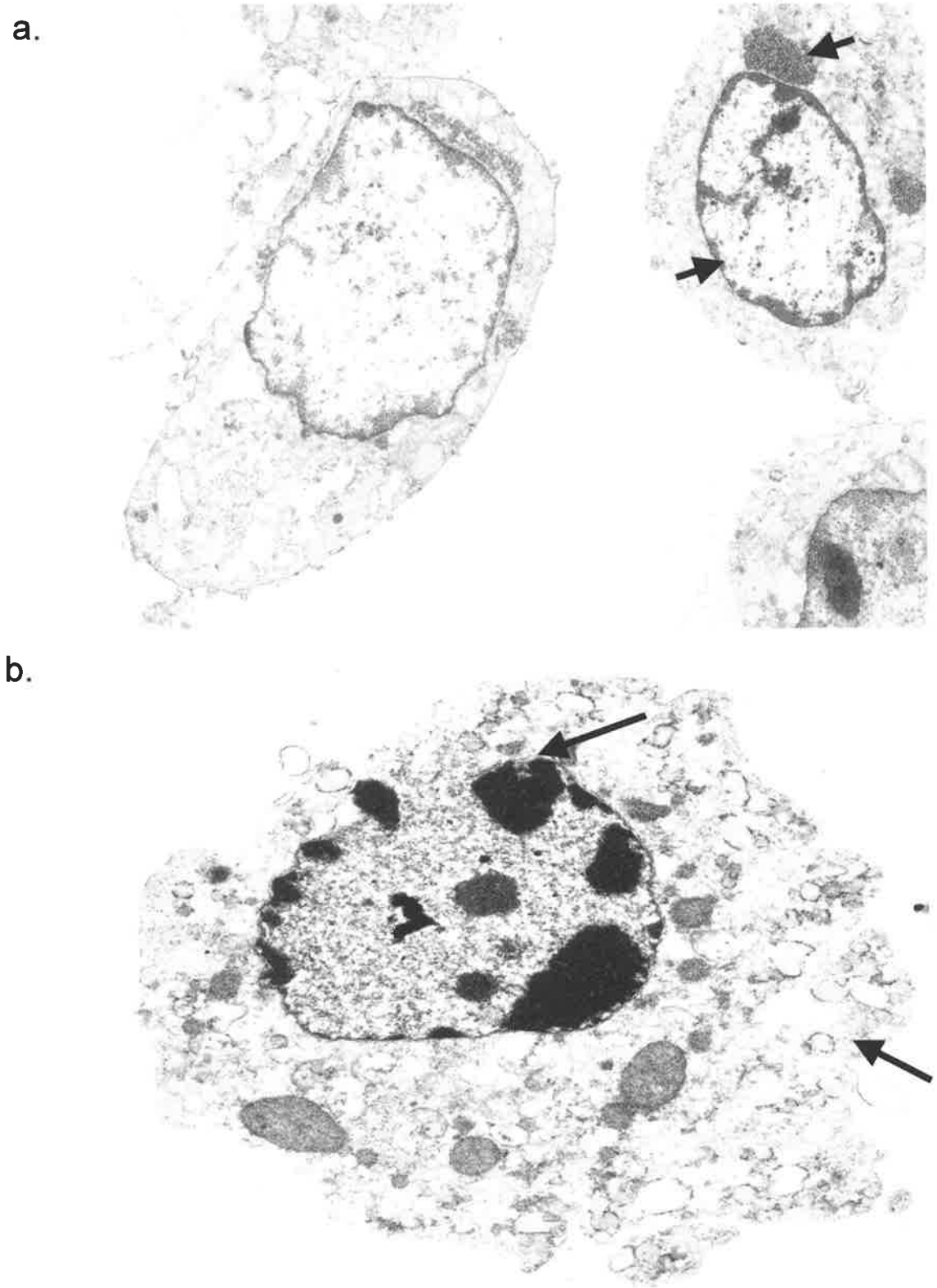


Figure 9-6

Unsorted and apoptotic AEC: EM (a) Unsorted AEC. arrows denote cells with features of apoptosis including cell shrinkage and chromatin condensation (b) Apoptotic AEC showing features of late apoptosis including chromatin condensation and early formation of apoptotic bodies with intact membrane.

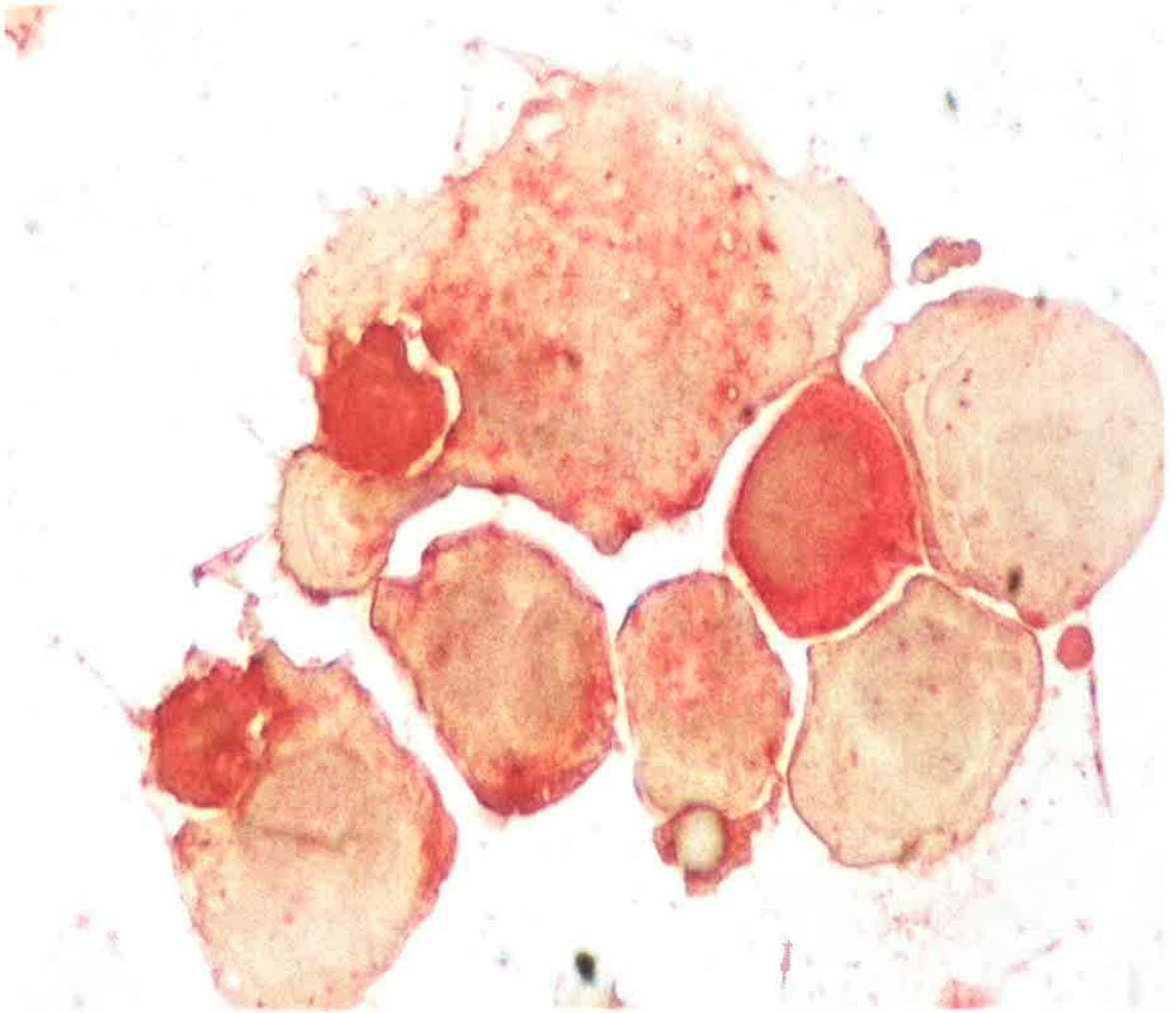


Figure 9-7

TGF-β in unsorted AEC: IHC

Smaller cells show increased staining for TGF-β and features of apoptosis, including cell shrinkage. Note the larger AEC appearing to engulf the smaller apoptotic cell. This was uncommonly seen, and correlates with reports in the literature of epithelial cells exhibiting phagocytic activity on apoptotic neighbours (Fadok VA. 1999).

9-3-5 Increased release of TGF-β by apoptotic AEC: ELISA

To further test my hypothesis, I investigated whether apoptotic 16HBE AEC released active TGF-β into culture medium after 24h incubation. Cultured apoptotic AEC secreted moreTGF-β than viable cells (383 pg/ml ± SD 90 vs 241 pg/ml ± SD 88; p=0.028). In contrast, in supernatants from viable cells, higher levels of soluble IL-6 were detected than for supernatants from apoptotic cells (106 pg/ml ± SD 66 vs 25 pg/ml ± SD 11; p=0.043).

9-3-6 Fas ligation: Increased TGF-β and IL-4 production and decreased IL-6 production

Between 70% and 85% of 16HBE cells, cultured in the presence of 500ng/ml aFas and brefeldin A for 48h, demonstrated positive staining with Annexin V, consistent with apoptosis. I investigated intracellular cytokine production by aFAS treated cells compared to those which had been cultured for 24h in the presence of brefeldin A alone. TGF-β and IL-4 production was higher for cells cultured in the presence of brefeldin A and aFas when compared to cells cultured in the presence of brefeldin A alone (TGF-β 15.5 % ± S.D. 0.6 vs 9.3% ± S.D.5.2; p=0.046, and IL-4 21.9% ± S.D. 6.3 vs 15.4% ± S.D.3.9; p=0.028). IL-6 production was lower in those cells cultured in the presence of aFas (25.7% ± S.D.8.8 vs 41.1% ± S.D. 4.8; p=0.030).

Chapter Nine Phase Two

9-4 Investigation of activation of NFκB in apoptotic AEC

9-4-1 Introduction

The next logical step was to investigate the production of cytokines by apoptotic AEC. Increased cytokine production results from increased gene expression, regulated by transcription factors (proteins that bind to DNA). Transcription factors are usually activated by phosphorylation by several types of kinases. This occurs under the influence of extracellular mediators, via surface receptors. Several families of transcription factors exist. Many are common to several cell types whereas others are cell specific (Barnes and Adcock, 1998). Common intracellular signaling pathways include the transcription factors nuclear factor-κB (NF-κB), activator protein-1 (AP-1) as well as the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) family (Barnes and Adcock, 1998) (**Figure 9-8).**

NFκB is a ubiquitously expressed transcription factor that regulates IL-6 genes, as well as those for IL-1α, IL-1β, IL-2, IL-3,IL-8 IL-12, GM-CSF, and MCP-1 (May and Ghosh, 1998) (**Figure 9-8**). NFκB is complexed to IκB in the cytoplasm. In response to stimuli such as cytokines, LPS and viral infections, phosphorylation of IκB induces dissociation of the NFκB/IκB complex. This allows free NFκB to translocate to the nucleus and function as a transcription factor that promotes the expression of a wide range of genes encoding cytokines. **Figure 9-8.**

a. NF-κB pathway

In unstimulated cells NF-κB is localised to the cytoplasm bound to IκB. When the cell is stimulated by a variety of inflammatory signals, including cytokines, chemokines, enzymes, adhesion molecules, viruses (rhinoviruses) and oxidative stress (O_3, NO_2, O_2), specific IκB kinases (IKK) phosphorylate IκB, inducing dissociation of the NFκB/IκB complex. This allows free NFκB to translocate to the nucleus where it binds to κB sites in the promoter regions of inflammatory genes encoding cytokines that include TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-8 IL-12, GM-CSF, and MCP-1.

These cytokines may then participate in an amplifying loop resulting in further activation of NFκB.

b. AP-1 pathway

Activation of map kinases (MEKK) leads to activation of Jun N-terminal kinase (JNK) and activation of AP-1. Activation may be induced by phorbol esters ((eg, PMA) via activation of protein kinase C (PKC)) and by various cytokines including TNF-α and IL-1β.

c. JAK-STAT pathway

Cytokines binding to their receptor results in the activation of Janus kinases (JAK) that phosphorylate intracellular domains of the receptor, resulting in phosphorylation of signal-transduction-activated transcription factors (STATs). Activated STATs dimerise and translocate to the nucleus where they bind to a recognition element on certain genes.

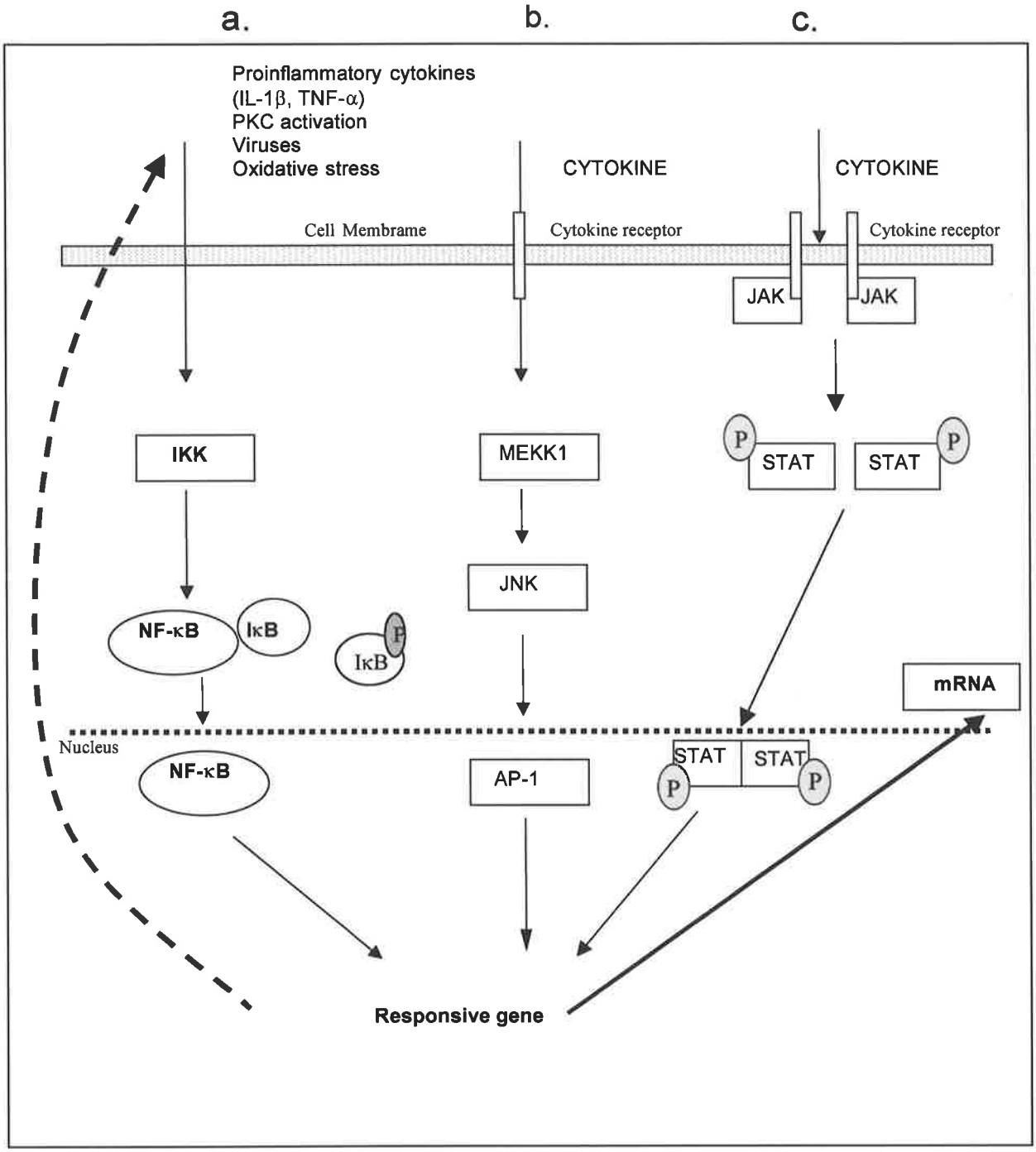


Figure 9-8

Pathways to cytokine production

Adapted from Barnes PJ. (1998) *Eur Respir J* **12**:224-225

Alternative pathways may be involved in production of TGF-β and IL-4 by AEC. Studies of the pathways to TGF-β gene expression in human fibroblasts, keratinocytes and endothelial cells (Martin et al. 1997) showed that TGF-β production was mediated by activation of the AP-1 in these cells (**Figure 9-8**). The AP-1 pathway has also been reported to mediate production of IL-4 in T-cells; production of IL-4 was regulated by cytoplasmic nuclear factor of activated T-cells (NF-AT). Activation of T-cells results in activation of this transcription factor, binding to the phosphatase calcineurin and transportation into the nucleus. Here, a transcriptional complex is formed with AP-1 that induces gene expression.

These families of transcription factors may interact with each other (Barnes and Adcock, 1998). For example, crosstalk between the AP-1 pathway and other cell-specific transcription factors may result in activation of the NFκB pathway (**Figure 9-9**).

Due to time restraints and access to antibodies, only one pathway, the NFκB pathway, was investigated, for viable and apoptotic AEC. As the published literature has identified this pathway for the production of IL-6 in viable cells, I was especially interested in comparing the nuclear and cytoplasmic expression of NFκB (as an indicator of nuclear translocation) with regard to the production of IL-6 by viable AEC. This pathway was of further interest with regard to the possible crosstalk between the AP-1 and NFκB pathways that might result in increased activation of the NFκB pathway in apoptotic AEC (in association with production of IL-4 and TGF-β).

Previous methods for investigation of NFκB activation have used immunofluorescence staining of PBMCs, Western Blot analysis or electrophoretic mobility shift assays (Segain et al. 2000; Pyatt et al. 1999). An alternative method to simultaneously identify cell type and NFκB activation would avoid cell purification techniques that have been shown to activate certain cell types resulting in upregulation of cytokine mRNA levels that may involve activation of NFκB (Hartel et al. 2001; Hodge et al. 2000).

Flow cytometry offers the ability to identify cell types and quantify specific cytokine production in a wide range of body fluids and cultured cells. Cell signal transduction molecules have been quantified using flow cytometry (Girdler et al. 2001; Chow et al. 2001). Specifically, NFκB activation has been identified by flow cytometry using a Mab that recognised an epitope within the NFκB nuclear localisation region that is exposed on activation (Pyatt et al. 1999).

Saponin has been reported to be a selective permeabilisation reagent for cell cytoplasmic membranes (Hughes et al. 1987) whereas other detergents such as Tween (utilised in commercial cell permeabilisation preparations such as Facsperm (BD)) permeabilise both nuclear and cytoplasmic membranes (Schmid et al. 1991) but maintain surface and intracellular antigenicity. I therefore developed a flow cytometric assay to measure simultaneous expression of nuclear and cytoplasmic NFκB (increased ratio of nuclear: cytoplasmic NFκB indicates activation and nuclear translocation of NFκB) in AEC. Using this assay, I investigated whether the NFκB pathway was involved in cytokine production by apoptotic and viable AEC.

Figure 9-9

Crosstalk between NF-κB and AP-1 transcription factors

a. TNF-α binding to TNFRII activates TNF-associated factor-2 (TRAF-2) which then activates NF-κB inducing kinase (NIK), which leads to activation of NF-κB via activation of IκB kinase complex (IKK) and phosphorylation of IκB. The free NF-κB then translocates to the nucleus, where it binds to κB sites in the promoter regions of inflammatory genes.

b. TRAF-2 may also activate a mitogen-activated protein (MAP) kinase enzyme, MEKK1, which leads to activation of Jun N-terminal kinase (JNK) and AP-1.

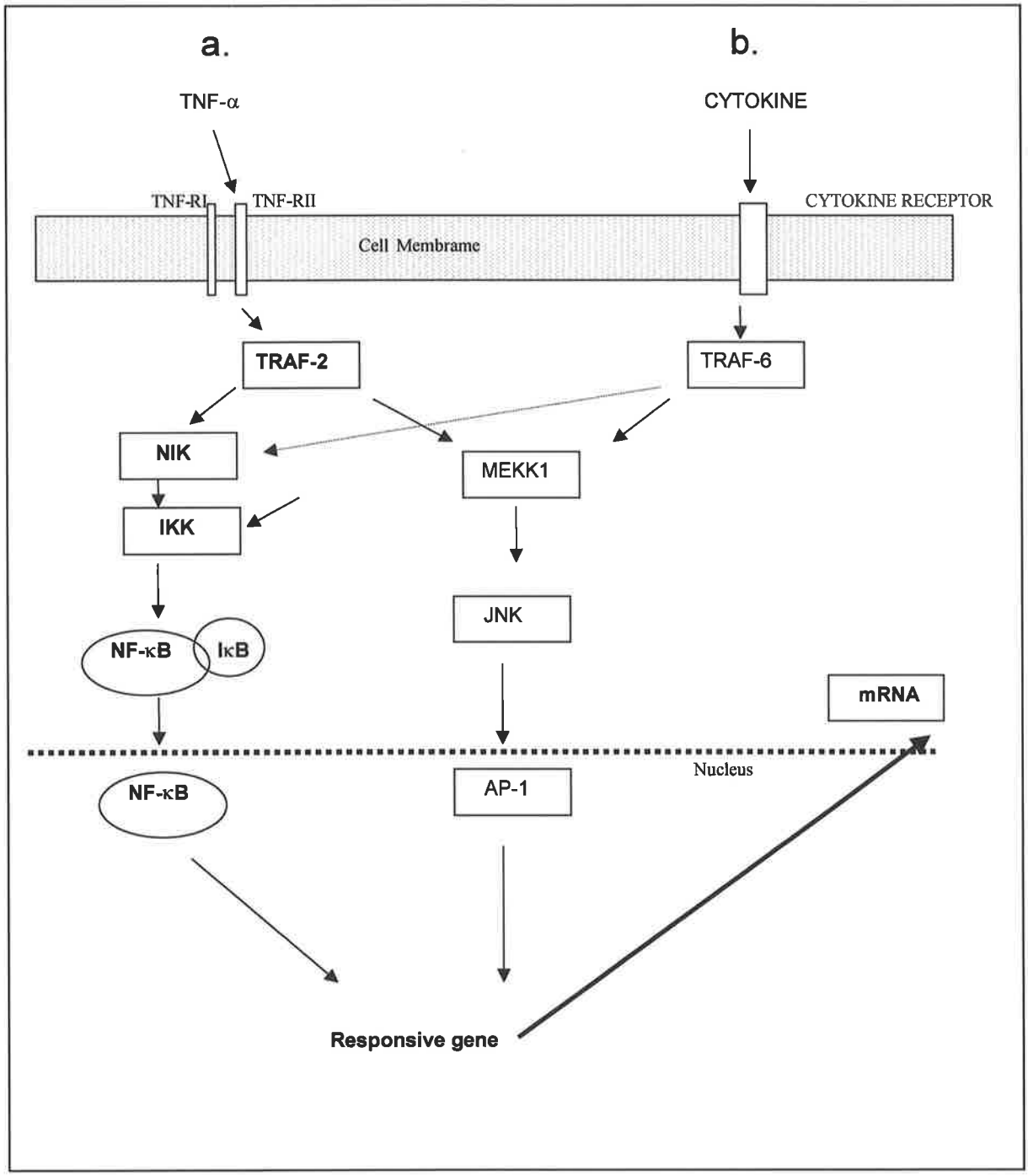


Figure 9-9

Crosstalk between NF-κB and AP-1 transcription factors

Adapted from Barnes PJ. (1998) *Eur Respir J* **12** 224-225

9-4-2 Methods

9-4-2 (a) Measurement of NFκB by flow cytometry

A 16HBE AEC line was used for these experiments. Cells were cultured and induced to apoptosis with 500 ng/ml aFas ± brefeldin A as described in the previous chapter.

The flow cytometric assay to measure cytoplasmic and nuclear NFκB is detailed in **Figure 9-10**.

AEC (500 μL) (apoptotic or viable) were washed with 2 ml 0.5% BSA/Isoton II, tubes centrifuged, supernatant was removed by suction and cells fixed with 200 μL 2% paraformaldehyde in Isoton II. Tubes were vortexed, left at room temperature for 10 min then washed with 2 ml 0.5% BSA/Isoton II. Cell membranes were permeabilised using 80 μL of a saponin-based Perm/Wash reagent (Pharmingen) then 1 μL rabbit anti-human p65 Mab (Santa Cruz, CA, USA) added for 10 min. Cells were washed by centrifugation with 2 ml Perm/Wash reagent. Donkey anti rabbit F(ab2) affinity purified Mab (FITC, 5 μL; Jackson Immuno Research Labs) was added, tubed vortexed and left for 10 min, at room temperature, in the dark.

For the flow cytometric assay to measure nuclear NFκB, cells were washed with 2 ml Perm/Wash reagent then 200 μL FACSPerm (BD) added to permeabilize the nuclear membrane. Tubes were vortexed and left for 10 min. Following a further wash with 2 ml 0.5% BSA/Isoton II, 1 μL p65 Mab was added, tubed vortexed and left for 10 min, at room temperature, in the dark. Cells were then washed with 2 ml 0.5% BSA/Isoton II. The secondary antibody, donkey anti rabbit F(ab2) AP PE (5 μL;

Jackson Immuno Research Labs)) was added and tubes again left for 10 min at room temperature, in the dark. Cells were washed with 2 ml 0.5% BSA/Isoton II, resuspend in 50 μL BSA/Isoton II and analysed by flow cytometry.

9-4-2(b) Statistical analysis

The Wilcoxon Signed Rank test was used to analyse the paired data from four separate experiments. This analysis was performed using SPSS software, p values <0.05 were considered significant.

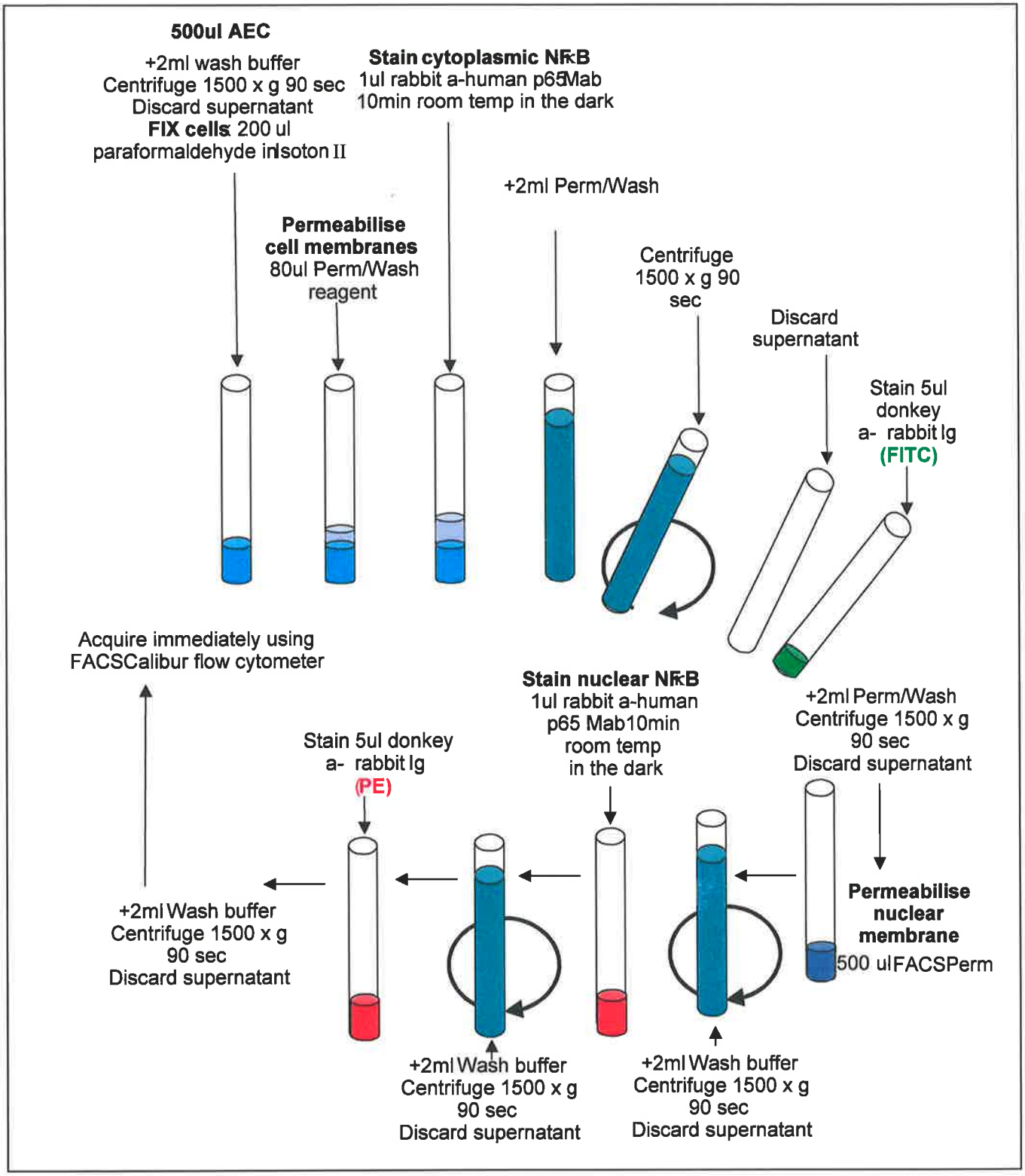


Figure 9-10

Measurement of NFκB by flow cytometry

9-5 Results: Phase Two

9-5-1 Cytokine production by viable and apoptotic AEC

To confirm that the viable and apoptotic cells in this study produced cytokines, IL-4, IL-6 and TGF-β production was investigated as outlined in Phase I (9-2-5) and Chapter Five. Viable cells produced significantly more IL-6 than apoptotic cells (Viable 41.1% ± 25.8% vs Apoptotic 25.8% ± 8.8%, p=0.030). As in Phase I of this chapter, production of IL-4 and TGF-β was significantly increased for apoptotic cells (IL-4 Viable 15.4 ± 3.9% vs Apoptotic 21.9% ± 6.3%, p=0.028; TGF-β Viable 9.3% ± 5.2% vs Apoptotic 15.6% ± 0.6%, p=0.047).

9-5-2 Cytoplasmic and nuclear NFkB expression by viable and apoptotic AEC

Flow cytometry was employed to measure the mean fluorescence intensity (MFI), a measure of the amount of NFkB (cytoplasmic or nuclear) expressed per cell.

Viable cells: MFI for cytoplasmic expression of NFkB for viable cells was 24 ± SD 104. The nuclear expression of NFkB was 119 ± SD 117, a significant increase in the nuclear:cytoplasmic ratio (p=0.038) indicating translocation of NFkB from cytoplasm to nucleus (**Figure 9-11**) (ie, that the IL-6 production noted in these cells in *10-5-3(a)* may have resulted from activation of the NFkB pathway).

Apoptotic cells: The MFI of cytoplasmic expression for apoptotic cells was 34 ± SD 127 and the MFI for nuclear expression was 87 ± 41; this was not statistically different, indicating that no detectable translocation had occurred from cytoplasm to

nucleus in apoptotic cells (ie, that the TGF-β and IL-4 production noted in these cells in *9-5-3(a)* did not result from activation of the NFkB pathway).

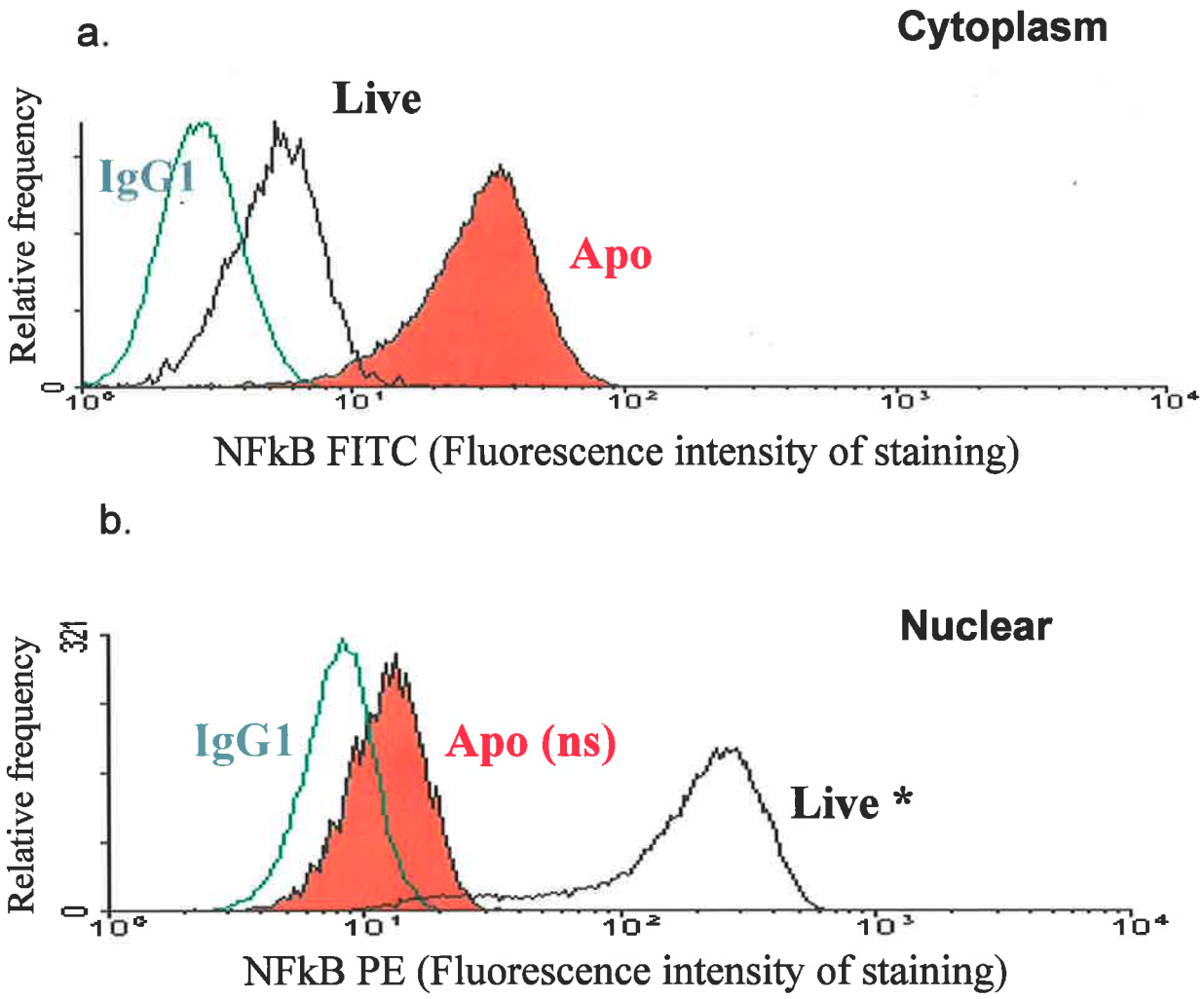


Figure 9-11

Cytoplasmic and nuclear expression of NFkB by viable and apoptotic AEC

Representative histograms show:

a. Cytoplasmic expression: Viable cells MFI=20, Apoptotic cells MFI=45

b. Nuclear expression: Viable cells MFI=150 (* a significant increase in nuclear expression (p=0.049) indicating translocation of NFkB from cytoplasm to nucleus); Apoptotic cells MFI = 20 (not significant).

9-6 Discussion

9-6-1 Apoptotic AEC produce cytokines

In this study I describe the novel finding that apoptotic human AEC produce IL-4 and TGF-β at higher levels than viable cells but reduced levels of production of the pro-inflammatory cytokine IL-6.

A bronchial AEC line, 16HBE, which retains many of the properties of human AEC, was used as an *in vitro* model (Cozens et al. 1994). Under 24h culture conditions, in the presence of brefeldin A, some cells spontaneously undergo apoptosis. This model is suitable for study of comparative properties and biological activities of apoptotic and viable human AEC. The validity of using a transformed cell line to investigate apoptosis has been established by previous studies (Hoshino et al. 2001; Aiba-Masago et al. 2001; Rorke et al. 2000).

Prior to sorting, 16HBE AEC were stained with Annexin V in the presence of calcium ions. The apoptotic and live populations were then sorted into calcium free cell culture buffer, washed to remove bound Annexin, and stained for intracellular cytokine production using flow cytometry. Annexin V is unsuitable for staining cells, which have been harvested using trypsin, due to membrane changes and PTS exposure in the outer membrane (Vermes et al. 2000). To avoid membrane damage, the cells were grown in, and attached to, polypropylene conical tubes for 24h prior to sorting. The cells could be detached, without enzymes or harsh scraping, by gentle pipetting with an ultrafine pipette (refer Appendix).

Flow cytometric investigation of intracellular production of cytokines may be influenced by the effect of dead cells, which often bind to antibodies non-specifically. I, therefore, applied several techniques to ensure that the increase in antibody binding reflected cytokine levels in apoptotic cells.

First, a region was drawn which excluded cells with ruptured membranes and debris based on forward and side light scatter characteristics. Events from this region were used for sorting.

Second, the apoptotic and live cells were sorted on the basis of Annexin V staining and negative cytokeratin 5/6 staining (shown to be absent from these cells, using histochemical techniques) and appropriate isotype matched controls used strictly as the basis for quadrant marker setting for analysis of cytokine production. The quadrant marker settings for live and apoptotic cell populations differed because apoptotic cells demonstrated more autofluorescence and non-specific staining than viable cells.

Third, blocking of binding sites on antibody to IL-4 with recombinant IL-4, resulted in abrogation of staining for IL-4 by apoptotic cells, confirming that the intracellular cytokine was not the result of non - specific binding.

Combination of Annexin V positive/ propidium iodide negative staining, applied post sorting and after further incubation showed that the proportion of necrotic cells was similar in both apoptotic and viable cell populations. Stein et al, reported that intracellular IL-4 expression was detectable in apoptotic but not necrotic cells (Stein et al. 2000). The inclusion of some necrotic cells in the 'apoptotic cell' population

may, therefore, have caused an underestimation of cytokine production by apoptotic cells, measured by flow cytometry.

Fourth, to further test my hypothesis, cells were sorted, re-cultured and the supernatant assayed for soluble TGF-β using ELISA.

While it is possible that cellular necrosis and resulting 'leakage' of cell contents may have contributed to some extent to TGF-β release, the proportion of necrotic cells was similar in both viable and apoptotic cell populations and it is unlikely that their presence could explain the significantly increased release of TGF-β by apoptotic cells. In addition, soluble IL-6 release by apoptotic cells measured by ELISA was not increased, consistent with intracellular cytokine production measured by flow cytometry.

Fifth, results were confirmed by semi-quantitative IHC staining and EM.

9-6-2 Investigation of mechanisms of cytokine production by apoptotic AEC

To understand how apoptotic AEC came to produce and secrete cytokines, I began to investigate the pathways involved in protein synthesis. Due to time restraints, only one pathway, the NFκB pathway (reported to be involved in IL-6 production), was investigated.

Current methods to evaluate NFκB activation involve measurement of active NFκB or phosphorylated IκB from cytoplasmic cell extracts or measurement of NFκB translocation from nuclear extracts by electrophoresis and Western Blotting. These

methods have problems of identification of the cell types associated with NFκB and require use of purified cell cultures or cell lines. Recently cell purification techniques have been shown to activate certain cell types and cause apoptosis of selected cell populations, resulting in upregulation of cytokine mRNA levels, which may lead to nuclear translocation of NFκB (Hartel et al. 2001; Hodge et al. 2000). Furthermore, a study from our laboratory has shown that culture of purified cells results in increased apoptosis (Hodge et al. 2000).

A novel flow cytometric assay was developed to measure NFκB in AEC. An affinity purified polyclonal Mab to human NFκB p65, and cytoplasmic permeabilisation using saponin, were used to identify cytoplasmic p65 in AEC. Subsequent nuclear membrane permeabilisation with Tween and further staining with anti-human NFκB p65 allowed determination of nuclear p65 (increased ratio of nuclear:cytoplasmic expression indicating activation of the NFκB pathway). To validate the new assay, future studies will compare these findings using 'gold standard' techniques such as electrophoretic mobility shift assays and confocal microscopy.

Using flow cytometry, I showed that the NFκB/ IκB pathway was not activated in apoptotic AEC, compared to viable cells. In contrast, in viable cells the pathway was activated and may therefore play a part in cell activation and production of IL-6 by these cells. As other transcription factors are most likely involved in production of TGF-β and IL-4 by apoptotic cells, these will be investigated in future studies.

There have been some studies of the pathways to TGF-β gene expression in human fibroblasts, keratinocytes and endothelial cells (Martin et al. 1997). TGF-β production was mediated by activation of AP-1 in these cells. Interestingly, the study

by Martin et al (1997) reported that ionising radiation (known to result in cell cycle arrest or apoptosis) resulted in increased TGF-β gene expression and heightened AP-1 activity. This may be explained by my findings from the previous chapter that apoptotic AEC produce TGF-β. However, there is little information about the regulation of transcription factors in the airways. AP-1 is a ubiquitous transcription factor that regulates large sets of genes. AP-1 is activated by various cytokines including TNF-α and IL-1β via activation of TNF-associated factors (TRAF) that subsequently activate MAP kinases. There is evidence that synergistic interaction of AP-1 and NFκB results in optimal expression of particular genes. It may therefore be necessary to have concurrent activation of several transcription factors in order to obtain maximum gene expression.

Most of the studies of IL-4 gene expression have addressed T-cells, as these cells are major producers of this cytokines. In T-cells, production of IL-4 and other T-cell-derived cytokines (IL-5 and IL-2) is regulated by cytoplasmic nuclear factor of activated T-cells (NF-AT). Activation of T-cells results in activation of this transcription factor, binding to the phosphatase calcineurin and transportation into the nucleus. Here, a transcriptional complex is formed with AP-1 that induces gene expression.

Thus, although there have been no specific studies of pathways for cytokine production in AEC, studies using other cell types suggest that AP-1 is active in production of both IL-4 and TGF-β. Investigation of this pathway for apoptotic and viable AEC may therefore provide useful information about how apoptotic AEC come to produce and release cytokines.

In conclusion, a novel flow cytometric assay for NFκB activation for viable and apoptotic AEC was applied to confirm that the NFκB pathway does not play a significant role in production of TGF-β and IL-4 by apoptotic AEC.

9-6-3 Implications in repair of injured epithelium in the normal lung

In this study, spontaneously apoptotic AEC and those in which apoptosis was induced by aFas produced significantly more IL-4 and TGF-β but less IL-6 than viable cells. The results are consistent with a recent report that TGF-β is released by apoptotic lymphocytes (Chen et al. 2001). The authors showed that apoptotic T-cells secrete not only latent but bio-active TGF-β. In the present study, I also found increased total (active + latent) TGF-β production by flow cytometry and increased active TGF-β by ELISA. Since only active TGF-β can function through the TGF-β receptor pathway (Letterio and Roberts, 1998) the presented data indicated that apoptotic cells may directly contribute to curtailing a potential inflammatory response by release of immunosuppressive cytokines.

In the human tracheobronchial epithelium, repair of acute mechanical injury induces hyperplasia (involving cell proliferation) and metaplasia (involving squamous differentiation), to temporarily cover the injured epithelium (McDowell et al. 1979). Both TGF-β and IL-4 are mediators thought to play key roles in regulation of airway repair. TGF-β mediates airway repair by inducing production of extracellular matrix components such as fibrinogen, inhibiting proliferation, inducing squamous metaplasia and inducing apoptosis of AEC (Romberger et al. 1992; Jetten et al. 1986; Rannels et al. 1982; Yoshida et al. 1992). Low levels of TGF-β in the early stage of epithelial cell repair would, therefore, facilitate the onset of hyperplasia.

Increasing levels of TGF-β, as a result of apoptosis as shown in the present study, could then act to mediate repair. Furthermore, TGF-β has been shown to induce its own production (Kim et al. 1989) and may act in this autocrine manner to further control proliferation.

IL-6, in contrast to IL-4 and TGF-β, has well documented pro-inflammatory effects (Huh et al. 1996). The observation that apoptotic AEC produce increased IL-4 and TGF-β and decreased IL-6 may further explain the anti-inflammatory nature of the apoptotic process. IL-4 has been shown to suppress IL-6, TNF-α and IL-1 release by LPS stimulated AM (Zissel et al. 1996).

The epithelium is generally regarded as a target for inflammatory mediators. There have been few reports of cytokine production by cells undergoing apoptosis (Gao et al. 1998; Stein et al. 2000; Chen et al. 2001) and no reports of apoptotic AEC producing cytokines. I have now shown that apoptotic AEC produce IL-4 and TGF-β. Production of these cytokines by apoptotic AEC may contribute to airway repair and resolution of inflammation following injury to the normal airway.

9-6-4 Implications for COPD

In previous chapters I demonstrated increased apoptosis of AEC and increased production of TGF-β in the airways in COPD. I have now demonstrated that apoptotic AEC produce TGF-β, providing a further explanation for the increased production of TGF-β in the airways in COPD.

This has significant implications in COPD. In normal repair situations, the production of TGF-β by apoptotic AEC may play a direct role in resolving inflammation and controlling proliferation of these cells. In COPD, however, excess production of TGF-β by inflammatory cells, AEC and apoptoic AEC may result in a feed-back loop (overwhelming the regulatory mechanisms as described in the previous chapter), characterised by increased AEC apoptosis, defective clearance, secondary necrosis and chronic inflammation.

Chapter Ten

Discussion and future directions

10-1 Discussion

Despite the high prevalence and debilitating nature of COPD and the clear need for effective therapies, development of new treatment strategies has been hampered by limited understanding of the basic pathogenesis of the disease.

When this project commenced, chronic inflammation and destruction of the extracellular matrix in COPD had been reported. However, there were relatively few studies addressing the mechanisms at basic cellular level to identify the mediators involved. The hypothesis that excess apoptosis and accumulation of apoptotic material in the airways are key factors in the pathogenesis of COPD formed the basis for the initial studies. This hypothesis was supported by the data obtained; increased rates of apoptosis were observed for AEC and lymphocytes in the airways in COPD. In addition, AM from COPD patients had a reduced phagocytic ability for apoptotic AEC. These findings have significant implications for understanding the reasons for the ineffective airway repair and chronic inflammation in COPD.

In the normal lung, cell turnover, repair of injured epithelium and resolution of inflammation are highly regulated processes whereby AEC, inflammatory leucocytes, and their potentially damaging contents, are removed by apoptosis and phagocytosis. This pathway in the normal airway is denoted in black in **Figure 10-1.**

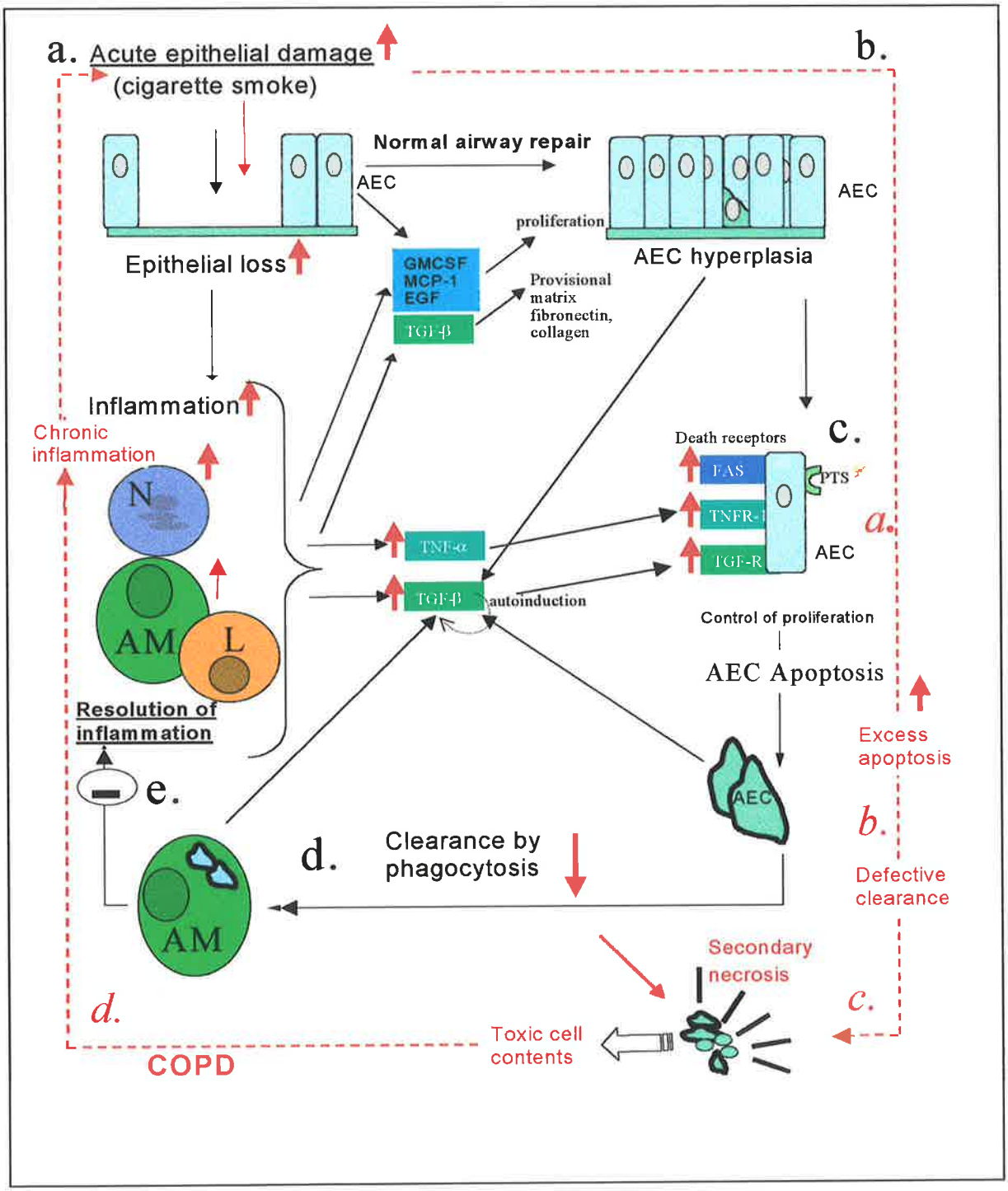


Figure 10-1

Defective repair of injured airway epithelium in COPD. Black arrows denote repair process following injury to the normal airway. Red arrows denote the findings from this thesis and proposed feed-back loop of chronic inflammation and defective repair in COPD. AM = alveolar macrophage, L = lymphocyte, N = neutrophil, AEC = airway epithelial cell

(a) Acute injury to the normal airway epithelium causes physical changes to the epithelium. Portions of the basement membrane are exposed as a result of cell loss.

(b) Residual basal cells release cytokines that initiate an inflammatory response with influx of AM, lymphocytes and neutrophils.

(c) AEC and leucocytes respond to these cytokines by producing additional cytokines (GMCSF, EGF, MCP-1) that induce de-differentiation and proliferation of AEC and TGF-β which instigates formation of a provisional matrix.

(d) If no further injury to the epithelium, excess AEC are removed by apoptosis (instigated by cytokines including TGF-β).

(e) Apoptotic AEC are cleared by phagocytosis by AM. The inflammatory response is resolved and normal epithelium restored.

The changes detected in COPD in this thesis are consistent with other studies of increased apoptosis associated with tissue destruction and inflammation in the airways, liver, skin and heart (Kasahara et al. 2001; Hagimoto et al. 1997; Kuwano et al. 1999; Vandivier et al. 2002; Hansen et al. 2000; Ogasawara et al. 1993; Hiramatsu et al. 1994; Trautman et al. 2000; Felzen et al. 1998). My findings and the implications for defective repair in COPD are shown in red in **Figure 10-1**.

(a) Increased production of apoptosis- inducing cytokines by AEC and leucocytes, as well as up-regulated expression of death receptors on AEC, result in increased apoptosis of AEC.

(b) Defects in AM phagocytic ability results in accumulation of uncleared apoptotic material, with *(c)* secondary necrosis and release of toxic cell contents.

(d) These changes result in chronic inflammation (compounded by chronic cigarette smoking and associated epithelial damage).

Thus, in COPD, increased apoptosis and defective clearance of AEC could result in a feed-back loop characterised by increased AEC apoptosis, defective clearance, secondary necrosis and chronic inflammation.

Novel assay systems utilising material obtained using the minimally invasive techniques of airway epithelial brushing and BAL are described in this thesis. Samples were obtained from airways 2-4 mm in diameter. It has been estimated that epithelium in these airways contributes substantially to the airway limitation that is the physiological hallmark of COPD. Thus, although the entire airway tree is involved in changes in COPD, useful and representative data was obtained from these studies. The use of flow cytometry allowed accurate quantitation of apoptosis in discrete cell subsets from the heterogenous populations obtained by the sampling procedures, which was not possible in previous studies that have used histochemical staining of tissue sections.

These findings are also significant because of new evidence that links apoptosis of pulmonary endothelial cells with the development of emphysema. There was an association between endothelial apoptosis, emphysema and downregulation of VEGFR (Kasahara et al. 2000; Kasahara et al. 2001). There may be a direct link between this phenomenon and smoking. Cigarette smoke extract decreased the expression of VEGFR in cultured cells and triggered apoptosis of pulmonary endothelial cells. Taken together with my findings, these studies point to a key, central role for increased apoptosis in the pathogenesis of COPD, broadening the concepts of pathogenesis in this disease beyond the traditional protease/anti-protease considerations. This knowledge may lead to development of novel anti-apoptotic therapies for patients with COPD, based on controlling apoptosis in the airways.

In the peripheral blood, as in the airways, activated T-cells are removed by apoptosis at the end of an inflammatory response in order to maintain cellular homeostasis. The link between T-cells in the peripheral blood and airways remains controversial. I, therefore, extended my studies and found increased apoptosis of stimulated T-cells in the peripheral blood in COPD, as I had found in the airways. Trafficking of T-cells occurs from the airways to the bloodstream. Lymphocytes from the bronchoalveolar space have been reported to re-enter the lung tissue, migrate to regional lymph nodes and re-join the systemic immune system (Schuster et al. 2000; Lehmann et al. 2001). Whether the findings presented in this thesis represent a systemic effect of COPD on peripheral cells or, alternatively, whether these cells have re-entered the circulation after passing through the airway epithelium requires further study.

The next logical step was to investigate possible mediators of apoptosis in the airways and peripheral blood. Strong evidence suggested that some of the changes associated with COPD (cigarette smoking, production of oxidants, disruption of lung extracellular matrix, release of enzymes and cytokines by neutrophils and AM) have the potential to cause increased apoptosis of AEC (Rahman et al. 2002; Hagimoto et al. 1997(1); Jyonouchi et al, 1998; D'Agostini et al. 2001; Vernooy et al. 2001). In the next phase of my study I investigated the hypothesis that increased activation of apoptotic pathways, mediated by TNFα/TNFR-I, TGF-β/TGF-R and Fas/FasL, would be detectable in the airways and peripheral blood from patients with COPD. These pathways could contribute to increased rates of apoptosis of AEC, infiltrating lymphocytes and peripheral blood T-cells in these patients.

That excess production of TGF-β plays a crucial role in increased apoptosis and defective repair in COPD is supported by substantial data presented in this thesis.

Evidence that TGF-β is produced by a variety of cells including AEC and leucocytes is presented. TGF-β produced by these cells may facilitate airway repair by inducing apoptosis and inhibiting AEC proliferation. Although several studies have reported investigation of TGF-β production in the airways (Aubert et al. 1994; Vignola et al. 1997; de Boer et al. 1998, Takizawa et al. 2001; Buhling et al, 1999), results presented have been conflicting, possibly due to limitations of methods such as ELISA or histochemical techniques which have not identified the types of cell producing TGF-β. Data presented in this thesis is the first report of the use of flow cytometric techniques and intracellular staining for the quantitation of TGF-β by a bronchial epithelial cell line. I then successfully applied this method to detect increased production of TGF-β by cells from peripheral blood, BAL and bronchial brushings in COPD.

In COPD, a failure to limit production of TGF-β and other pro-apoptotic cytokines (e.g., by continuous injury) may contribute to the feed-back loop of increased apoptosis of AEC, defective clearance, secondary necrosis and chronic inflammation. AM and AEC demonstrated increased production of TGF-β in COPD. Furthermore, TGF-β has been shown to induce its own production and may contribute, in this autocrine manner, to the increased production of TGF-β in COPD (Kim et al. 1989). In contrast to its effects on AEC, TGF-β prolongs survival of neutrophils (Lagraoui et al. 1997) which may result in their persistence and increased tissue damage (resulting from release of neutrophil cellular contents) and chronic inflammation.

An interesting cause for the increase in TGF-β in COPD was found. Apoptotic AEC were shown to produce cytokines, including TGF-β, in vitro. In normal repair

situations, the production of TGF-β by apoptotic AEC may play a direct role in resolving inflammation by inducing apoptosis and controlling proliferation of these cells. In COPD, however, excess production of TGF-β may result from the increased numbers of apoptotic cells that are present in the airways in COPD.

Considerable efforts have been made toward improving the understanding of the apoptotic process in recent years. In this regard, apoptosis may involve an increase in expression of key pro-apoptotic genes (such as Bax and p53) as well as decreased expression of anti-apoptotic genes (e.g., Bcl2, NF-κB) and cytokines (e.g., IL-7 and IL-2). I used flow cytometry to confirm decreased expression of the IL-7 receptor and increased p53 in the peripheral blood in COPD, again findings consistent with increased apoptosis. Given the tight relationship between Bcl-2 and Bax, it will be important in future studies to investigate Bax expression. It has been suggested that the ratio of Bax/Bcl-2 may be more important than the individual values for determining apoptosis (Stoetzer et al. 1996). This ratio will therefore be explored in future studies. It is probable that similar changes in apoptosis-related proteins occur in the airways. Thus, the investigation of these intracellular signaling pathways in airway cells will also form the basis of further studies.

The fate of apoptotic AEC in airways, gut and kidney is that of rapid recognition and phagocytosis by neighbouring cells that function as professional phagocytes (Vandivier et al, 2002(1); Wiegand et al, 2001) . In the airways, a critical component of the highly regulated process of airway repair is the effective removal of apoptotic material by AM. Apoptosis is rapid and usually occurs within the epithelial layer. Some apoptotic cells 'slough off', especially under pathological conditions, such as defective clearance or overwhelming numbers of apoptotic cells (Ren et al. 1995). Previous studies on phagocytic ability of AM in COPD have reported decreased

phagocytic capacity using opsonised yeast, E. coli and Candida albicans as the targets for phagocytosis (Ferrara et al. 1996; Meloni et al. 1996; Prieto et al. 2001).

Therefore, to further investigate the reasons for increased apoptosis of AEC in COPD I hypothesised that, in addition to an absolute increase in pro-apoptotic mechanisms, impaired capacity for phagocytic clearance of apoptotic material may be an important factor in the pathogenesis of COPD. To investigate this hypothesis I developed a novel in vitro flow-cytometric technique to quantitate phagocytosis of apoptotic AEC by BAL-derived AM. In this assay, I induced apoptosis in cultured AEC which were then exposed to BAL-derived obtained by BAL. Phagocytic cells were identified by flow cytometry using macrophage markers and apoptotic cells were identified with epithelial markers. The use of apoptotic AEC in this way was physiologically relevant for the study of ineffective repair in COPD. In addition, the flow cytometric assay had the advantages of allowing accurate quantitation of the uptake of apoptotic AEC by AM, rather than relying on less accurate manual counting techniques. The method also incorporated parallel analyses of the capacity of AM to ingest FITC labelled microbeads, to enable the investigation of broad deficiencies in AM function as well as recognition of apoptotic targets. Using these techniques, AM from subjects with or without COPD were compared with respect to their ex vivo phagocytic activity with either apoptotic AEC or microbeads.

Importantly, I found that AM from COPD subjects had a reduced phagocytic ability for apoptotic AEC. No difference was observed between AM from COPD patients compared to normal subjects when microbeads were used as phagocytic targets. These findings suggest that there are recognition problems relating to the clearance of apoptotic AEC in COPD. Whether these problems involve AEC or AM or both is still not clear, and this question provides a basis for future studies.

Traditional anti-inflammatory approaches, such as the use of potent corticosteroids, have failed to control epithelial destruction or alter the gradual decline of lung function associated with COPD. Significantly, corticosteroids induce apoptosis of AEC, resulting in AEC loss and airway damage (Dorscheid et al. 2001). This commonly used treatment may, therefore, contribute to the progression of COPD.

10-2 Future directions

There is evidence that B2 agonists (e.g. salbutamol and salmeterol), phosphodiesterase inhibitors (particular newer PDE4 inhibitors) and some antibiotics, in particular macrolides have anti-inflammatory properties. The effect of these agents on AEC apoptosis and AM phagocytosis has not been studied, and forms a basis for my proposed future experiments. The new assay systems reported here will be applied to investigate several potentially new, as well as currently used pharmaceutical agents. I will also compare the action of short and long acting B2 agonists, anticholinergics and phosphodiesterase inhibitors, as they are the most widely prescribed agents for the treatment of COPD and their effects on apoptosis have not been studied.

16HBE AEC will be cultured in the presence/absence of therapeutic agents, including PDE4 inhibitors, macrolide antibiotics and agents routinely used in the treatment of COPD (steroids and short and long acting β2 agonists), to investigate the effect on apoptosis and expression of the relevant associated mediators. Apoptosis, necrosis and expression of associated mediators (cytokines TGF-β, TNF-α, IL-7 and their receptors, gene products Bcl2, NF-κB, p53 and Bax) will be investigated using methods outlined above. Dose response experiments will be

carried out on all agents and optimal concentrations used. This work will then be extended to primary cultures of airway epithelial cells from COPD and control patients. ELISA (e.g., for TGF-β) and Western Blot (e.g., Bcl2/Bax) will be used as supporting techniques.

The studies presented here form a basis for a search for alternative therapies for COPD. In this regard I will assess the use of fetuin. This ubiquitous glycoprotein, conserved throughout most species, not only has anti-inflammatory properties but also has been recently demonstrated to increase the uptake of apoptotic leucocytes by human AM (H. Jersmann, personal communication). What makes this protein even more interesting in the context of TGF-β contributing to AEC death is the fact that fetuin acts as a naturally occurring antagonist of this cytokine (Dziegielewska and Andersen, 1998). Furthermore, fetuin has recently been successfully used as an anti-inflammatory agent in an in vivo model (Ombrellino et al. 2001).

More novel approaches are proposed to extend these findings. The recently-developed in vitro assays of phagocytosis of apoptotic cells have enormous potential for assessment of the modulation of AM phagocytic function and screening of proposed therapeutic interventions. Investigation of the effects of drugs commonly used in the treatment of COPD on AM phagocytic function have been reported (Vecchiarelli et al. 1994; Capelli et al. 1993). These studies have demonstrated increased phagocytic activity by AM in the presence of antibiotics and drugs that are long-acting beta 2 agonists. However, these studies relied on opsonised zymosan, beads or C. albicans as in vitro targets for phagocytosis. Using the physiologically relevant system for assay of phagocytosis, I will investigate commonly used and novel therapeutic agents, as outlined above.

This will permit assessment of the feasibility of genetic manipulation of AM ex vivo. To achieve this I propose to use adenoviral (Ad) vectors to deliver genes known to enhance phagocytic activity. An Ad vector containing the CD36 gene will be constructed under the control of the CMV promoter. CD36 is the leading candidate based on literature reports showing that such transfection can improve the phagocytic capacity of other cells (Ren et al. 1995). If successful in establishing the concept that AM function can be improved by ex vivo gene transfer, one could envisage a therapeutic approach whereby individual patients' macrophages are harvested, manipulated, then re-instilled into the patient to achieve improved airway clearance. The investigation of highly novel approaches such as this are needed if COPD therapy is to significantly progress.

In summary, this thesis describes the important contribution of increased apoptosis, and defective clearance of apoptotic cells by phagocytosis, to the pathogenesis of COPD. These findings are particularly relevant in relation to future experimental approaches that may help to design novel treatment strategies for COPD.

Chapter Eleven

Appendix

A-1 Optimisation of techniques

A1-1 Effect of removal of cells from culture plates on membrane damage

Trypsin treatment to remove cells from culture plates has been reported to cause transient or permanent membrane damage (H. Shapiro, personal communication: Purdue Cytometry Mailing List, April 12.2000). These changes may result in artefactual staining with apoptosis markers such as PI or Annexin V. A 16HBE AEC line was used extensively in my studies. To investigate whether trypsin (or other agents) caused artefactual membrane damage to these cells, apoptosis (or membrane damage) was measured following removal of cells from tissue culture plates with these agents.

Methods

16HBE AECs were grown until 80% confluent in tissue culture flasks (Corning). Cells were removed using

(1) 0.1% trypsin + 0.2g/l disodium EDTA*, for 5 min or

(2) Citric saline * for 5 and 30 min (L Barber, personal communication: Purdue Cytometry Mailing List, 13/4/00) or

(3) 20mM EDTA for 5 min (P Poncelet, personal communication: Purdue Cytometry Mailing List, 13/6/00) or

(4) 2 mM EDTA for 5 min then 30 min

Apoptosis (or membrane damage) was measured by PI staining immediately following removal from plates and washing, as described in Chapter Four.

Preparation of reagents

(1) *Trypsin / EDTA*

Trypsin (1.0 g; Gibco cat. No 840-7072 1M trypsin 1:250) and EDTA (di sodium salt, 0.2g/l) were dissolved in 1x PBS and sterilised by filtration.

(2) *Citric saline*

This method has been reported to detach cells with excellent viability (L. Barber, personal communication: Purdue Cytometry Mailing List, April 13.2000).

For a 10x stock solution, potassium chloride (1.35 M) and sodium citrate (0.15 M) were dissolved in sterile distilled water, stored at 4^0C and diluted 1:10 before use.

Results

Trypsin + EDTA resulted in less membrane damage than other agents (assessed by positive staining with Pi). Citrate saline and EDTA resulted in high levels of staining with PI (**Table A1**).

Table A1

Apoptosis of 16HBE AEC after removal from culture plates with various agents. (mean values (±SD) of two experiments carried out in duplicate)

	Time	**Percent PI staining**
Citrate	5 min	Minimal detachment
Citrate	30 min	68.9 ± 11.2
EDTA 20 mM	5 min	72.6 ± 4.9
EDTA 2 mM	5 min	Minimal detachment
EDTA 2 mM	30 min	49.6 ± 12.8
Trypsin + EDTA	5 min	24.8 ± 0.6

Conclusion

Trypsin + EDTA for removal of 16HBE AEC from culture plates resulted in less PI staining than other agents and was thereafter used for all in vitro experiments described in this thesis.

A1-2 Investigation of transience of membrane damage

(1) To determine whether the positive PI staining noted in the previous experiments was due to transient membrane damage (that would recover with re-culture), 16HBE AEC were removed from culture plates with trypsin/ EDTA, then re-incubated for various times in culture tubes and stained with PI (as described in Chapter Four).

(2) To determine the percentage of cells that were apoptotic prior to detachment from culture plates, 1 ml of 50 μg/ml PI was added directly to the culture plates for 30min. The cells were washed with PBS then removed from the plate with trypsin / EDTA and analysed by flow cytometry. To compare the total apoptosis (including possible membrane damage by removal of cells), trypsinised cells from a second culture plate were stained with PI and analysed in parallel.

Results

1. The data suggest that treating 16HBE cells with trypsin / EDTA does not result in transient membrane damage, as there was a very small, non-significant, reduction in the mean percent of cells that stained with PI (ie, re-establishment of intact cell membrane) after 2h re-culture. The percent of PI positive cells then increased up to 48h (**Table A2**).

Table A2

Transience of membrane damage after trypsin treatment of AEC

Time	Percent PI staining
0	20.0 ± SD 9.9
2h	18.4 ± SD 8.2
4h	28.0 ± SD 10.0
24h	23.7 ± SD 8.9
48h	28.2 ± SD 10.3

2. PI staining of 16HBE AEC showed that removal of 16HBE AEC from culture plates with trypsin / EDTA did not significantly effect the cell membrane, as PI staining of cells before and after trypsin treatment was not significantly different.

Table A3

PI staining of apoptotic 16HBE AEC after trypsin treatment of AEC

PI added before trypsin	PI added after trypsin
17.7 ± 1.0	24.8 ± 0.6

Conclusion

16HBE AEC can be successfully removed from culture plates with trypsin / EDTA without significant transient membrane damage.

A-1-3 Comparison of methods for re-suspending cultured AEC

In vitro experiments described in Chapters Nine and Ten required 24h culture of 16HBE AEC. This was carried out in polypropylene conical tubes at 37^0C for 24h. To establish the optimal method to re-suspend these cells (with minimum membrane damage), two methods were assessed.

1. Cells were vortexed for 10 sec.
2. Cells were resuspended by gentle pipetting with an ultrafine (BD) syringe.

Results

Re-suspension of cells with an ultrafine pipette resulted in less membrane damage than cells re-suspended using vortexing (Vortex 27.6 ± 9.9 vs ultrafine syringe 24.2 ± 5.0; (Mean values of four experiments ± SD)), although the difference was not statistically different (Wilcoxon).

Conclusion

Re-suspension of cells with an ultrafine pipette was thereafter used for all *in vitro* experiments described in this thesis.

A-1-4 Establishment of optimal cell culture techniques

In vitro experiments described in Chapters Nine and Ten required 24h culture of 16HBE AEC. To establish the optimal cell culture techniques,1 ml aliquots of 16HBE AEC (5 x 10^5 cells per ml) were cultured in six well culture plates or 10 ml culture tubes for 24h. Cells were removed by gentle pipetting then apoptosis assessed by positive staining with PI as described in Chapter 4.

Results

Cells cultured in 6 well plated exhibited higher staining with PI than those cultured in polypropylene tubes (Well: 29.8% PI positive ± SD 12.0 vs tube: 24.2% PI positive ± SD 5.0, n = 3). The difference was not statistically different (Wilcoxon).

Conclusion

Culture of 16HBE AEC in polypropylene tubes was thereafter used for all *in vitro* experiments described in this thesis.

A-1-5 Titration of Mabs

All Mabs used in this study were titrated to determine optimal concentrations for use. The concentrations varied for different cell types.

The following figure shows a representative experiment investigating the optimal concentration of Mabs for flow cytometric detection of peripheral blood-derived monocyte cytokine production. The optimal dilution of 1:20 was thereafter employed.

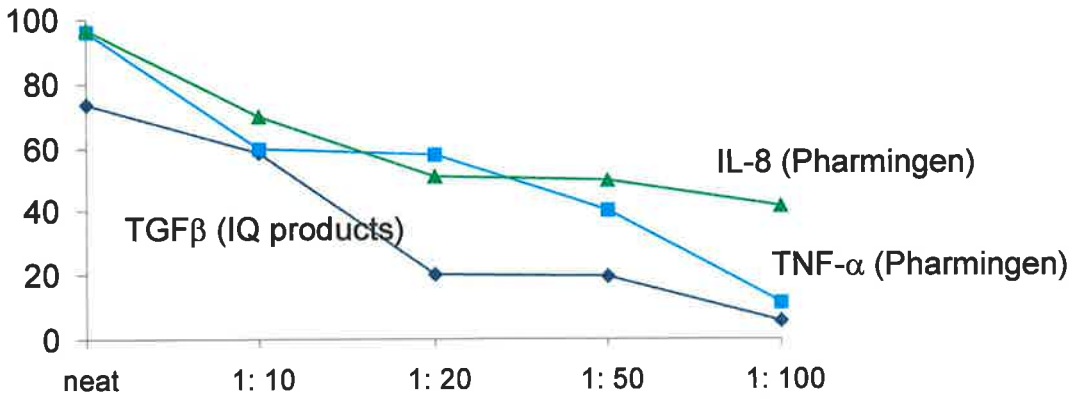


Figure A-1 Optimal concentrations of Mabs for flow cytometry

Early experiments investigating TGF-β production used an indirect flow-cytometric method and unconjugated Mab to TGF-β. During the course of this study, a directly-conjugated Mab for TGF-β (IQ products) became available. This Mab was titrated for optimal concentration as shown above and used for all subsequent experiments.

Some early experiments utilised a Mab to TNF-α obtained from BD. During the course of the study, a Mab to TNF-α was purchased from Pharmingen. The latter Mab showed superior sensitivity for detection of TNF-α in AEC and BAL-derived cells so was thereafter employed for all subsequent experiments.

A-1-6 Optimisation of stimuli for cytokine production

A range of stimuli was investigated for optimal cytokine production by AEC and peripheral blood-derived T-cells and monocytes. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Results

(a) AEC

Bronchial brushing-derived AEC production of cytokines at 24h (results show % cells producing cytokine) (representative experiment). Optimal conditions chosen for cytokine production shown in red.

	IL-4	IL-6	IL-8	TGF-b	GMCSF
BA, no stimuli	10.5	31	57	4	27
100 ng/ml LPS	**11**	**31**	**62**	**13**	**33**
50 ng/ml PMA + 1 μg/ml ionomycin	10.5	36	55	6	28

(b) I6HBE

16HBE AEC production of cytokines at 24h (results show % cells producing cytokine) (representative experiment). Optimal conditions chosen for cytokine production shown in red.

	IL-4	IL-6	TGF-b
BA, no stimuli	4	22	18
100 ng/ml LPS	**6**	**26**	**22**
50 ng/ml PMA + 1 μg/ml Ionomycin	3	20	18
100 ng/ml TNF-α	6	26	15

(c) Peripheral blood

T cell production of cytokines at 24h (results show % cells producing cytokine).

Optimal conditions chosen for cytokine production shown in red.

	TNF-α	IL-4
BA, no stimulus	<2	<2
1 μg/ml PHA	4	<2
10 μg/ml PHA	11	<2
50 ng/ml PMA	28	<2
50 ng/ml PMA + 1 μg/ml Ionomycin	**42**	**5**

Monocyte production of cytokines at 24h (results show % cells producing cytokine).

Optimal conditions chosen for cytokine production shown in red.

	TNF-α	IL-6	IL-8
BA, no stimulus	<2	<2	<2
10 ng/ml LPS	43	41	45
100 ng/ml LPS	67	51	**61**
1 μg/ml LPS**	**70**	**55**	**59**
10 μg/ml LPS	•	•	•
50 ng/ml PMA	•	•	•
50 ng/ml PMA + 1 μg/ml Ionomycin	•	•	•

• denotes loss of CD14 from cells

(**1 μg/ml LPS used for all experiments in this thesis)

A-1-7 Phenotyping of 16HBE AEC

The 16HBE bronchial epithelial cell line was used extensively in this study. Baseline phenotyping of cytokine receptors, recognition molecules, adhesion molecules and costimulatory molecules was performed.

Results

Table A-5 Phenotyping of 16HBE AEC (nd: not detectable, notable expression indicated in bold)

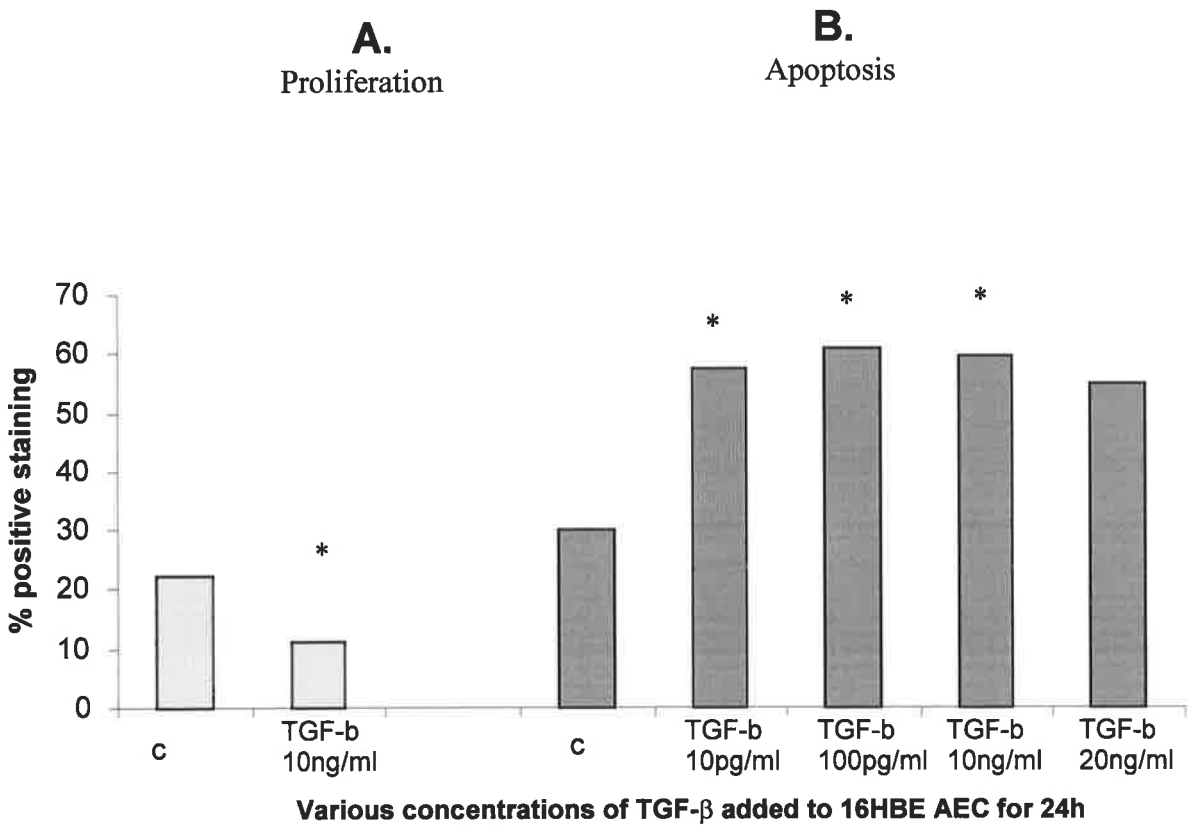
	n	Mean	SD	Function
TGF-Receptor	7	22.0	11.0	Cytokine receptors
CD124 (IL-4R)	13	3.1	2.3	
CD126 (IL-6R)	9	nd	-	
CD130 (IL-6R)	8	57.2	2.0	
CD25 (IL-2Rα)	5	nd	-	
CD122 (IL-2Rβ)	5	nd	-	
CD132 (IL-2Rγ)	10	5.1	8.3	
TNFRI	5	4.5	3.3	
TNFRII	5	5.4	2.1	
CD127 (IL-7R)	5	nd	-	
CD51 (αv integrin)	5	90.2	2.6	Recognition by phagocytes
CD61 (β3 integrin)	5	nd	-	
CD36	6	7.3	3.6	
CD31	5	3.4	2.0	
CD95	5	72.9	10.8	Fas
CD49B (α2 integrin)	6	93.1	6.0	Adhesion molecules
CD49D (α4 integrin)	7	nd	-	
CD49E (α5 integrin)	7	14.2	7.5	
CD49F (α6 integrin)	6	62.4	28.3	
CD106 (VCAM)	5	nd	-	
CD54 (ICAM)	7	90.1	11.6	
CD44 (HCAM)	6	32.6	16.8	
CD40	5	93.5	6.1	Costimulatory molecules
CD80	5	nd	-	
CD86	5	3.6	3.3	
HLA	6	95.9	2.0	Antigen recognition molecules
HLADR	5	nd	-	
CD71	5	94.3	4.9	Cell activation (transferrin receptor)
CD69	5	nd	-	Cell activation

A1-1-8 TGF-β: Effects on apoptosis and proliferation of 16HBE AEC

Several concentrations of rhTGF-β were added to cultured 16HBE AEC. The effects of TGF-β on apoptosis (assessed by Annexin V staining) and proliferation (assessed by proliferating cell nuclear antigen (PCNA) staining) are presented in the following chart. These experiments were performed to validate the use of 1 ng/ml of TGF-β for experiments described in this thesis.

Results

TGF-β at an optimal concentration of 10ng/ml significantly inhibited proliferation and increased apoptosis of 16HBE AEC.



Appendix 2. Information Sheets and Consent Forms for ex vivo studies

A2.1 Information sheet for bronchoscopy

ROYAL ADELAIDE HOSPITAL

INFORMATION SHEET FOR BRONCHOSCOPY FOR RESEARCH PROJECT

TITLE OF PROJECT

The role of apoptosis and its modification in the pathogenesis and progression of chronic obstructive pulmonary disease.

INVESTIGATORS

M D Holmes, PN Reynolds, R Scicchitano, S Hodge.

GENERAL INFORMATION

1. The purpose of this study is to assess the role of the clearance mechanisms in the lungs for the removal of dead and dying cells from the airways, in chronic bronchitis and emphysema (COPD). There is evidence to suggest that these mechanisms are abnormal in COPD and contribute to the disease. If this is the case, this knowledge can be used to design new treatments for chronic airways disease. Studies are needed to clarify this.

2. To look at this possibility, we are studying human lungs by taking small scrapings (brushings) of the lining of the breathing tubes (bronchi) during bronchoscopy. In addition we will wash sterile saline solution into and out of the lungs (bronchoalveolar lavage) to obtain cells from the lung substance to study. Bronchoscopy is a routine diagnostic test in our Unit and 15-20 are performed every week.

3. We will principally be looking at patients with chronic bronchitis and for comparison, normal volunteers who do not have lung disease.

4. It is possible, though by no means certain, that this study may identify important differences between chronic bronchitics and normal volunteers, and that the cells we obtain may respond to investigative treatments to improve their function (when tested in the laboratory).

5. You will not personally benefit from being in this study although we hope that the results of the study will help improve the understanding and treatment of airways disease.

6. You have been asked to participate in this study:-

a) Because you have chronic bronchitis

or

b) You are a normal volunteer, a group need to establish what is normal for the things we are studying.

WHAT PARTICIPATING IN THIS STUDY INVOLVES

You will be asked to give written informed consent to Fibreoptic Bronchoscopy.

1. **WHAT IS A BRONCHOSCOPY?**

A bronchoscopy is a test in which a small flexible tube called a bronchoscope is passed through the airways into the lung. This enables the doctor to see directly inside the lung and to take various samples. This is usually performed under local anaesthetic.

2. **WHY IS A BRONCHOSCOPY USUALLY NECESSARY?**

A bronchoscopy is usually performed when your doctor suspects something is wrong with your lung but is unable to diagnose your condition without further examining the lung. In our department approximately 15 - 20 bronchoscopies are done each week. In this study the bronchoscopy enables us to obtain specimens for research.

3. **PREPARATION FOR A BRONCHOSCOPY**

You must have nothing to eat or drink after midnight the night before the bronchoscopy. If you regularly take medicines in the morning it will most likely be recommended that you take these with a small sip of water at least 4 hours before the bronchoscopy is done – this will be discussed with you by the doctor informing you about the study. An intramuscular injection may be ordered and will be given to you approximately one hour before the bronchoscopy. This injection is a drug called atropine, which helps to reduce secretions in the upper airways and helps to stop you from feeling faint. This injection often makes you feel very dry in the mouth.

4. **WHAT HAPPENS DURING A BRONCHOSCOPY?**

On arrival to theatre you will be met by staff. The bronchoscopy staff further prepare you for the bronchoscopy by anaesthetising the throat and upper airways, using a mouthwash and a spray you are asked to inhale. This will make it difficult for you to swallow but it takes away the unpleasant sensations from the mouth. If you are someone with COPD and have a tendency to asthma, you will be given a dose of Ventolin to inhale. A small needle will be placed into a vein in the back of your hand so that you can be given a sedative which will cause you to become drowsy, making it easier for you to relax. The needle will stay in your hand during the test, just in case you become too drowsy and need an antidote to the sedative. The bronchoscope is usually passed through the nose after the bronchoscopy nurse has inserted a local anaesthetic gel to allow the instrument to pass easily without too much discomfort. The bronchoscope is then passed into the lung and gently maneuvered through the lung passages; this procedure will usually take about 10 - 15 minutes. During this time brushings of the breathing tube lining will be obtained as well as bronchoalveolar lavage. Throughout the test the oxygen levels in your blood will be constantly monitored with a clip-on finger probe, and some extra oxygen will be given to you by little tubes placed on your lips. In some people, the combination of the sedative and the slight reduction in airflow due to the bronchoscope being present in the airways or the instillation of fluid during

brochoalveolar lavage can lead to a lowering of oxygen levels. This is why we monitor oxygen levels closely and give everyone a little extra.

5. **WHAT HAPPENS AFTER YOUR BRONCHOSCOPY?**

You are advised not to eat or drink until instructed by recovery staff. You will continue to receive extra oxygen until you fully wake up. This is because the normal reflexes that protect the airway have been numbed by local anaesthetic and food and fluid may pass into the windpipe. You should also not drive a car or operate any complex machinery within 12 hours of completing the test (due to the effects of the sedative). We prefer that you have someone else in the house with you on your first night at home, mainly in case you happen to suffer from fever and need attention. One of the researchers will contact you the next day to see if there have been any problems.

POTENTIAL RISKS OF BRONCHOSCOPY

Bronchoscopy is a routine test and has been shown to be quite safe.

1. **Drug reactions** - the drugs used for bronchoscopy include-

(a) Lignocaine - as a local anaesthetic. In the dose used (<150mg), this is very safe

(b) Atropine - given to dry the mouth and airways - this may cause a feeling of fast heart rate. People with glaucoma or difficulty passing urine should alert the doctor.

(c) Midazolam - a sedative like Valium, a dose is given to make you drowsy. If too much is given you could stop breathing however we watch very closely and it is very unusual in individuals like yourself. An antidote to midazolam is always readily on hand in the theatre, should someone unexpectedly become too drowsy.

(d) Fentanyl - this is a sedative like Morphine and is given to make you drowsy and reduce cough. If too much is given you could stop breathing however we watch very closely and it is very unusual in individuals like yourself. An antidote to Fentanyl is always readily on hand in the theatre.

(e) Ventolin - before bronchoscopy to help avoid an attack asthmatics are given Ventolin.

2. **Coughing blood** - after the bronchoscopy you will usually cough up a small amount of blood due to the brushings. This is expected and will settle quickly. It is rarely a problem.

3. **Fever** - some patients (approximately one in ten) develop a fever after bronchoscopy. This usually occurs four to eight hours after the procedure and settles with Panadol.

4. **Lung infection** - this is a rare complication of bronchoscopy and will settle quickly with antibiotics.

5. **Acute asthma** - this can occur, but is uncommon in mild asthmatics particularly with Ventolin pre-medication.

6. **Hypoxia (low oxygen levels)**: - This could occur due to accidental over-sedation or as a result of bronchoalveolar lavage. There is almost always some drop in oxygen levels for a brief period with lavage, however you have been selected for the study because your baseline levels of oxygen are high enough to permit the test to be done safely. Everyone is given extra oxygen via small tubes during and after the test, and xygen levels are monitored constantly during the test to ensure levels are adequate.

7. **Aspiration :** This refers to the accidental inhaling of material that has been vomited up from the stomach. This is very rare if you have followed directions and not had anything to eat or drink since midnight the night before. In the unlikely event this does happen, the bronchoscope will be used to suck out as much material as possible from the lungs, oxygen levels will be maintained with high flow oxygen and you will stay in hospital for observation and antibiotics until stable.

8. **Failure of instruments :** The instruments currently in use are not known to cause problems by breaking and parts getting stuck in the lungs. Failure has been seem with older, re-usable brushes, but the current brushes are single use and no problems have been seen during the time they have been used here in Australia (over 10 years).

9. **Other :** Mishaps such as falling off the operating table and so forth while sedated are extremely unlikely, the attending nursing staff are highly trained in assisting with bronchoscopies and perform several tests each week.

For further specific information about this study please contact:

Dr Mark Holmes	phone 8222 5487 or
Dr Paul N. Reynolds	phone 8222 5376 or
Dr Raffaele Schicchitano	phone 8222 5487

For general information please contact Dr Michael James, Chairman of the Research Ethics Committee, phone (08) 8222 4139.

A2.2 Consent form for bronchoscopy

ROYAL ADELAIDE HOSPITAL
CONSENT FORM

PROTOCOL NAME: The role of apoptosis and its modification in the pathogenesis and progression of chronic obstructive pulmonary disease.

INVESTIGATORS: Dr Mark D. Holmes, Dr Paul N. Reynolds, A/Prof. R. Scicchitano, Ms S. Hodge

1. The nature and purpose of the research project has been explained to me. I understand it, and agree to take part.
2. I understand that I may not directly benefit from taking part in the trial.
3. I understand that, while information gained during the study may be published, I will not be identified and my personal results will remain confidential.
4. I understand that I can withdraw from the study at any stage and that this will not affect my medical care, now or in the future.
5. I understand that I should not become pregnant during the course of this trial. In the event of a pregnancy occurring, I agree to notify the investigator as soon as is practically possible."
6. I understand the statement concerning payment to me for taking part in this study, which is contained in the Information Sheet.
7. I have had the opportunity to discuss taking part in this investigation with a family member or friend.

Name of Subject: _____

Signed: _____

Dated: _____

I certify that I have explained the study to the patient/volunteer and consider that he/she understands what is involved.

Signed: _____
(Investigator)

A2.3 Information sheet for peripheral blood collection

ROYAL ADELAIDE HOSPITAL

INFORMATION SHEET FOR BLOOD COLLECTION

TITLE
The role of cytokines and apoptosis in the pathogenesis of chronic airways inflammation.

GENERAL INFORMATION

1. The purpose of this study is to assess the role of naturally occurring "hormone like" cytokines and other functional molecules in chronic bronchitis and emphysema (COPD) and other lung diseases. There is evidence to suggest that these molecules may play a role in lung disease. If this is the case they may lead to new treatments for chronic airways disease. Studies are needed to clarify this.

2. To begin to look at this possibility, we are studying human peripheral blood. We will be collecting 10ml of your blood into a container. This research will be carried out, where possible, on surplus specimens, already collected for your diagnostic testing. On occasions where the specimens are not sent for diagnostic testing, then they will be used for research purposes only. The doctor will tell you whether the specimens are to be used for diagnostic or research purposes.

3. We will principally be looking at patients with COPD and for comparison, patients with other chest problems.

4. It is possible, though by no means certain, that this study may identify differences between COPD before and after treatment and between COPD and patients with some other chest problems

5. You will not personally benefit from being in this study although we hope that the results of the study will help improve the understanding and treatment of airways disease

6. You have been asked to participate in this study: -
   a) You have COPD **OR**
   b) You are having blood taken for other reasons [you are a non-smoker and do not suffer from COPD], so have been asked to be part of a control group (needed to establish what is normal for the molecules we are studying).

For further specific information about this study please contact:

Dr Mark Holmes	phone 8222 5487 or
Dr Paul N. Reynolds	phone 8222 5376 or
Dr Raffaele Schicchitano	phone 8222 5487

For general information please contact Dr Michael James, Chairman of the Research Ethics Committee, phone (08) 8222 4139.

A2.4 Consent form for blood collection

ROYAL ADELAIDE HOSPITAL
CONSENT FORM FOR BLOOD COLLECTION

TITLE OF PROJECT
The role of apoptosis and cytokines in the pathogenesis of chronic airways inflammation.

INVESTIGATORS
S Hodge, R Scicchitano, M D Holmes, P N Reynolds

1. The nature and purpose of the research project has been explained to me. I understand and agree to take part.

2. I understand that I may not directly benefit from taking part in the trial.

3. I understand that while information gained during my study may be published, I will not be identified and my personal results will remain confidential.

4. I understand that I can withdraw from the study at any stage and that this will not affect my medical care now, or in the future.

5. I have had the opportunity to discuss taking part in this investigation with an independent person.

Name of Subject: _____

Signed: _____

Date: _____

I certify that I have explained the study to the subject and consider they understand what is involved.

Signed ..... Print Name:
.....
(Investigator)

Appendix Three: Reagents and equipment

All reagents were of analytical grade.

A3-1 Reagents and immunological kits

Reagent		Source	
TGF-β Receptor Kit	FLUOROKINE™	R&D	NFTGO
IL-6	EIA kit 96/well	R&D	DB100
TGF-β	EIA kit 96/well	R&D	
Isoton 2		Coulter-Immunotech	
Intragam		CSL, Australia	
Mitotracker green	-	Molecular Probes	M7514
Polystyrene microbeads	FITC	Polysciences	22958
APAP	-	Dakopatts	-
E. coli LPS	Serotype 0111:B4	Sigma	L2654
PHA	Used at 5μg/ml	Sigma	L-902
PMA	Used at 0.1mg/ml;	Sigma	P-8139
Ionomycin	Used at 1ug/ml	Sigma	1-0634
Methyl Prednisolone	Used at 10^{-5}M	Faulding, Australia	4620A
Dexamethasone	Used at 10^{-5}M	David Bull Labs. Australia	
Afas	Used at 500ng/ml	Sigma	F4424
Brefeldin A,	Used at 1-5μg/ml	Sigma	B6542
n-octyl β-D- galactopyranoside		Sigma	026 1
EDTA	20nM (.744g/100ml PBS)	Sigma	
Lymphoprep		Trace Scientific	211900100V
Blood collection containers containing:			
(a) Lithium Heparin	Used at 50IU/ 5ml blood	Disposable products	
(b) K3 EDTA		BD	362753
Sodium Heparin	Used at 500IU/10ml blood	David Bull Labs.	492445A
CaliBRITE beads		BD	34950
Paraformaldehyde		Sigma	P6148
Permeabilising Solution (FACSperm)	Diluted 1/10 with d.w.	BD	340457
Digitonin		Sigma	D5628
Crystal Violet		Gurrs	-
Fast red TR salt		Sigma	C137085
Perm/wash reagent		Phamingen	2090KZ
Lysing Solution (FACS brand)	diluted 1/10 with d.w.	BD	349202

A3-2 Immunological reagents (Antibodies were stored at 4^0C. Exposure to light was minimised by covering tubes when not in use).

Mab (or ligand)	Fluorochrome	Supplier	Details
Annexin V	FITC	Pharmingen	S56420
7AAD	-	Sigma	A9400
PI	-	Sigma	556420
SSDNA	FITC	Chemicon	MAB3299
Caspace	FITC	Promega	G746A
CD45/CD14 (leucogate)	FITC/PE	BD	340040
CD3	Cy-5	Dako	C7067
CD4	PerCP	BD	347324
CD14	PE	Dako	R0864
CD14	PECy-5	Immunotech	IM2640
CD3	FITC	Dako	RO818
CD3	PE	Dako	RO810
CD3	PE-CY-5	Immunotech	IM2635
CD4	FITC	Dako	F0766
CD8	FITC	BD	347313
CD33	FITC	BD	340533
CD33	PE-CY5	Immunotech	IM2647
CD33	PE	BD	347787
CD36	FITC	Immunotech	0766
CD45	PE	Immunotech	2078
CD45	PE CY5	Immunotech	IM2653
CD49B	FITC	Immunotech	1425
CD51	FITC	Immunotech	1855
CD61	FITC	BD	348093
CD95	PE	Pharmingen	555674
CD122	FITC	Immunotech	6604931
CD127	PE	Immunotech	IM1980
CD130	PE	Pharmingen	MO31585
CD132	PE	Pharmingen	35195B
Epithelial cell antigen	FITC	Dako	F0860
a-cytokeratin 5,6	Unconjugated	Boehringer Mannheim	-
PCNA	FITC	Dako	F0863
TNF-R1	PE	R&D	FAB225
TNF-R2	FITC	R&D	FAB226F
a-bcl-2	FITC	Dako	F7053
a-p53	PE	Novo Castra Labs	1801
Goat anti-mouse IgM	FITC	Rockland	610-1207
Rat-a-mouse IgG1	PE	BD	340270
Rat-a-mouse IgG1	FITC	BD	
Rabbit-a-mouse Ig	-	Dakopatts	Z259
Rabbit a-human p65	Unconjugated	Santa Cruz	
Donkey anti-rabbit F(ab2)AP	FITC	Jackson Immuno Res. Labs	711096152
Donkey anti-rabbit	PE	Jackson Immuno	711116152
a-TGF-β	Unconjugated	R&D	MAB240
a-TGF-β	PE	IQ products	IQP169P
TNF-a	PE	BD	340512
TNF-a	FITC	BD	340511
a-IL-2	FITC	BD	340448
a-IL-4	PE	Pharmingen	18655A
a-IL-6	PE	Pharmingen	18875A
a-IL-8	FITC	BD	340509
a-IL-8	PE	Pharmingen	554720
IgG1/IgG1	FITC/PE	BD	349526

A3-3 Equipment

Item	Supplier
Polystyrene tubes suitable for use on a FACSCalibur (FACS tubes)	Evergreen Scientific
Sterile 6ml Facs tubes for sorting	Falcon
10 ml conical polypropylene tubes (Culture tubes)	Johns Professional Products
Finnpipette 0.5-10ul	Labsystems
Finnpipette 5-40ul	Labsystems
Micropipettor - Eppendorf 'repeater' 1-5ml with plastic tips	Eppendorf
Centrifuge with swinging bucket rotor (16x100mm tube carriers)	Heraeus (carriers: Sepatech)
Sorvall RT 600D centrifuge	Du Pont
"Dark Box" with plastic lining	'Home made'
Technicon HIE	Bayer Diagnostics
FACSCalibur Flow Cytometer	BD
Cellquest Software	BD
AutoCOMP software	BD
Water bath	Grant Instruments, Cambridge
ELISA plate washer	SLT Labinstruments, France
ELISA plate reader	Hybaid Ltd., U.K.
Staining Machine	Shandon Veristat Southern Products
305nm transilluninator UV source	UVP
25cm2 tissue culture plates	Corning-Costar
Untrafine insulin syringe (29 Gauge)	BD

A3-4 Preparation of buffers and reagents

1. *Phosphate buffered saline (PBS) (0.1M, pH7.4)*

Method

Dissolve 8.0g sodium chloride, 0.2g potassium chloride, 1.44g disodium hydrogen orthophosphate (dihydrate) and 0.2g potassium dihydrogen orthophosphate in 1L purified water. Adjust pH to 7.4.

2. *Dulbecco's PBS (DPBS) + BSA ("wash buffer") without magnesium or calcium*

Method

Dissolve 16g sodium chloride, 0.4g potassium chloride, 2.3g disodium hydrogen orthophosphate and 0.4g potassium dihydrogen orthophosphate in 2L purified water. Adjust pH to 7.4-7.6.

Add BSA to a final concentration of 1%.

3. *RPMI + 10% FCS + Pen/Strep*

2 pkt	RPMI 1640 Powder Gibco Cat No. 31800-022
2 vials	Penicillin/Streptomycin Gibco Cat No. 15075-0130
100 ml FCS	
2 litres	Sterile water for injection or distilled deionised water
2 litre	glass bottle/flask labelled appropriately
2 litre	glass measuring cylinder

Method

Measure out water. Use 2x 20 ml water to reconstitute Pen/Strep vials.
Pour 1800 mls water into the glass container.
Add powdered media to the water with gentle mixing. DO NOT HEAT.
Use rest of the water to rinse out the packets and add to the flask.
Add Pen/Strep vial contents. Add FCS. Adjust pH to ~ 7.1
Keep container covered until filtration. Sterile filter. Store 4°C

4. *HEPES buffer*

HEPES	10 mmol/l	NaCl	150 mmol/l
KCl	5 mmol/l	$MgCl_2$	1.8 mmol/l
$CaCl_2$	1.8 mmol/l		

Method

Make up in sterile water for injection or distilled deionised water

Adjust pH to 7.0 with 1M NaOH

Check osmolality. Sterile filter

Store room temperature or4^{0}C

5. Mononuclear cell separation- Lymphoprep

Mononuclear cells have a lower buoyant density than erythrocytes and polymorphonuclear cells. Mononuclear cells can therefore be isolated by centrifugation on an iso-osmotic medium with a density close to 1.077g/ml (Lymphoprep) which allows the erythrocytes and granulocytes to sediment through the medium while retaining the mononuclear cells at the sample medium interface. The specimen should be diluted with culture medium prior to layering on Lymphoprep. Method should be performed aseptically in a laminar flow hood.

Requirements

Lymphoprep (NYCOMED)

Alcohol swab

21G needle

10ml syringe

Mixing cannula

10ml centrifuge tube

Tissue Culture Medium (TCM)

eg Hank's Balance Salt Solution without Ca/Mg (HBSS)

Sterile transfer pipettes

Waste beaker

Method

Swab cap of Lymphoprep bottle with alcohol swab and aseptically withdraw 3 to 4 ml of Lymphoprep with a 21G needle and syringe.

Replace the needle with a mixing cannula and gently underlayer the specimen with the Lymphoprep.

Centrifuge the tube at 400g (Sorvall centrifuge 1500rpm) for 25 minutes at 15^{O}C.

Being careful not to disturb the layers aspirate off the supernatant with a sterile transfer pipette and discard into the waste beaker.
Collect the mononuclear cells at the interface above the Lymphoprep and transfer into a centrifuge tube.
Wash twice with at least double the volume of tissue culture medium.
Centrifuge at 800g (Sorvall centrifuge 2000rpm) for 10 minutes.
Decant or aspirate off supernatant.
Resuspend cell pellet in about 2 to 3 ml of TCM.

6. Ionomycin

1 mg dissolve in 2ml absolute ethanol
Add 18ml DPBS
Aliquot 200ul lots -20^0C
Use 20ul/ 1ml cells or whole blood = 1ug/ml

7. Brefeldin A

5mg Sigma B6542 ; dissolve in 1 ml DMSO [sterile] 5mg/ml = 5ug/ul
Store in 20ul aliquots -20^0C
Add 180 ul DPBS = 0.5ug/ul
Use 20ul/ml = 10ug / ml cells

8. PMA

1mg Sigma P1839
Dissolve in 1ml sterile DMSO = 1mg/ml
Dilute to 10ml = 0.1mg/ml
Store 20ul aliquots -20^0C
Dilute 1:50 by adding 980ul DPBS to 20ul PMA
Use 25ul/ml cells = 25ng (50ug/ml diluted 1:50= 1ng/ul so 25ul=25ng)

9. *Sodium Heparin* 5000 IU in 1 ml (David Bull Labs. Victoria, Ausralia).
Collect 10 ml whole blood into 100µl (50 IU) Heparin and mix well

10. Reagents for Papanicolou staining

(a) Acid-alcohol (0.6%)
6ml conc. HCL in 1000ml 70% alcohol

(b) Sodium bicarbonate (0.25%)

10g in 4000ml water (approx 10 heaped scoops of large flat spatula

(c) 70% alcohol: 300ml absolute alcohol in 700ml water

96% alcohol: 40ml absolute alcohol in 960ml water

(d) Absolute alcohol

(e) OG-6

(f) EA-50

(g) Xylene substitute

11. DAPPI staining

Fix cells in cold acetone/alcohol kept at -70^0C for 1 min.

Add 1 drop DAPPI for I min.

Wash in slide wash 5 min.

Mount in antifade. Protect from light.

Slide wash (Tris buffer)

0.05M TRIS (24.23g)

0.15M NaCl (35g)

0.01% Triton X-100 (16 drops)

Make up to 4L with distilled water.

Adjust pH to 7.6 using 1NHCl

Anti-Fade Media

Combine:

2 g n-propyl gallate (shelf powder)

40 ml glycerol (shelf liquid)

10 ml 1X PBS

Stir these on a stir plate with low or medium heat until the n-propyl gallate is completely dissolved (this may take up to an hour).

Adjust the pH to 7.6, using NaOH and HCl.

Store in the refrigerator in screw-top vials covered with foil (to protect from light).

Anti-Fade media is very viscous, but does not harden. To use, apply enough media to the slide so that the space between the coverslip and the slide is completely filled. Vacuum off the excess anti-fade media from the edges, and seal with nail polish.

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