The Role of NF-κB p65 and Brd4 in Oxidative Stress Driven Inflammation

A thesis submitted for the degree of

Doctor of Philosophy (PhD)

Younis M. Khan

January 2013

Airway Disease Section, National Heart & Lung institute,

Faculty of Medicine, Imperial College London,

Dovehouse Street SW3 6LY UK.

Declaration

I hereby declare that the work presented in this thesis is of my own investigation and written by myself.

Candidate: Younis M. Khan

Signature: *Younis Khan*

Abstract

Chronic obstructive pulmonary disease (COPD) is caused by prolonged cigarette smoke (CS) exposure. Increased oxidative stress produced by elevated oxidants, derived from cells and CS, or reduced anti-oxidant response is reported in COPD patients. Chronic inflammation is another key characteristic of COPD which persists long after smoking cessation. This suggests that oxidative stress may affect the course of inflammatory gene expression through changes in epigenetic modifications such as DNA and histone methylation and histone acetylation. Hydrogen peroxide (H_2O_2) is one of many oxidants reported to be elevated in breath condensates of COPD patients and is associated with enhanced inflammation. Inhaled corticosteroids (ICS) are frequently used in the management of inflammation in COPD patients; however, they provide little or no benefits to COPD patients. Understanding the mechanisms underlying this enhanced inflammatory drive and the lack of ICS response may provide novel potential therapeutic targets. Abnormal histone acetylation profiles have been linked to COPD and to relative steroid unresponsiveness. In mononuclear cells, BET bromodomain inhibitors can block acetylation-induced inflammatory responses.

H_2O_2, an oxidant, was used to establish both an acute (2 hours) and a Chronic (low concentration exposure for 5, 10 and 15 days) oxidative stress model. In the acute model, H_2O_2 (100μM) enhanced TNF-α induced IL-6 and CXCL8 expression in BEAS-2B cells whereas H_2O_2 alone had no effect on inflammatory gene expression. This confirms that oxidative stress is involved in the enhanced induction of inflammation. Continued exposure of cells to lower concentrations of H_2O_2 (50μM) for 5 days results in a similar enhancement of TNF-α-induced IL-6 and CXCL8 expression but this effect was lost over 10 and 15 days due to induction of antioxidant genes. We extended the acute model with slight modification by using IL-1β instead of TNF-α as it gave us better IL-6 and CXCL8 induction. Intracellular studies showed that oxidative stress-driven cytokine induction is mediated via activation of the NF-κB pathway and increasing NF-κB p65 promoter binding. This was associated with increased histone H3 acetylation at κB response elements in both the *IL-6* and *IL-8* promoter

regions. Chromatin immunoprecipitation assays also confirmed recruitment of bromodomain protein 4 (Brd4) to the same sites. Inhibition of Brd4 by JQ1 and PFI-1 significantly reduced the expression of IL-6 and CXCL8 cytokines.

H_2O_2 enhanced IL-1β-induced IL-6 and CXCL8 expression by activating the NF-κB pathway. The induction of these cytokines was reduced by the BET bromodomain inhibitors JQ1 and PFI1. Our findings demonstrate that bromodomain inhibitors could potentially be used as new therapeutic agents in inflammatory diseases whereas ICS are ineffective.

Acknowledgement

I am immensely grateful to my primary supervisor, Prof. Ian M. Adcock, for his tireless supervision, guidance, advice and sharing his invaluable knowledge with me. I admire his enthusiasm, dedication and energy. I am thankful to my secondary supervisor, Dr. Paul Kirkham, for his supervision and advice. My gratitudes are extended to Prof. Peter J. Barnes for providing me with the opportunity to carry out research at Airway Disease Section. I would also like to thank Medical Reserch Council for funding both my MRes and PhD.

I thank everyone who helped me in the lab at the Airways Disease Section over the years in particular Amir, Colin, Elen, Ada, Lucy, Kiran, Nadia, Pai-Chen, Po-Jui Chang, Nico and Charis.

Finally, I dedicate this PhD thesis to my mum, brother, sisters and wife to be. I wouldn't have been able to finish this PhD without their love, prayers and support. Thank you so much!

"Always dream and shoot higher than you know you can do. Do not bother just to be better than your contemporaries or predecessors. Try to be better than yourself." William Faulkner

Table of contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Abbreviations

AAT	Alpha-1 antitrypsin
ABECs	Airway Bronchial Epithelial Cells
AP-1	Activator protein-1
ASMCs	Airway smooth muscle cells
BALF	Bronchoalveolar lavage fluid
BET	Bromodomain and extra terminal domain
BEAS-2B	Transformed human bronchial epithelial cell line
BPE	Bovine pituitary extract
Brd2	Bromodomain-containing protein 2
Brd4	Bromodomain-containing protein 4
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CITED2	CBP/p300-interacting transactivator-2
COPD	Chronic obstructive pulmonary disease
Co-IP	Co-immunoprecipitation
CpG	Cytosine-phosphate-Guanine
CS	Cigarette smoke
CT	Computed tomography
DNMT	DNA methyltransferase
DNP	Dinitrophenylhydrazine
DoH	Department of Health
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene-di-amine-tetraacetic acid
FCS	Foetal calf serum
FACS	Fluoroscence activated cell sorter
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity

GC	Glucocorticosteroid
GM-CSF	Granulocyte-macrophages colony-stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
GWA	Genomic-wide association
H_2O_2	Hydrogen peroxide
HMT	Histone methyl-transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HO-1	Haemoxygenase-1
4-HNE	4-hydroxy-2-nonenal
ICS	Inhaled corticosteroids
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL-1β	Interleukin 1 beta
IP	Immunoprecipitation
JQ1	Jun-Qui 1 (Bromodomain inhibitor)
Kac	Acetylated lysine
LVRS	Lung volume reduction surgery
MDA	Malondialdehyde
MAPK	Mitogen activated protein kinase
MnSOD	Manganese Superoxide dismutase
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor-kappa B
PAFs	Platelet-activating factors
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCAF	P300/CBP-associated protein
PFI-1	Pfizer inhibitor 1 (Bromodomain inhibitor)

PTMs	Post-translation modifications
QPCR	Quantitative polymerase chain reaction
rEGF	Recombinant epidermal growth factor
RIPA	Radioimmunoprecipitation assay
RNA Pol II	RNA polymerase II
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TAD	Transactivation domain
TAF2	TBP-associated factor 2
TBP	TATA-binding protein
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour Necrosis Factor-alpha
TSS	Transcription starting site

Chapter 1

General Introduction

1.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles and gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients" (GOLD 2011). This progressive worsening of the lung function is caused by narrowing of the small airways due to increased mucus production, proliferation of the smooth muscle cells and fibrosis. This is also accompanied by the destruction of alveoli structure (emphysema) and chronic bronchitis (Fig. 1.1)(Barnes 2007). Weight loss, nutritional abnormalities and skeletal muscle dysfunction are also recognised symptoms of COPD (Vogelmeier and Bals 2007). Physical manifestations of the disease include breathlessness (dyspnea), chronic cough with/without sputum, wheezing and intolerance to exercise (Qaseem, Wilt et al. 2011).

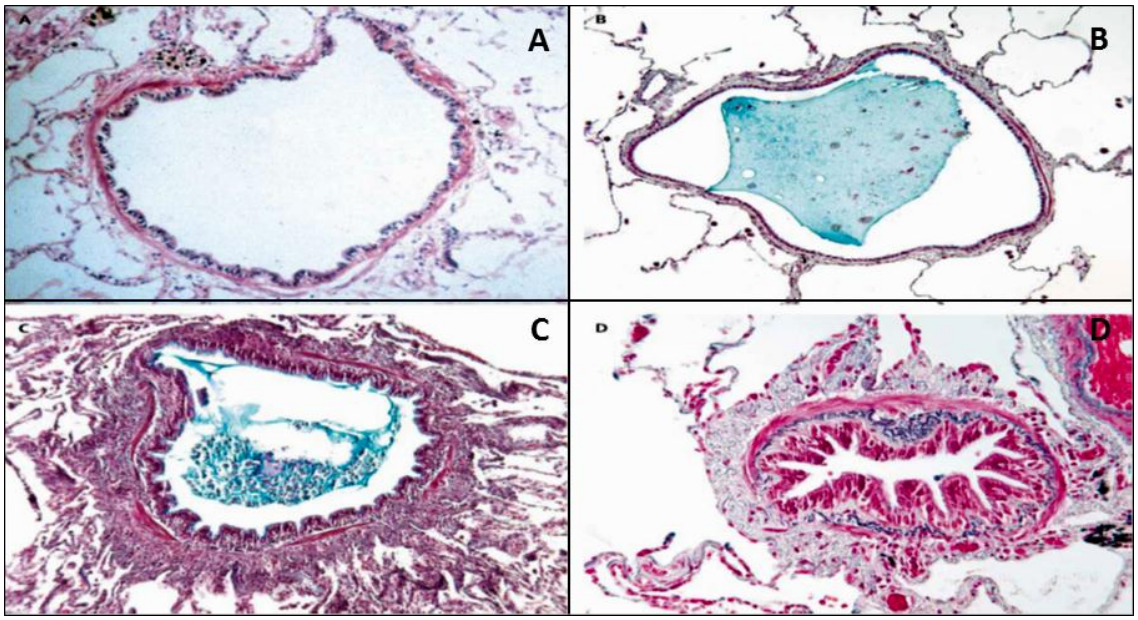


Figure 1.1: Histopathology of COPD small airways (A) Normal airway with open lumen **(B)** Small airway lumen filled with mucus **(C)** Acute inflammation and mucus surrounded by thick airway wall **(D)** Chronic inflammation: fibrosis and hyperplasia of smooth muscle cells accompanied by fibrosis resulting in the obstruction of the airway. Adapted from Hogg (2004).

1.1.2 Epidemiology and prevalence

COPD is ranked as the fifth most common cause of death globally and predicted to be the fourth leading killer by 2030. It is just below ischemic heart disease, cerebrovascular disease, and HIV/AIDS (Mathers and Loncar 2006). It is believed that the lifetime risk of developing COPD is 28% by age 80 (Gershon, Warner et al. 2011). The World Health Organisation (WHO) projected that it could become the 3rd biggest cause of mortality by 2020 worldwide (Fig. 1.2) (Buist, McBurnie et al. 2007). The prevalence of COPD among the world population counts for about 10%. According to WHO, there are more than 64 million people affected by COPD (Mathers and Fat 2008). However, this is underestimated and the actual number is higher than 600 million (Sin and Vestbo 2009). Most COPD patients are either misdiagnosed or unrecognised even long after severely disabled (Barnes 2007; WHO 2008). The rate of COPD has increased in the affluent countries in comparison with the developing countries mainly due to aging population and better management of other major illnesses such as cancer and cardiovascular diseases (Nowak, Berger et al. 2005). One quarter of general adult population age 40 or above have mild airflow obstruction (Buist, McBurnie et al. 2007; Mannino and Buist 2007; Menezes, Perez-Padilla et al. 2008). For example, there is an estimated 12.2 million (14.3%) people aged ≥ 40 suffers from mild airflow limitation in the five major cities of Latin American countries (Brazil, Mexico, Uruguay, Chile and Mexico) (Menezes, Perez-Padilla et al. 2008). Epidemiological studies have shown that prevalence of COPD in USA has doubled between 1979 and 2002 (Decramer, Janssens et al. 2012). It affects 5% of adult population and costs the economy $49.9 billion per annum (Qaseem, Wilt et al. 2011). In Canada, a 2% increase in the numbers of females with COPD was observed between 1996 and 2007 and 9.5% of the Canadian population suffer from COPD (Gershon, Wang et al. 2010). There are still many parts of the world, for example Africa and Asia, from which detailed epidemiological data are yet to be reported (Soriano and Rodriguez-Roisin 2011).

According to Department of Health (DoH), 25,000 people die from COPD a year in England and Wales. COPD accounted for 4.8% of all deaths in England between 2007 and 2009. The DoH reveals

that around 835,000 people suffer from COPD in the UK and an estimate 2.2 million (13% of population of England) aged 35 and above remain undiagnosed (Department of Health 2011). COPD also places an enormous financial burden on health system and economy as a direct result of hospitalisation and time lost from work (Barnes 2007; Gruffydd-Jones 2008). It has been reported that GP consultation and hospitalisation has increased for COPD compared with cardiovascular diseases, resulting in a greater than 3-fold increase in healthcare costs (Barnes 2007). An estimated cost for COPD is over £800 million annually in addition to £2.7 billion in lost working days in the UK alone (Department of Health 2010). It is clear that COPD poses a serious problem socially and economically that needs to be addressed.

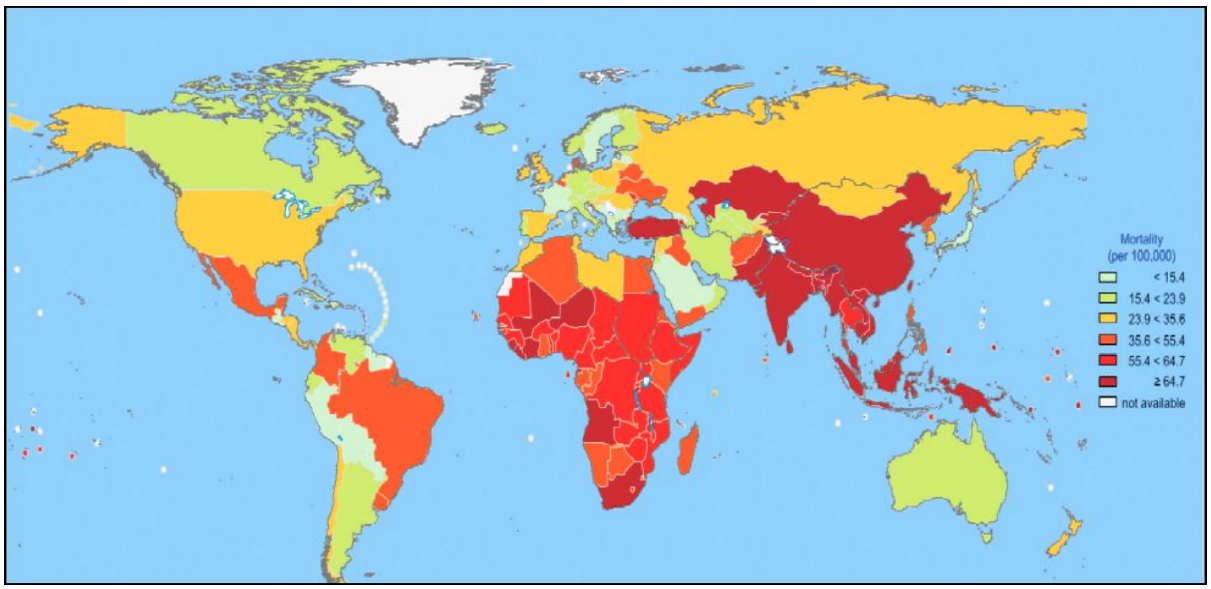


Figure 1.2: World map of estimate mortality rate caused by COPD in male patients per 100,000 (WHO updated 2004) (Mathers and Fat 2008; Bhome 2012).

1.1.3 Aetiology

Tobacco smoking is the primary risk factor linked to COPD in prosperous Westernised countries and environmental pollution, especially indoor biomass smoke, is associated in the developing world (Decramer, Janssens et al. 2012). Cigarette smoke (CS) is a complex mixture of noxious particles, chemicals and reactive oxidant species (ROS) (Rahman and Adcock 2006; Min, Bodas et al. 2011). Physical barriers and immune system protect the human lung from harmful environmental agents

such as pollution, bacteria, viruses and fungi (Nikota and Stampfli 2012). However, continuous cigarette smoking affects these barriers by increasing epithelial permeability and impairing mucociliary clearance (van der Vaart, Postma et al. 2004; Forteza, Casalino-Matsuda et al. 2012). In addition, both COPD and smokers are susceptible to respiratory infection, which is the main cause of exacerbations in COPD patients especially during later stages of the disease (GOLD III-IV) (Arcavi and Benowitz 2004; Churg, Cosio et al. 2008).

Not all smokers develop COPD and there have been reports of COPD in non-smokers. A number of risk factors have been identified, other than cigarette smoking, such as age, occupational exposure to organic dust, asthma, respiratory infection, biomass fuel combustion and poor socioeconomic status (Fig. 1.3) (Salvi and Barnes 2009; Clancy and Nobes 2012; Forteza, Casalino-Matsuda et al. 2012). In addition, susceptibility to COPD is also influenced by genetic risk factors. It is long known that deficiency in α_1-antitrypsin due to mutation in the gene serpin peptidase inhibitor, clade A, member 1 (*SERPINA1*) leads to COPD and accounts for 1-2% of COPD cases (Kukkonen, Tiili et al. 2011; Clancy and Nobes 2012).

Recent studies of genomic-wide association (GWA), that examine association between common genetic variation and associated phenotype between group of individuals, have identified a number of candidate genes which are associated with COPD (Berndt, Leme et al. 2012; Bosse 2012; Foreman, Campos et al. 2012). Among these, COPD patients show a correlation between lung function and single nucleotide polymorphisms (SNPs) in three loci (*GSTCD*, *TNS1* and *HTR4*) (Soler Artigas, Wain et al. 2011). *GSTCD* gene encodes for glutathione s-transferase C-terminal domain-containing protein. GSTCD protein catalyses glutathione (GSH) binding to oxidative stress products and regulates lipid mediators (prostaglandins and leukotrienes); thereby prevents cellular damage (Hayes, Flanagan et al. 2005). GSH is antioxidant that donates an electron (H^++e^-) to unstable molecules such as ROS to become oxidised glutathione disulphide (GSSG) (Flohe 2012). *TNS1* gene encodes for tensin-1 adhesion protein that show binding preference to actin with SH2 (Src homology 2) domain. Tensin-1 is associated with cell signalling and migration (Hall, Balsbaugh et al. 2010). *HTR4* gene encodes for

5-hydroxytryptamin 4 (5-HT) receptor which is a member of G-protein-coupled receptors (GPCRs) and expressed in neurones and epithelial type cells (Ghavami, Stark et al. 1999; Wilk, Shrine et al. 2012). GPCRs are generally expressed in phagocytes for chemokines and chemoattractants (Sun and Ye 2012). Similarly, *BICD1* (Bicaudal D homology 1) is another gene which has been studied in telomere shortening and cellular senescence (Savale, Chaouat et al. 2009). Leukocytes from COPD patients have reduced telomere lengths compared with healthy smokers and non-smokers (Savale, Chaouat et al. 2009). This premature reduction is associated with oxidative stress and inflammatory damage (Kawanishi and Oikawa 2004). SNP analysis of *BICD1* reveals genetic variation between emphysematous patients and healthy control (Kong, Cho et al. 2011). These loci may provide some explanation to the underlying cause of COPD but it is ultimately the interaction between nature (environmental risk factors) and nurture (genotype) that leads to COPD (phenotype) (Ober, Butte et al. 2010).

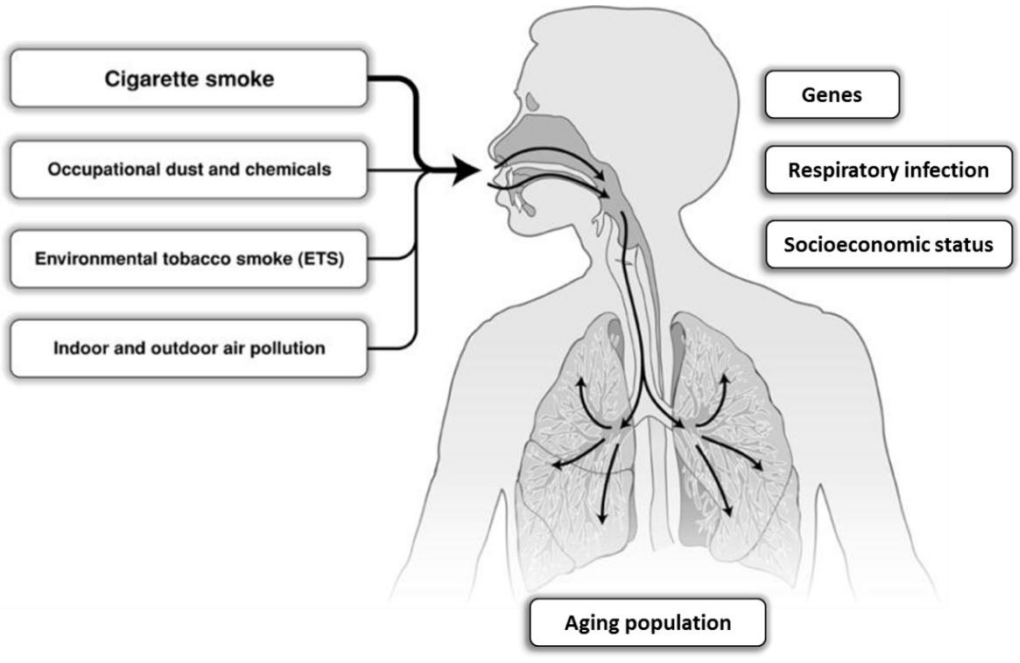


Figure 1.3: Risk factors associated with COPD. Tobacco smoking is the primary risk factor for COPD in about 90% of cases. However, susceptibility of an individual to COPD also depends on others environmental and genetic risk factors (Salvi and Barnes 2009; Berndt, Leme et al. 2012). Depiction obtained and modified from the Global Initiative for Chronic Obstructive Lung Disease (2011).

1.1.4 Pathophysiology of COPD

COPD is a generic term to describe a number of diseased conditions including emphysema, chronic bronchitis and small airways disease. Each condition has its unique clinical features but they all contribute to the continuous restriction of airflow throughout respiratory airways (Barnes 2007; Vestbo, Hurd et al. 2012). The normal function of the lung is to provide continuous supply of oxygen and remove carbon dioxide. It is achieved through simple diffusion which occurs between alveoli and blood. This exchange mechanism is vital for survival (Clancy and Nobes 2012). Lung function decreases with age but it is accelerated in COPD patients due to cigarette smoke (CS) (Fig. 1.4) (Fletcher and Peto 1977; Bednarek, Gorecka et al. 2006). Noxious particles in CS cause irritation of the airways which results in enlargement of mucus gland, mucus hypersecretion, ciliary dysfunction and epithelial-cells hyperplasia in the early stage of the disease. This is accompanied by increased in goblet cells, smooth muscle cells mass, and fibrosis (Barnes 2004; Rogers 2005).

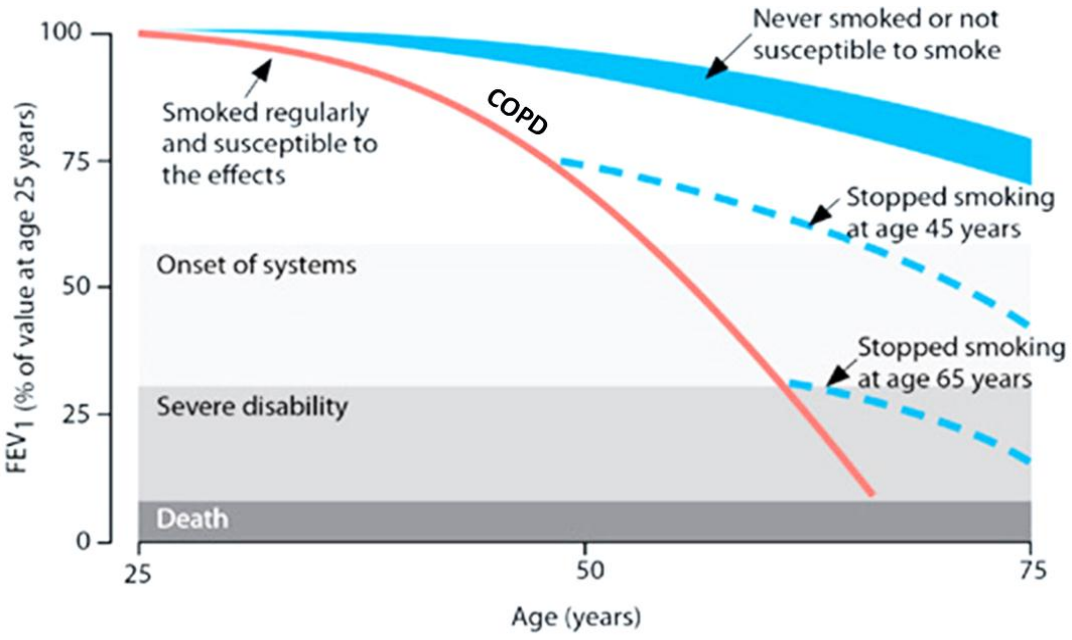


Figure 1.4: The effect of cigarette smoking on lung function. Lung function decreases with senescence, however, this is rapidly decreased in in COPD patients. Adapted from Fletcher and Peto (1977) and Bednarek, Gorecka et al. (2006).

CS and other irritants trigger inflammatory reaction in the small airways, lung parenchyma and elsewhere in the body (systemic) (Barnes 2008; Voelkel, Gomez-Arroyo et al. 2011). Inflammation occurs not only in COPD patients but also in healthy smokers (Willemse, Postma et al. 2004). However, the degree of intensity varies depending on individual susceptibility, genetics and the magnitude of CS exposure (Dewar and Curry 2006). Persistent inflammation and on-going repair of the airways leads to remodelling involving decrease in airway lumen and increased in the surrounding tissues (Fig. 1.5). This remodelling is accompanied by excess mucus in the airways, causing airflow obstruction (Decramer, Janssens et al. 2012). In addition, chronic inflammation and increase proteinases such as elastase and matrix metalloproteinases (MMPs) released from macrophages and neutrophils cause the disappearance of surrounding alveoli, leaving behind abnormal enlarged airspace-termed emphysema. This has an impact on the O_2 and CO_2 exchange mechanism (Min, Bodas et al. 2011; Voelkel, Gomez-Arroyo et al. 2011).

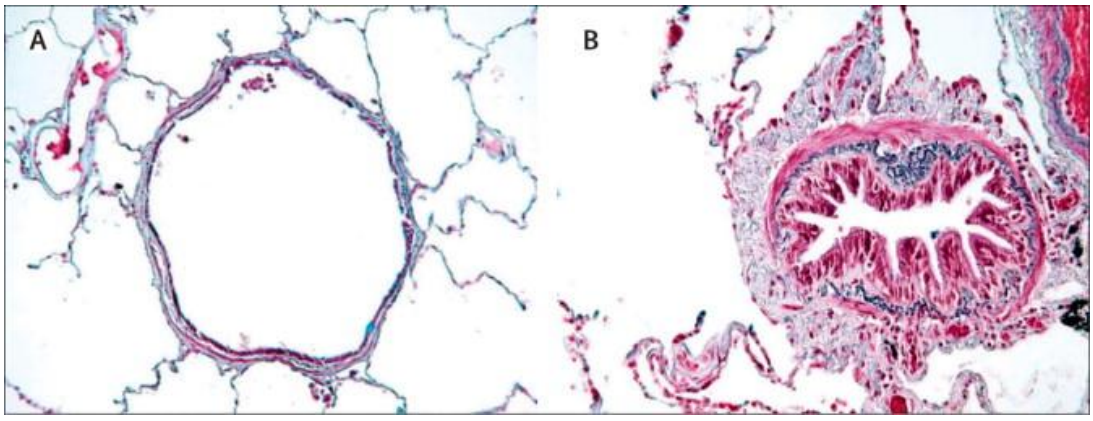
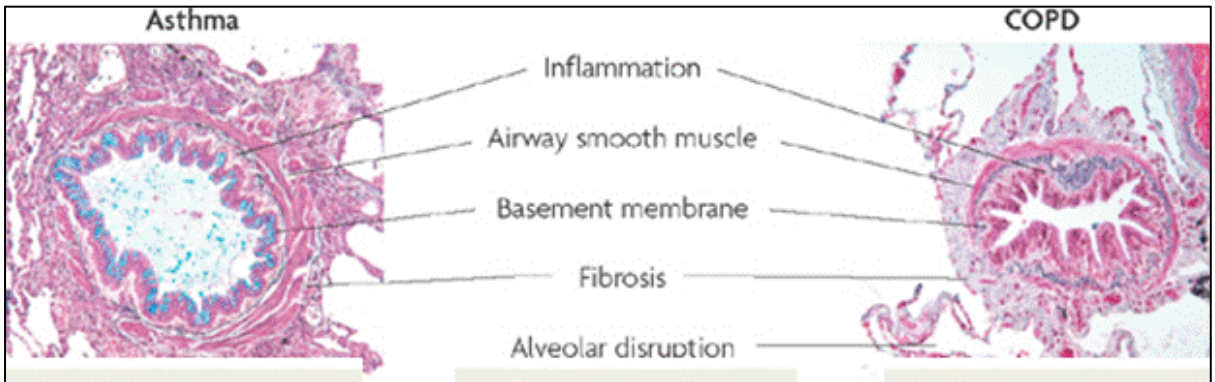


Figure 1.5: Comparison between healthy and COPD airways. (A) Normal airway: Large lumen and thin structural walls **(B)** COPD airway: Lumen is narrowed and surrounded by inflammed tissues (Decramer, Janssens et al. 2012).

COPD and asthma share some similar clinical features but there are major differences. Both are inflammatory disorders of respiratory tract and cause narrowing of the airways. However, asthma often starts in childhood and inflammation is localised to larger airways, whereas, COPD develops in age above 40 and associated with inflammation of small airways (Barnes 2008; Barnes 2008). The inflammatory response in asthma is mainly triggered by allergen and mediated by dendritic cells,

eosinophils, activated mast cells, CD^+4 T cells (Th2 cells). In contrast, COPD is associated with CS and recruit macrophages and neutrophils and CD^+ 8 T cells at site of inflammation (Fig. 1.6) (Barnes 2008; Zanini, Chetta et al. 2010). Steroids are the most effective anti-inflammatory drugs and are frequently used in asthma. However, they are ineffective in most patients with COPD (Barnes 2006).



+++	Inflammation	+++
+++	Airway smooth muscle	+
++	Basement membrane	–
+ (subepithelial)	Fibrosis	+++ (peribronchiolar)
–	Alveolar disruption	+++
++	Airway vessels	No change
++ (and activated)	Mast cells	Normal
++	Dendritic cells	ND
++	Eosinophils	Normal
Normal	Neutrophils	++
T_H2 type	Lymphocytes	T_H1 and T_C1 type
Often shed	Epithelium	Pseudostratified
++	Goblet cells	++

Figure 1.6: The difference between asthma and COPD. There are some similarities and differences between asthma and COPD including both histopathological and immunological changes which are highlighted above. Adapted and modified from Barnes (2008).

1.1.5 Diagnosis

COPD is diagnosed depending on signs, symptoms and medical history. However, a spirometry test is frequently used to assess lung function. Spirometry test shows an irreversible decrease in forced expiratory volume in 1 second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FEV_1/FVC) (Mannino and Buist 2007; 2011). The severity of the disease is classed into 4 categories depending on FEV_1/FVC ratio (Table 1.). X-ray and quantitative computed tomography (CT) are also utilised to evaluate lung function and structure (Mets, de Jong et al. 2012). The chest CT scans assess lung density, which is proportional to lung airspace enlargement otherwise known as emphysema (Berndt, Leme et al. 2012).

Classification of COPD severity	Classification based on post bronchodilator lung function
GOLD 1 (mild)	FEV_1/FVC <0·70 and FEV_1 ≥80% predicted
GOLD 2 (moderate)	FEV_1/FVC <0·70 and 80% >FEV_1 ≥ 50% predicted
GOLD 3 (severe)	FEV_1/FVC <0·70 and 50% >FEV_1 ≥ 30% predicted
GOLD 4 (very severe)	FEV_1/FVC <0·70 and FEV_1 <30% predicted or FEV_1 <50% predicted plus chronic respiratory failure

Table 1: Spirometric classification of COPD severity. Spirometry test is used to classify the severity of the disease. FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity. Adapted from Mannino and Buist (2007).

1.1.6 Treatments

The multifaceted nature of COPD has proved challenging to develop a treatment that can target all the components of the disease (Barnes 2010). Currently, combinations of pharmacological and non-pharmacological strategies are used in the management of COPD. Pharmacological options include bronchodilators, inhaled corticosteroids (ICS), combination therapy and long-term oxygen therapy (LTOT). Non-pharmacological interventions are smoking cessation, pulmonary rehabilitations,

mechanical ventilation and lung-volume-reduction surgery (LVRS) (Hanania, Ambrosino et al. 2005). Among these treatments, bronchodilators and ICS are frequently prescribed (Barnes and Stockley 2005).

Bronchodilators are used for symptomatic relief, which consists of short-acting β_2-agonists (Salbutamol) and long-acting (Salmeterol and Formoterol) or anti-muscarinic drugs like Tiotropium. They improve FEV_1 in some patients, but, the effect on lung function, reduction in inflammation and exacerbation remain poor (Hanania, Ambrosino et al. 2005; Barnes 2010; Bhome 2012). ICS are also used either alone or in combination with bronchodilators in the management of inflammation in COPD patients. However, they provide little or no benefits to COPD patients (Hakim, Adcock et al. 2012). It has minimal significant effects on some of the key inflammatory mediators such as CXCL8/IL-8, tumour necrosis factor-alpha (TNF-α) and MMPs (Barnes 2007). ICS and antibiotics are recommended by GOLD for symptomatic treatments in patients with exacerbation and whose FEV_1<50% of predicted (GOLD 2011; Mackay and Hurst 2012). Glucocorticoids are the most effective and widely used therapy in many of inflammatory and immune diseases with exception of COPD (Barnes and Adcock 2009). This lack of response is partially explained by cigarette smoke and oxidative stress, reducing HDAC2 activity and expression as well as impairing GR translocation (Barnes and Adcock 2003; Barnes 2009). However, it may also be due to other epigenetic changes, including alteration in DNA and histones status, under oxidative stress (Adcock, Tsaprouni et al. 2007; Adcock, Chou et al. 2009).

1.2 Chronic Inflammation

Inflammation is the core component of COPD and drives the pathogenesis of the disease, resulting in remodelling of small airways and destruction of lung parenchyma (Barnes 2008). Inflammation is a critical physiological response against infections, physical injuries, and irritants which is characterised by loss of function, redness, pain, swelling and heat (Fig. 1.7) (Lawrence, Willoughby et

al. 2002). Inflammation is vital for healing, pathogen removal and limiting harmful stimuli (Winkelman 2008). Inflammation is usually a temporary process that entails increased blood flow, fluids, leukocytes and inflammatory mediators to the site of injury. This results in quarantining and elimination of the harmful entities along with repairing the affected tissue. This short-lived inflammation is described as acute inflammation (Feghali and Wright 1997). However, continuous and unchecked inflammation, known as chronic inflammation, can result in a number of conditions including septic shock, asthma, atopic rhinitis, atherosclerosis, rheumatoid arthritis and COPD (Pitchford 2007; Fox, Hegde et al. 2010).

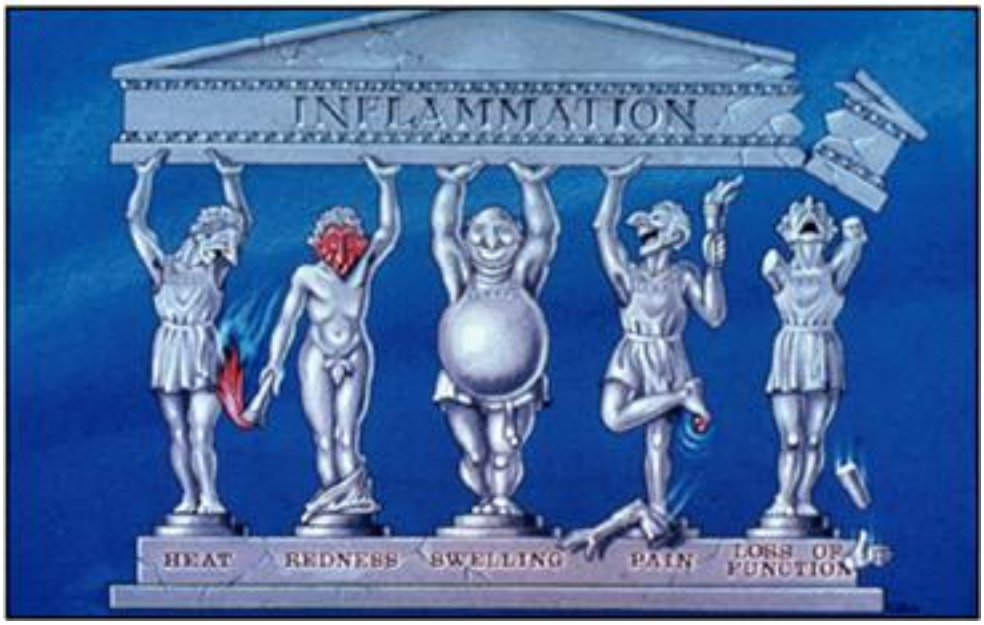


Figure 1.7: Characteristics of inflammation. Inflammation is a normal physiological response against infections, injuries and irritants which is chracterised by heat, redness, swelling, pain and loss of function (Lawrence, Willoughby et al. 2002).

1.2.1 Inflammatory Cells

Chronic inflammation is a complex disorder that involves several types of cells in a COPD (Barnes 2004). Irritants and chemicals, derived from cigarrete smoke (CS), activate a number of structural and immune cells that produce multiple inflammatory mediators, which orchestrate inflammation in COPD (Tamimi, Serdarevic et al. 2012). Structural cells maintain the integrity of bronchial wall and form a first line of defence against inhaled harmful agents. The inflammatory responses, initiated by

structural cells in the airways, are potentiated by immune cells, resulting in airway remodelling and obstruction (Panettieri 2004; Tamimi, Serdarevic et al. 2012).

(A) Structural cells

Structural cells play an important role in the induction of inflammation and airway remodelling in COPD. Some these cells are briefly described below.

I) Epithelial cells

Airway epithelial cells (AECs) line the airways and form a barrier against the pathogens and noxious particles (Wang, Bai et al. 2008). The main function of epithelium is to maintain the integrity and structure of the airways. Airway inflammation and injury may cause epithelial damage and abnormal activation of repair and/or activation process (Sacco, Silvestri et al. 2004; Wen, Reid et al. 2010). Following injury, the epithelium repairs and regenerates itself in order to restore its function through proliferation and differentiation (Fig. 1.8) (Di Stefano, Caramori et al. 2004). The epithelium consist of a number of specialised epithelial cells including ciliated epithelial and mucous (goblet) cells. A layer of mucus covering the ciliated epithelial cells traps and removes the inhaled particles from the airways, a process termed mucociliary clearance (Rogers 2005). In COPD, mucociliary function is impaired as a result of CS. This causes the production of excess mucus and its retention, leading to obstruction and infection of the airways (Koblizek, Tomsova et al. 2011; Liu and Di 2012). It has been shown that the airway epithelium is differentiated (squamous metaplasia) in chronic bronchitis and COPD due to increased proliferation (Barnes, Shapiro et al. 2003). In addition, AECs are an integral part of the innate and adaptive immune response. They produce antimicrobial peptides such as mucins, defesins and antiprotease (SLPI: secretory leukoprotease inhibitor) (Barnes 2004). They also produce pro-inflammatory factors, which recruit and activate phagocytes to the site of infection (Li, Wang et al. 2012). CS and other noxious particles may modify these beneficial

roles of AECs, making COPD patients more susceptible to respiratory infection and exacerbation (Dacydchenko and Bova 2007; Hurst, Vestbo et al. 2010).

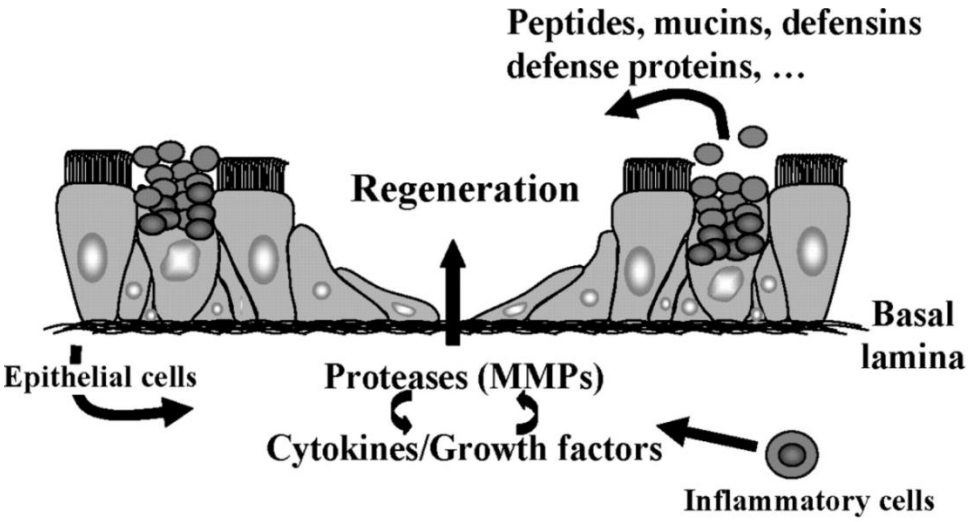


Figure 1.8: The role of epithelial cells in aiways. Epithelial cells protect and maintain the integity of the aiways. The epithelium consists of speciliased cells such as ciliated epithlial and goblet cells, which together are involved in mucociliary clearance. Epithelial cells also have the ability to regenerate following injury. Epithelial cells are an integral part of adaptive and innate immune response by secreting several antimicrobial peptides, proteases and inflammatory mediators. Adapted and modified from Puchelle, Zahm et al. (2006).

II) Airway smooth muscle cells

Airway smooth muscle cells (ASMCs) encircle the entire bronchial airways in the circular orientation below the epithelial basement membrane (Fig. 1.9) (Matthay and Clements 2004). Beside maintaining airway structure, their possible functions include controlling the size of airway lumen, expelling foreign materials through coughing or forced expiration and assisting in lung contraction (James and Carroll 2000). Evidence also suggests that in addition to their contractile abilities, they produce several inflammatory and growth factors which not only contribute to inflammation but also alter contractile properties and calcium homeostasis, resulting in airway wall remodelling (Panettieri 2004; Chang, Bhavsar et al. 2012). ASMCs account for bronchial hyper-responsiveness in asthma and COPD which is characterised by a heightened response to direct or indirect challenges and also the reduced bronchodilator response seen in COPD patients (Chung and Sterk 2000). The small airways in COPD patients become thicker and rigid due to increased ASMCs proliferation (Knox

1994). The changes in airway smooth muscle are more prominent in the small airways than in large airways and correlate with airflow limitation in COPD (Chung 2005; Camoretti-Mercado 2009).

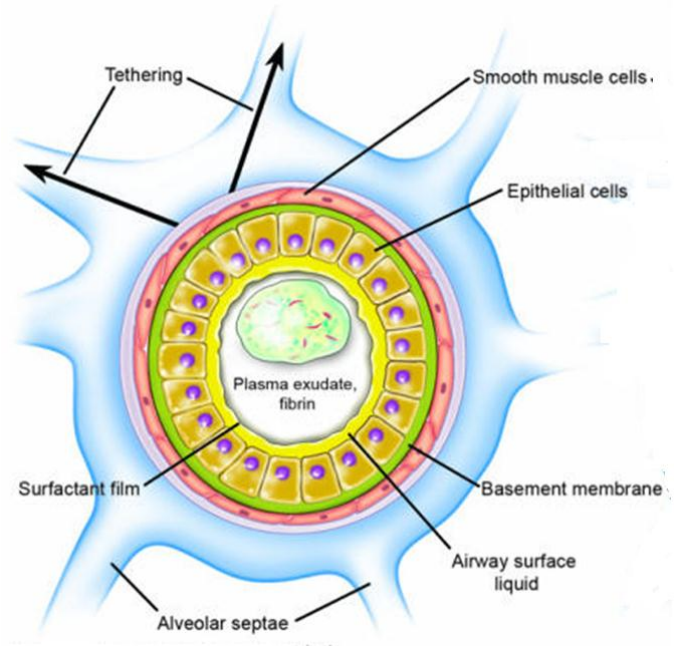


Figure 1.9: The stucture of the bronchial airway. The figure illustates the structure of the aiway with smooth muscles cells encircling the airway underneathe the epithelial basmement membrane (Matthay and Clements 2004).

III) Fibroblasts

Airway fibroblasts are responsible for extracellular matrix (ECM) regeneration and maintenance. They are major producers of ECM proteins (laminin, fibronectin, and fibrinogen), collagens proteoglycans and inflammatory mediators (Ingram, Huggins et al. 2011; Zhang, Wu et al. 2011). Fibroblast are found in the extracellular matrix under the epithelial basement membrane, interacting with the sheath of smooth muscle cells (Fig. 1.10) (Knight 2001). The ability of epithelial cells to repair the damage in the airways is not only dependent on their own actions but also on inflammatory mediators, growth factors and ECM proteins, released by other cells in particular fibroblasts (Sacco, Silvestri et al. 2004). Fibrosis is a the key feature of airway remodelling, characterised by the excess deposition of collagen fibres and proteoglycans under the basement membrane, which is seen in COPD and asthmatic patients (Brightling, Gupta et al. 2011; Rennard and

Wachenfeldt 2011). Fibroblasts are an important source of transforming growth factor (TGF-β), which is associated with the induction of local fibrosis (Huang, Sharma et al. 2002; Wells, Kruglov et al. 2004). TGF-β, released from the fibroblast of COPD patients, modulates the production of proteoglycans and ECM proteins (Konigshoff, Kneidinger et al. 2009). It has been shown that proteoglycans are differentially expressed in COPD Lungs (Hallgren, Nihlberg et al. 2010). A study has also demonstrated that fibroblasts from COPD patients manifest diminished repair responses as compared to healthy controls and smokers, attributed to the development of emphysema (Togo, Holz et al. 2008).

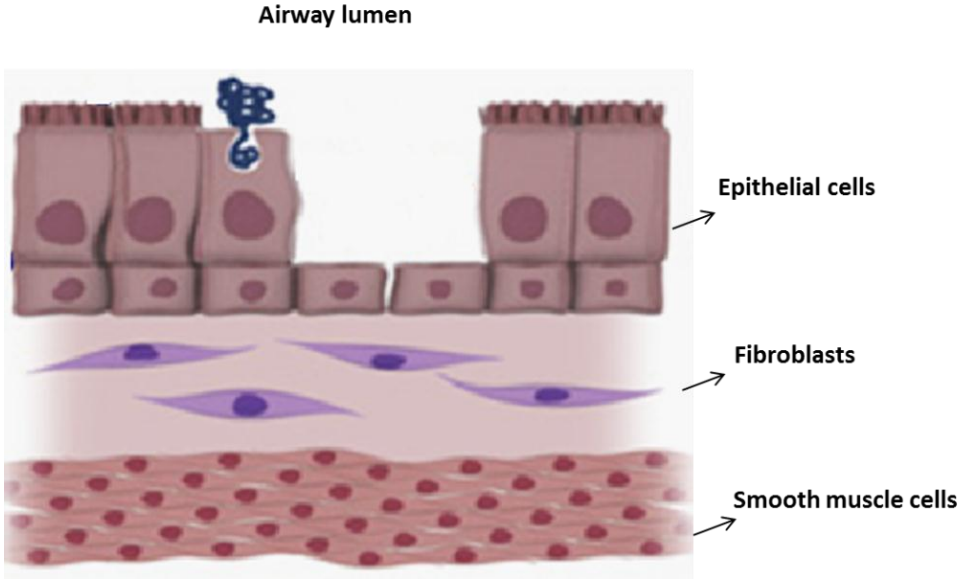


Figure 1.10: Fibroblasts in the lung airways. Fibroblast are found in the extracellular matrix beneath the epithelial basement membrane, interacting with the smooth muscle cells. Fibroblasts are major producers of, growth factors, collagens and proteoglycans which contribute to fibrosis and airway remodelling following inflammation and injury. Adapted and modified from Holgate (2007)

B) Immune cells

Chronic inflammation in COPD is associated with the accumulation of several immune cells in the small airways and the severity of the disease correlates with the degree of airway inflammation (Chung and Adcock 2008). Some of these cells are discussed below.

I) Macrophages

Macrophages are one of the most important effector and inflammatory regulatory cells in COPD and play a central role in the innate and acquired immune system (Barnes 2004). They are involved in the clearance of pathogens, necrotic cellular debris and apoptotic neutrophils from respiratory tract through phagocytosis (Donnelly and Barnes 2012). Macrophages are activated by CS and secrete several inflammatory proteins that orchestrate inflammation in COPD (Yao and Rahman 2009). Studies have shown that there is an increased presence of macrophages in the sputum, bronchoalveolar lavage fluid (BALF), and small airways of COPD patients which increases with disease severity (Tetley 2002; Barnes 2004; Frankenberger, Menzel et al. 2004). Macrophages are differentiated tissue monocytes that originate from stem cells in the bone marrow. They are considered as phagocytic cells that reside in lymphoid and non-lymphoid tissues (Geissmann, Manz et al. 2010). This higher numbers of macrophages in smokers and COPD patients may be due to increased recruitment of monocytes from circulation into the inflamed tissue or increased proliferation and survival in the lung (Rosseau, Selhorst et al. 2000; Barnes 2004).

Activated macrophages produce a range of effector molecules including ROS, lipid mediators, cytokines, chemokines and proteases (Fig. 1. 11) (Wood and Stockley 2006; Chung and Adcock 2008). Corticosteroids are ineffective in suppression of inflammation in COPD (Adcock, Ito et al. 2004; Hayashi, Wada et al. 2004). In accordance, the release of inflammatory mediators (CXCL8, TNF-α and MMP-9) from COPD macrophages failed to be suppressed by dexamethasone following stimulation with IL-1β (Culpitt, Rogers et al. 2003). There is a positive correlation between the numbers of macrophages in the alveolar space of patients with emphysema compared with normal smokers (Retamales, Elliott et al. 2001; Meshi, Vitalis et al. 2002). This may be due to increased production of elastolytic enzymes (MMPs, cathepsins, elastase) by macrophages and neutrophils that cause alveolar walls destruction (Abboud and Vimalanathan 2008; Betsuyaku, Fuke et al. 2012). Studies have shown that elastolytic activity of alveolar macrophages (AMs) from patients with emphysema is greater in comparison with patient with other lung diseases (Ofulue and Ko 1999;

Russell, Culpitt et al. 2002; Abboud and Vimalanathan 2008). This is supported by significantly greater AM cell counts in the BALF of emphysematous patients compared with healthy smokers (Abboud, Ofulue et al. 1998; Tetley 2002). Overall, COPD macrophages overexpress inflammatory proteins and proteases, are less mobile and have a reduced phagocytic capacity compared to those from control subjects (Donnelly and Barnes 2012).

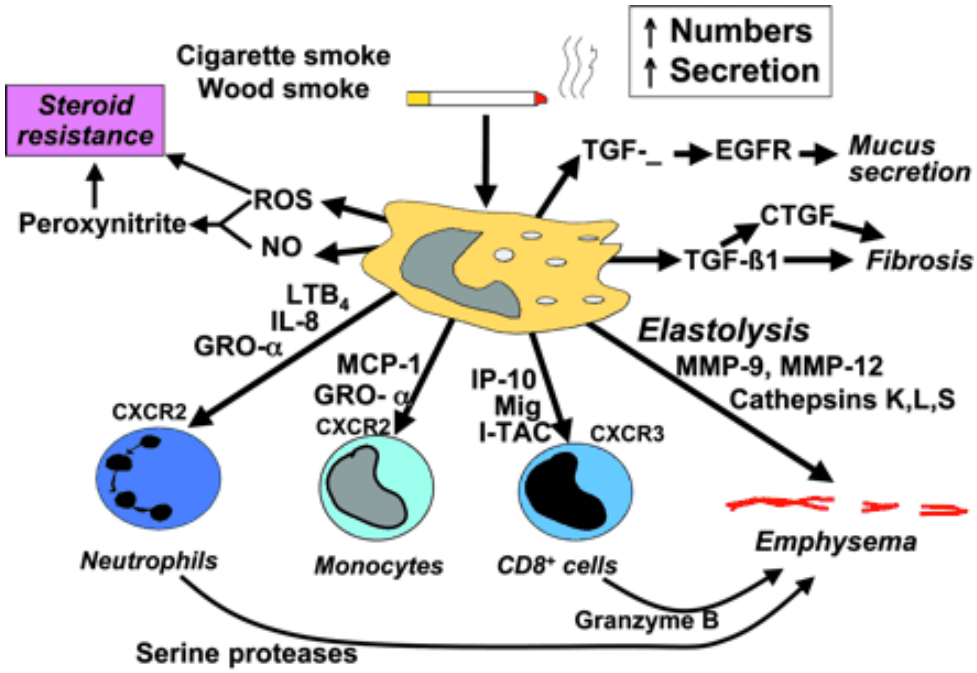


Figure 1.11: The activation of a macrophage by cigarette smoke extract. Cigarette smoke activates macropahges to prudeuce a variety of inflammatory proteins and proteolytic enzymes that contribute to the pathophysilogy of COPD, including emphysema, fibrosis, hypermucus secretions, steroid resistence and activation of other inflammatoy cells (Barnes 2004).

II) Neutrophils

Neutrophils have been proposed as the primary effector cells in COPD and the major cellular sources of inflammatory mediators (Barnes 2008). Neutrophils develop from stem cells in the bone marrow into fully mature neutrophils before they are released into the blood circulation. Upon activation, neutrophils migrate from blood vessels into the connective tissues at the site of injury/infection. They attach to endothelial cells along the vessel wall using adhesion glycoproteins such as selectin and integrin (Fig. 1.12) (Borregaard 2010; Bugl, Wirths et al. 2012). Activated neutrophils kill and eliminate microorganisms or harmful stimuli through phagocytosis, respiratory burst (generation of

ROS) and the release of proteases from their granules. They also secrete several chemoattractants and inflammatory mediators to recruit other inflammatory cells at the damaged site (Pettersen and Adler 2002). As inflammation resolves, neutrophils undergo apoptosis and are removed by macrophages. Programmed cell death (apoptosis) reduces the leakage of the cytotoxic content into the extracellular space as supposed to necrosis (Milot and Filep 2011; Zhou, Dai et al. 2012).

Sputum from COPD patients show increased neutrophilia which is correlated with the severity of peripheral airway dysfunction (O'Donnell, Peebles et al. 2004; Singh, Edwards et al. 2010). Neutrophil elastase and several MMPs are recognised as major proteinases released by neutrophils during inflammation (Shapiro 2002; Zhou, Dai et al. 2012). COPD patients, with deficient or low levels of α_1-antitrypsin, inadequately inhibit these proteinases, leading to excess tissue damage seen as emphysema (Shapiro 2002; Kukkonen, Tiili et al. 2011). Neutrophils from COPD patients show delayed apoptosis, increased chemotaxis and proteolytic activity when compared with healthy controls (Noguera, Batle et al. 2001; Milara, Juan et al. 2012; Zhang, He et al. 2012). This is indicative of the persistent inflammation seen in COPD.

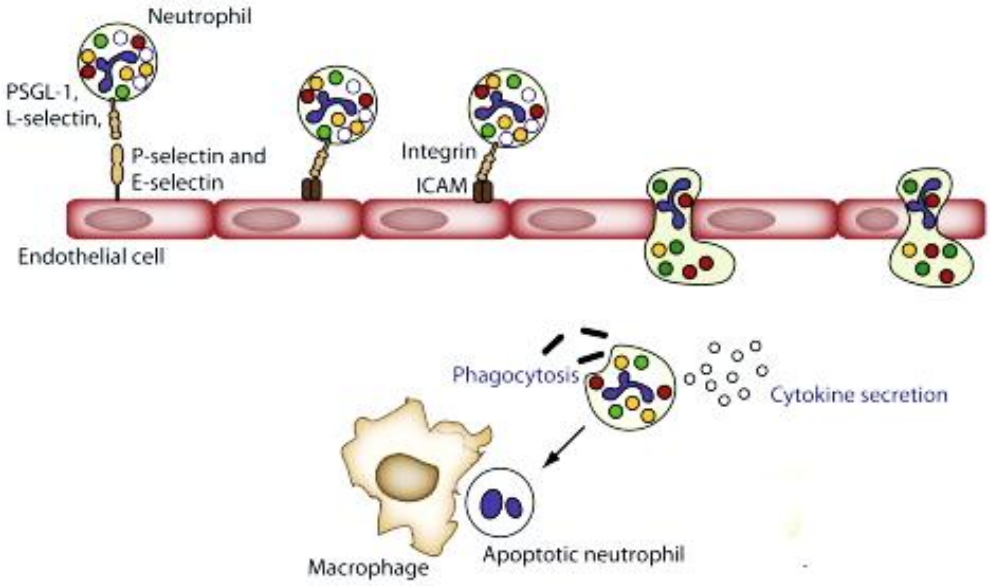


Figure 1.12: The migration of neutrophils from bloodstream to the site of injury. Under normal conditions, nueutrophils move along the blood vessel wall. Once activated by the stimuli, neutrophil attaches to the endothelial cells using adhesion moleucles (selectin, integrim, ICAM, PSGL-1) and migrate through the microvascular endothelial barrier to the site of injury. This allows it to phagocytose the microorganisms and release inflammatory cytokines and chemokines to attract

other inflammtory cells. Once the damged is limited, the neutrophil undergoes apoptosis and is removed by macrophages. PSGL-1: Neutrophil P-selectin glycoprotein ligand1; ICAM: intercellular adhesion molecule. Adapted and modified from Borregaard (2010).

III) T Lymphocytes

T cells (TCs) are group of lymphocytes that differ from other lymphocytes (Natural killer and B cells) by expressing T cell receptors (TCRs). They originate from stem cells in the bone marrow and mature in the thymus. TCs are important part of adaptive immune response and are involved in cell mediated immunity. They are generally divided into CD4+ cells (T-helper) and CD8+ cells (T cytotoxic) (Brusselle, Joos et al. 2011). Both CD4+ and CD8+ cells are present in the peripheral airways and lung parenchyma of COPD patients. However, the prevalence is skewed towards CD8+ cells in COPD (Saetta, Di Stefano et al. 1998; Paats, Bergen et al. 2012). COPD patients have increased number of CD8+ T cells in the central and peripheral airways when compared with smokers and non-smokers with healthy lung functions. This accumulation is partially explained by reduced apoptosis of CD8+ T cells and existence of chemoattractants secreted by resident cells (Siena, Gjomarkaj et al. 2011; Borger, Oliver et al. 2012). Using flow cytometry analysis, it has been shown that a significant proportion of both natural killer and CD8+ T cells are higher in COPD sputum than smokers and release higher levels of proteolytic enzymes such as perforin and granzyme-B than smokers (Urbanowicz, Lamb et al. 2010). Similarly, levels of perforin in the epithelial lining fluid, obtained from the peripheral airways of COPD patients, are significantly higher and correlate with lung dysfunction when compared with healthy smokers and non-smokers (Shiratsuchi, Asai et al. 2011). The ability of T lymphocytes to survive longer in the lung and release proteolytic enzymes may be contributing to tissue cytotoxicity and emphysema (Fig. 1.11) (Barnes 2004).

IV) B cells

B cells are group of lymphocytes that are primarily involved in the induction of humoral immune response (part of adaptive immune system). They are distinguished from other lymphocytes by expressing B cell receptors (BCRs) on their cell surface membrane. BCRs enable B cells to bind to

specific antigens and produce specific antibodies against that antigen (Brusselle, Joos et al. 2011; Almqvist and Martensson 2012). Bronchial biopsies of large airways from COPD patients have shown increased number of B cells (Gosman, Willemse et al. 2006; Polverino, Baraldo et al. 2010). Furthermore, they appear in small airways and lung parenchyma of COPD patients as a part of organised lymphoid follicles, consisting of B-cells, T lymphocytes and dendritic cells (Fig. 1.13) (Plumb, Smyth et al. 2009; Olloquequi, Montes et al. 2011).

Accumulating evidence suggests that COPD, particularly in late stage disease (Gold stage III and IV), shares many clinical and pathological attributes of autoimmune disease. All smokers have some degree of inflammation; however, it is amplified in COPD and persists long after smoking cessation, suggesting the presence of a similar self-perpetuating mechanism to that which occurs in other autoimmune diseases (Hogg 2006; Putova, Dostal et al. 2007; Tzouvelekis, Kostikas et al. 2012). It has been postulated that perhaps CS down regulates immune suppressor cells and exposes intracellular hidden epitopes by damaging lung tissues (Agusti, MacNee et al. 2003; Taylor 2010). Our group has shown that oxidative stress induces unique post-translational modifications that make proteins highly immunogenic, thereby breaking the immune tolerance and inducing the presence of autoantibodies (Kirkham, Caramori et al. 2011). T-helper 17 cells, a subset of CD4+T cells, are elevated in COPD, which are important in autoimmune response and activation of B cells (Di Stefano, Caramori et al. 2009; Plumb, Smyth et al. 2009). Several studies have reported the prevalence of autoantibodies in COPD patients (Lee, Goswami et al. 2007; Feghali-Bostwick, Gadgil et al. 2008; Bonarius, Brandsma et al. 2011; ; Kirkham, Caramori et al. 2011; Rinaldi, Lehouck et al. 2012).

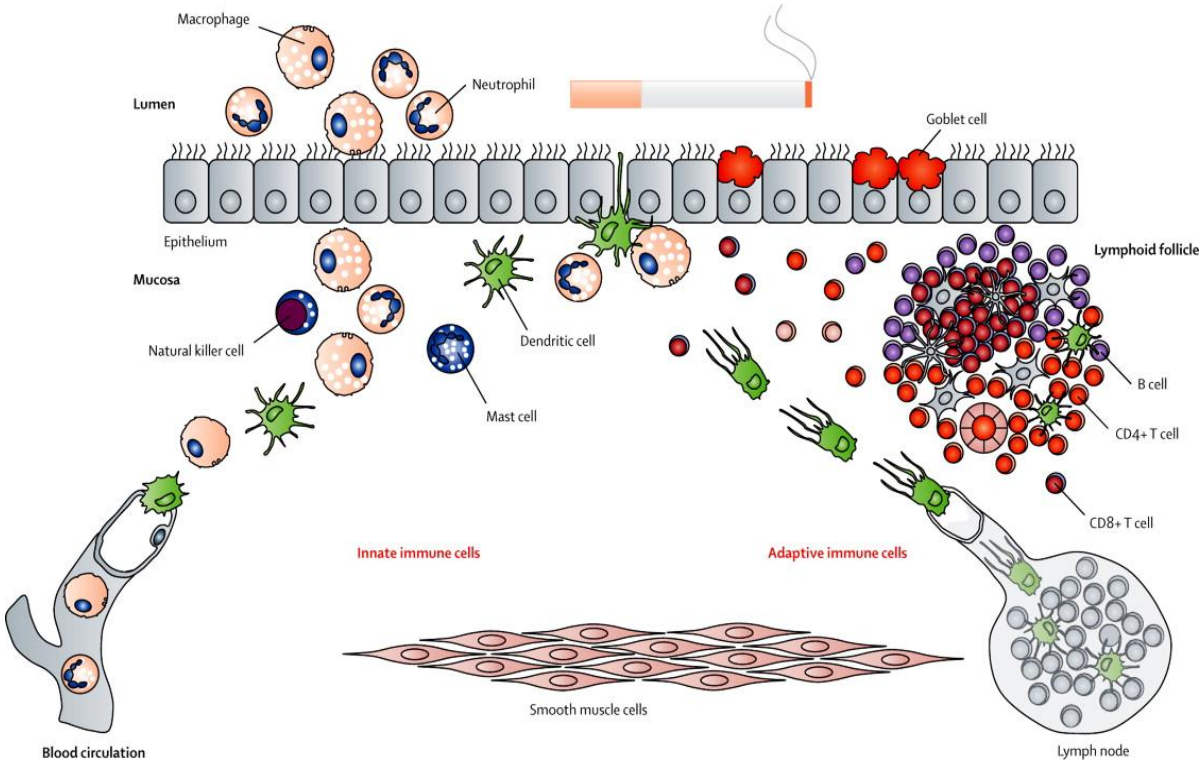


Figure 1.13: The innate and adaptive immune responses in COPD. Inflammation remains for years after smoking cessation in COPD. It is well documented that the innate immune system (Epithelial cells, macrophages, neutrophils, smooth musclses cells etc.) are pivatal in orchestrating inflammation in COPD. However, the involvement of the adaptive immune sytem (T lymphoctes, B cells, dentritic cells, autoantidodies etc.) has also been implicated in the pathophysiology of the disease, adding another dimension to the complexity of the disease (Brusselle, Joos et al. 2011).

1.2.2 Inflammatory mediators

Activated inflammatory cells secrete a cocktail of molecules that drive and maintain inflammation locally and systemically. These molecules include cytokines, chemokines, proteases and lipid mediators, which results in the disease pathophysiology, including fibrosis, emphysema, excess mucus production and chronic inflammation (Fig. 1.14) (Barnes 2009). Some of these mediators are discussed in the following paragraphs.

A) Cytokines

Cytokines are regulatory proteins that are produce by structural and immune cells in response to infection, injury and inflammation (Dinarello 2000). Multiple proinflammatory genes are expressed

in COPD, leading to increased production of inflammatory cytokines, which is linked to disease severity (Barnes 2004).

Increased levels of TNF-α and interleukin (IL)-6 are reported in the serum and sputum of COPD patients, which are secreted by inflammatory cells such as epithelial cells, macrophages, airway smooth muscle cells, and T lymphocytes (Tsoumakidou, Tzanakis et al. 2003; Sin and Man 2008; Grubek-Jaworska, Paplinska et al. 2012). TNF-α is associated with cachexia (weight loss) in COPD and negatively correlates with FEV_1 (Deveci, Deveci et al. 2010; Grubek-Jaworska, Paplinska et al. 2012). IL-6 is recognised as important pro-inflammatory cytokine, involved in the differentiation and activation of CD4+ T cells and regulatory T cells (T reg cells) (Neurath and Finotto 2011). Several inflammatory stimuli e.g. IL-1β and TNF-α can trigger the expression of IL-6 in various cell types including macrophages, epithelial cells and T lymphocytes (Thorley and Tetley 2007). There is an increased level of IL-6 in COPD serum and lung (Chaouat, Savale et al. 2009). SNP analysis of *IL-6* gene shows variation in COPD patients when compared to control subjects and decline in lung function (He, Foreman et al. 2009). There is marked elevation of IL-1β and reduced levels of endogenous IL-1 antagonists in the sputum of COPD patients (Sapey, Ahmad et al. 2009). IL-1β is an endogenous pyrogen, secreted by a variety of cells in particular macrophages. It promotes leucocytosis especially the release of neutrophils from bone marrow and the induction of other inflammatory cytokines and adhesion molecules (Chung 2001). IL-17 is another potent inflammatory cytokine, strongly associated with Th-17, CD4+ and CD8+ lymphocytes and recruits macrophages and neutrophils to the site of inflammation (Eustace, Smyth et al. 2011; Vanaudenaerde, Verleden et al. 2011). Sputum and peripheral lung tissues from COPD show up regulation of IL-17 expression (Zhang, Lao et al. 2010; Vargas-Rojas, Ramirez-Venegas et al. 2011; Cazzola and Matera 2012). There are other cytokines that are also considered to be important drivers of inflammation in COPD such as thymic stromal lymphopoietin (TSLP), IL-18 and IL-32 (Barnes 2009; Vargas-Rojas, Ramirez-Venegas et al. 2011).

B) Chemokines

Chemokines are proteins that act as chemoattractants to recruit and guide the migration of inflammatory cells to the site of inflammation such as airways in COPD (Barnes 2008). Chemokines are generally divided into four groups, C, CC, CXC and CX_3C chemokines. This classification is based on the disulfide bond between first two cysteine residues, for example, if the first two cysteine residues are adjacent to each other then it is grouped as CC chemokine (β-chemokine) or if there is intervening amino acid between these two cysteine residues then it is classed as CXC chemokine (α-chemokine) (Fernandez and Lolis 2002). CCL2, which is also known as monocyte chemoattractant protein-1 (MCP-1), attracts monocytes from bloodstream to affected tissue to become macrophages (Barnes 2003). CCL2 levels are elevated in the sputum of COPD and correlates with decreased in FEV_1 (Traves, Culpitt et al. 2002). Patients with chronic bronchitis exhibit increased concentrations of CCL3 (or macrophage inflammatory protein-1; MIP-1), which is one of main chemoattractants of macrophages (Capelli, Di Stefano et al. 1999). There are other inflammatory cells which also express the receptor (CCR5) for CCL3 chemokine including, T lymphocytes, epithelial cells and eosinophils (Barnes 2008; Barnes 2009). CCL5 (or RANTES) is a chemotactic for a number of inflammatory cells including T cells and blood monocytes. It is responsible for increased number of macrophages and CD8+ T cells in the lung of COPD patients (Conti and DiGioacchino 2001; Di Stefano, Caramori et al. 2009). CXCL8 (or IL-8), also known as neutrophil chemotactic factor, induces chemotaxis of neutrophils and phagocytosis (Broaddus, Hebert et al. 1992; Williams and Jose 2001). It is released by a number of cells including macrophages and airway epithelial cells (Williams and Jose 2001). CXCL8 is significantly increased in the sputum of COPD patients and correlates with increased numbers of neutrophils (Keatings, Collins et al. 1996; Malerba, Ricciardolo et al. 2006). The concentrations of CXCL8 and number of neutrophils are further increased in the sputum of COPD patients during acute exacerbated (Crooks, Bayley et al. 2000; Aaron, Angel et al. 2001; Fujimoto, Yasuo et al. 2005). High expression of CXCL8 plays a key role in COPD pathogenesis and has led to the development of CCR1/2 agonist strategies for COPD (Mukaida 2003; Donnelly and Barnes 2006;

Struthers and Pasternak 2010). Clinical trials have shown a mixture of both disappointing and positive results so far (Lazaar, Sweeney et al. 2011; Lebre, Vergunst et al. 2011).

C) Lipid mediators

There is increased evidence that lipid mediators, derived from polyunsaturated fatty acids, promote and regulate inflammation (Calder 2009). Increased prostaglandins have been measured in the exhaled breath condensate of COPD patients and this correlates with airflow limitation and disease severity (Montuschi, Kharitonov et al. 2003; Chen, Chen et al. 2008). Prostaglandins are bronchoconstrictors and induce coughing in COPD by activating airway sensory nerves (Maher, Birrell et al. 2009). Leukotrienes B_4 (LTB_4) is another lipid mediator released by macrophages and neutrophils. It is a potent chemoattractant of neutrophils in the airways. It is elevated in the exhaled breath condensate and sputum of COPD patients (Montuschi, Kharitonov et al. 2003; Montuschi 2005). Several other lipid mediators have been reported to be important in the induction of inflammation in COPD, including thromboxane, cysteinyl-leukotrienes and platelet-activating factors (PAFs) (Barnes 2009; Calder 2009). However, not all lipid mediators are associated with inflammation, for example, lipoxins, resolvins and protectins are believed to be protective and help resolving inflammation (Kohli and Levy 2009). It seems that there is a balance between pro and anti-inflammatory lipid mediators to maintain homeostasis.

D) Protease/anti-protease

Proteases (also referred as proteinase) are enzymes that are important in the killing of microbial pathogens; however, their deregulation can lead to undesirable effects such as inflammation and tissue destructions (emphysema). The existence of endogenous anti-proteinases can halt and dampen the unwanted properties of these enzymes (Abboud and Vimalanathan 2008). In COPD, there is an increased level of proteases over anti-proteases (Gadek, Fells et al. 1981). This could have a potentially positive impact on infection, however, unless a perfect balance is achieved, the effect can be damaging (Inoue, Takano et al. 2005). A number of proteases are implicating in the

pathophysiology of the COPD including neutrophil elastase (NE) and MMPs (Inoue, Takano et al. 2005). For example, there is a 3-12-fold increase in MMP-1, -8 and -9 levels in the sputum of COPD patients, which negatively correlates with FEV_1 (Calder 2009). Pro-inflammatory mediators such as IL-1β activate macrophages and neutrophils to secrete MMP-9 and NE, respectively (Chung 2001; Culpitt, Rogers et al. 2003). Neutrophils derived NE has pluripotent properties. It can regulate the expression of cathpsin B and MMP2 in macrophages and it can also activate pro-MMP-2, -7 (Greene and McElvaney 2009). It is also regarded as proinflammtory mediator (Carroll, Greene et al. 2005). There are increased number of neutrophils in the sputum and peripheral lung airways of COPD patients which is associated with increased NE and impaired lung airways (O'Donnell, Peebles et al. 2004; Singh, Edwards et al. 2010). α_1-antitrypsin is a protease inhibitor, synthesised in liver and secreted into blood, where it diffuses into lung interstitium (supporting tissues) and alveolar lining fluid. It inactivates NE and prevents lung damage from proteases (Brode, Ling et al. 2012). Its deficiency is linked to emphysema in COPD patients (Shapiro 2002; Kukkonen, Tiili et al. 2011).

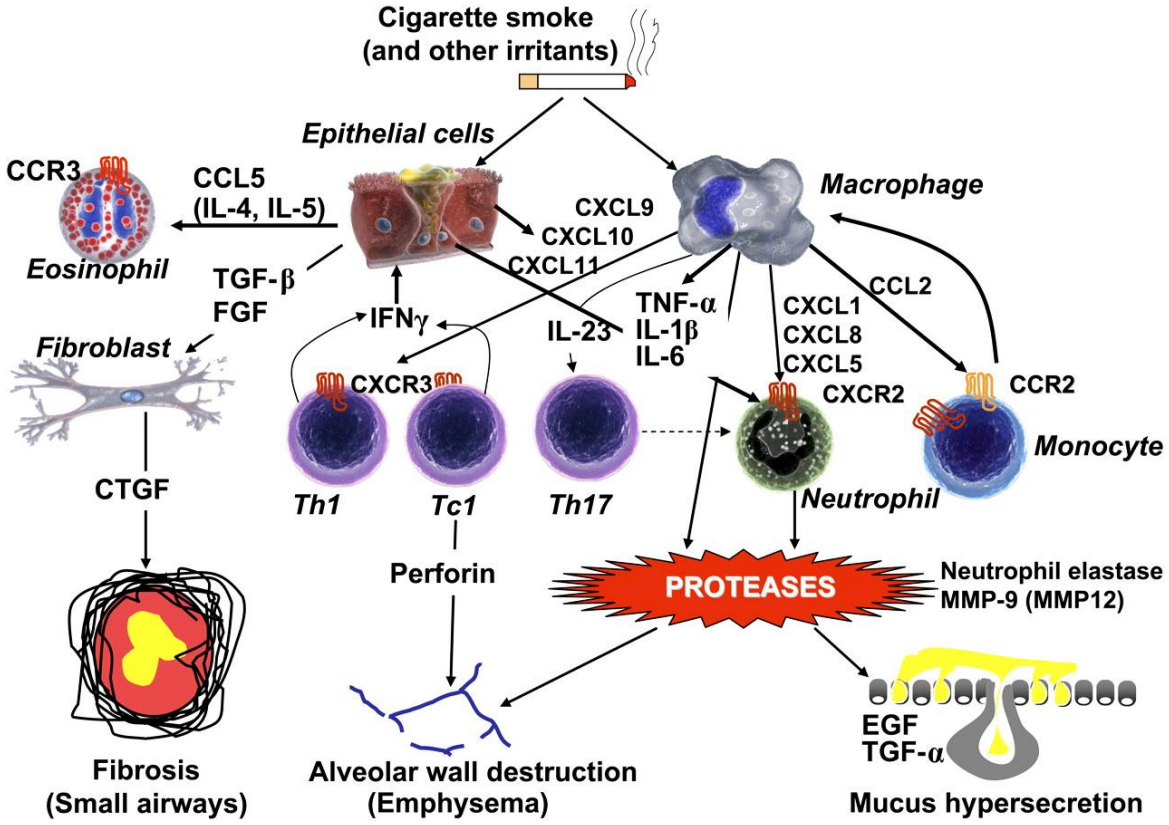


Figure 1.14:The role of inflamamtory cells and mediators in the pathophysiology of COPD. Inflammation is the core component of COPD. Cigarette smoke activates several structural and immune cells to produce a plethora of cytokines, chemokines, lipid mediators and proteases, resulting in the pathophysiological features of the disease such as fibrosis, emphysema, excess mucus production and airway limitation (Barnes 2009).

1.3 The NF-κB inflammatory pathway

The nuclear factor-kappa B (NF-κB) inflammatory pathway is one of the main signalling pathways activated in inflammatory cells and is the master transcription regulator of several inflammatory mediators including cytokines, chemokines, growth factors and proteases (Schreck and Baeuerle 1990; Salminen, Huuskonen et al. 2008). NF-κB is activated in COPD and its activity increases furthermore during exacerbation (Kersul, Iglesias et al. 2011). It is also involved in the innate and adaptive immune system (Watters, Kenny et al. 2007). The NF-κB transcription complex comprises of two families of proteins, the Rel family (RelA/p65, c-Rel and RelB) and NF-κB family (p100 and p105) (Li and Verma 2002). The NF-κB proteins are cleaved upon activation (p100 → p52 and p105

→ p50), following post-translational modifications (PTMs), through proteolysis in order to become active DNA-binding proteins (Gilmore 2006). The Rel family proteins contain C-terminal transcription activation domains (TADs) that enable gene transcription, in contrast, NF-κB proteins do not have a TAD and form a heterodimer with Rel proteins to act as transcriptional activators (Hayden, West et al. 2006). The NF-κB heterodimer binds to a 9-10 base pair of DNA site called the κB response element that has great variability in sequence. This variability contributes to the regulation of diverse sets of genes in a cell and context-dependent manner (Gilmore 2006; Morgan and Liu 2011). The NF-κB transcription factor is found as an inactive complex in the cytoplasm of most cells and can be activated by two pathways, the classical (canonical) or the alternative (non-canonical) (Cai, Jiang et al. 2011). These two pathways, alone or together, can be activated by specific set of stimuli to target the expression of a distinct spectrum of genes (Adcock 1997; Brown, Claudio et al. 2008). In the following, I discuss each pathway briefly.

1.3.1 The classical pathway

In non-stimulated cells, the NF-κB heterodimer (p65/RelA and p50 subunits) reside in the cytoplasm, bound to the inhibitory IκBα protein. The binding of an inflammatory stimulus such as TNF-α, IL-1β or LPS to its respective receptor leads to a phosphorylation cascade resulting in activation of the IκB kinase (IKK) complex. This consists of three subunits, IKKα, IKKβ and IKKγ/Nemo (regulatory molecule) (Liu, Yang et al. 2005; Hayden, West et al. 2006). The IKK complex phosphorylates IκBα (on Ser32 and Ser36) which is targeted for ubiquination and degradation by the E3 ubiquitin-ligase (E3RS IκB) and 26S proteasome, respectively (Edwards, Bartlett et al. 2009). The degradation of IκBα allows the translocation of the NF-κB p65/p50 dimer into the nucleus where it binds to promoter regions of inflammatory genes and also to co-activators such as p300, cyclic AMP response element binding protein (CBP), p300/CBP-associated protine (PCAF), and TATA-binding protein(TBP)-associated factor 2 (TAF-2). Co-activator molecules have intrinsic histone acetyltransferase (HAT) activity and subsequently acetylate core histones (Barnes, Adcock et al. 2005; Barnes and Adcock

2009). Classically, this results in disruption of the electro-static attraction between histones and DNA. Acetylated histones signal the recruitment of DNA remodelling factors (SW1/SNF), bromodomain-containing proteins and transcriptional co-activators, leading to local unwinding of DNA (Adcock, Ito et al. 2004; Yang, Yik et al. 2005). This confers access to RNA polymerase II to the transcriptional start site, leading to the expression of several inflammatory mediators (Fig. 1.15) (Rahman and Adcock 2006). However, recent findings suggest that the binding of all transcription factors is associated with open chromatin and that the ligand-induced active chromatin remodelling process is linked to about 10% regulated genes. For example, AP-1 transcription factor maintain accessibility of glucocorticoid receptor (GR) to GR binding site (Biddie, John et al. 2011). The classical pathway is generally activated when a ligand binds to TCR, BCR, Toll-like receptor (TLR), and IL-1 receptor family (Hayden and Ghosh 2004).

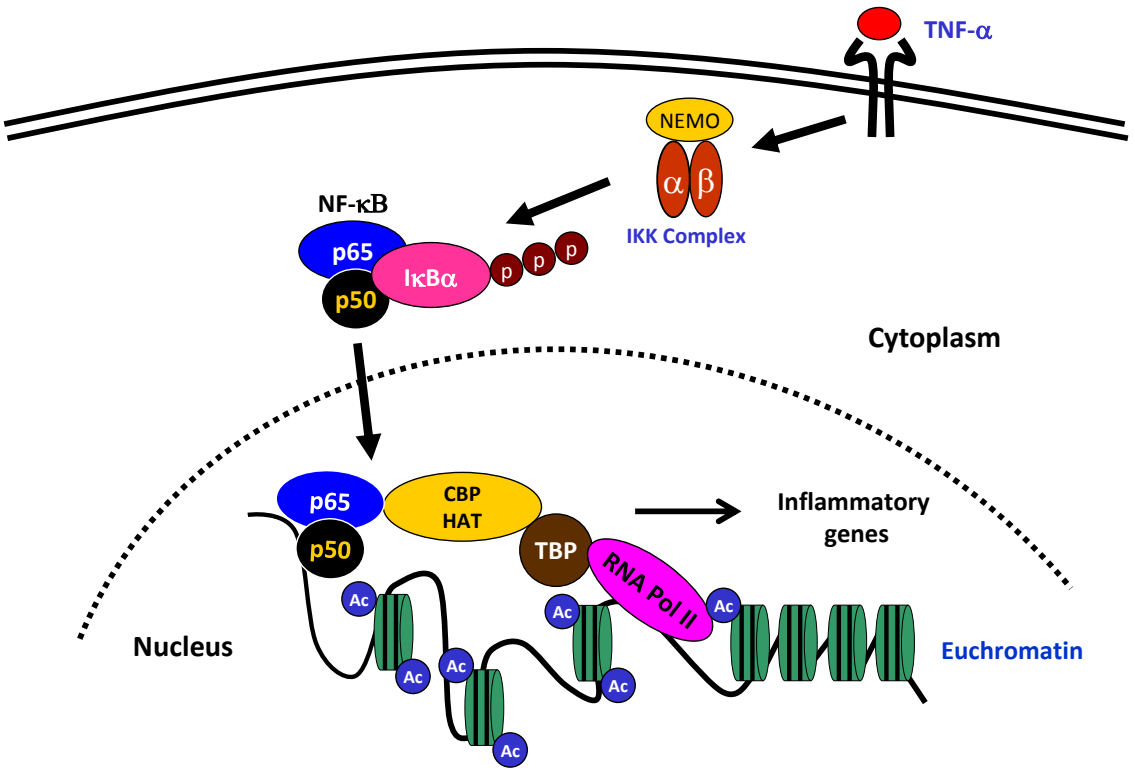


Figure 1.15: The activation of NF-κB classical pathway. The binding of inflammatory (TNF-α/IL-1β) stimuli to their respective receptors, induces the IKK complex to phosphorylate the NF-κB IκBα subunit. The IκBα protein is subsequently degraded by the proteasome, allowing the translocation of p65/p50 heterodimer into the nucleus and thereby activation of inflammatory genes (Barnes and Adcock 2009).

1.3.2 The alternative pathway

The alternative pathway is activated in the development of lymphoid organs and the adaptive immune system (Gerondakis and Siebenlist 2010). Following stimuli, the NF-κB-inducing kinase (NIK) phosphorylates the IKK complex which consists of two IKKα proteins and does not require the regulatory IKKγ/NEMO protein (Dejardin 2006). The IKKα homodimer phosphorylates the C-terminal domain of p100 protein, which is found in complex with RelB protein in the cytoplasm. The phosphorylated p100 protein undergoes proteolysis and becomes active p52 protein in this process (Fig. 1.16). The newly formed p52 and RelB heterodimer translocate into the nucleus, activating target genes (Dejardin 2006; Gilmore 2006). The alternative pathway can be activated in cells expressing lymphotoxin B recepotors (LTβR), B-cell activating factor receptors (BCAFRs) and CD40 receptors. These stimuli (LTβ, BCAF and CD40) can also activate the classical pathway (Hayden and Ghosh 2004).

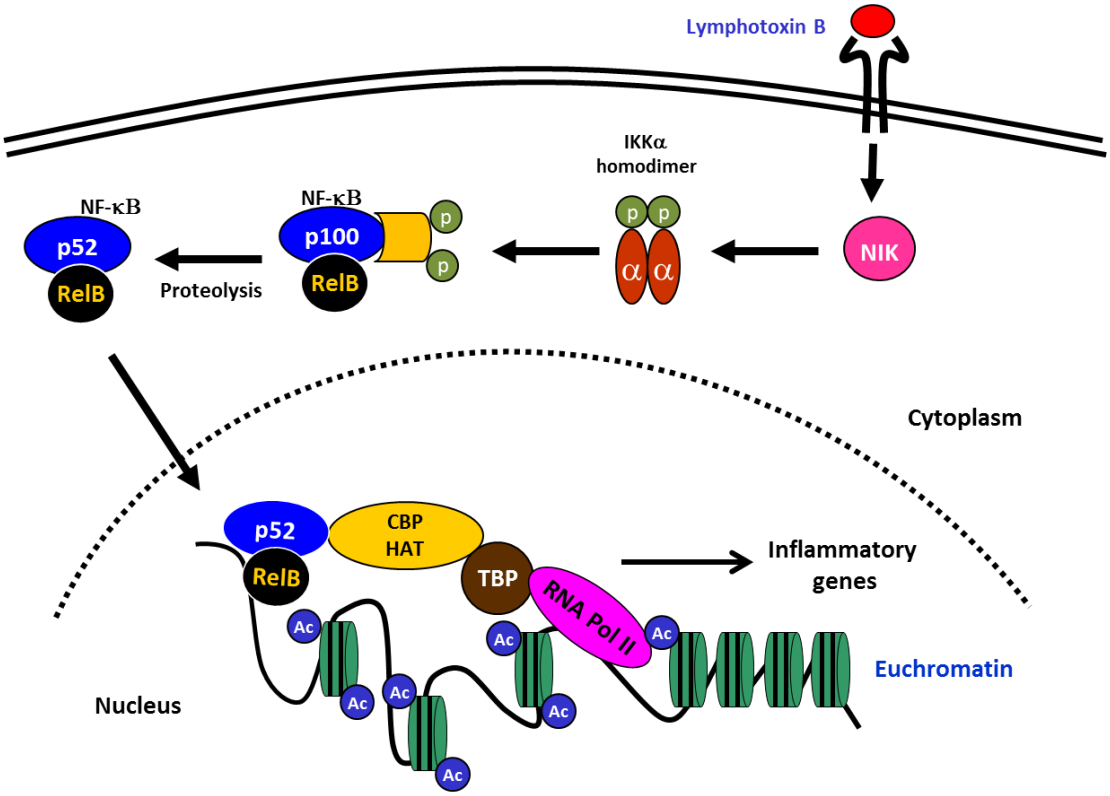


Figure 1.16: The activation of the alternative NF-κB pathway. Following stimuli, NIK induces phosphorylation of IKKα homodimers which in turn phosphorylates the p100/RelB complex. The precursor p100 undergoes proteolysis following phosphorylation to become active p52 protein. This enables the p52/RelB complex to translocate into the nucleus, inducing specific sets of target genes (Hayden and Ghosh 2004).

1.3.3 The regulation of the NF-κB pathway

The NF-κB transcription factor modulates the transcription of several genes that are entailed in immune response, development, cancer and inflammation. Therefore, its biological activity has to be tightly regulated in order to maintain a cellular homeostasis (Liu, Yang et al. 2005). This balance is achieved though several negative regulatory mechanisms. During transcription, NF-κB also transcribes its negative regulatory proteins IκBα and A20 (Vereecke, Beyaert et al. 2009). The newly synthesised IκBα protein moves into the nucleus where it binds to the NF-κB heterodimer, inhibiting its function and shuttling it back into the cytosol (Ruland 2011). A20 is an ubiquitin modifying enzyme that is directly induced by NF-κB. A20 deubiquitinates the IKK complex resulting in its

destabilisation and thereby, preventing phosphorylation and degradation of IκBα protein and NF-κB activation (Boone, Turer et al. 2004; Enesa, Ito et al. 2008). Studies have shown that PTMs such as acetylation and phosphorylation of p65/RelA is essential for its optimal transcription activity (Huang, Yang et al. 2009; Huang, Yang et al. 2010; Zhang, Liu et al. 2012; Zhang, Liu et al. 2012). Several lysine residues on p65 have been identified to be acetylated by CBP/p300 acetyltransferase (Oeckinghaus and Ghosh 2009). CBP/p300-interacting transactivator-2 (CITED2) protein has been shown to inhibit p65 acetylation and interaction with CBP/p300 acetyltransferase, leading to reduced DNA binding affinity (Fig. 1.17) (Lou, Sun et al. 2011).

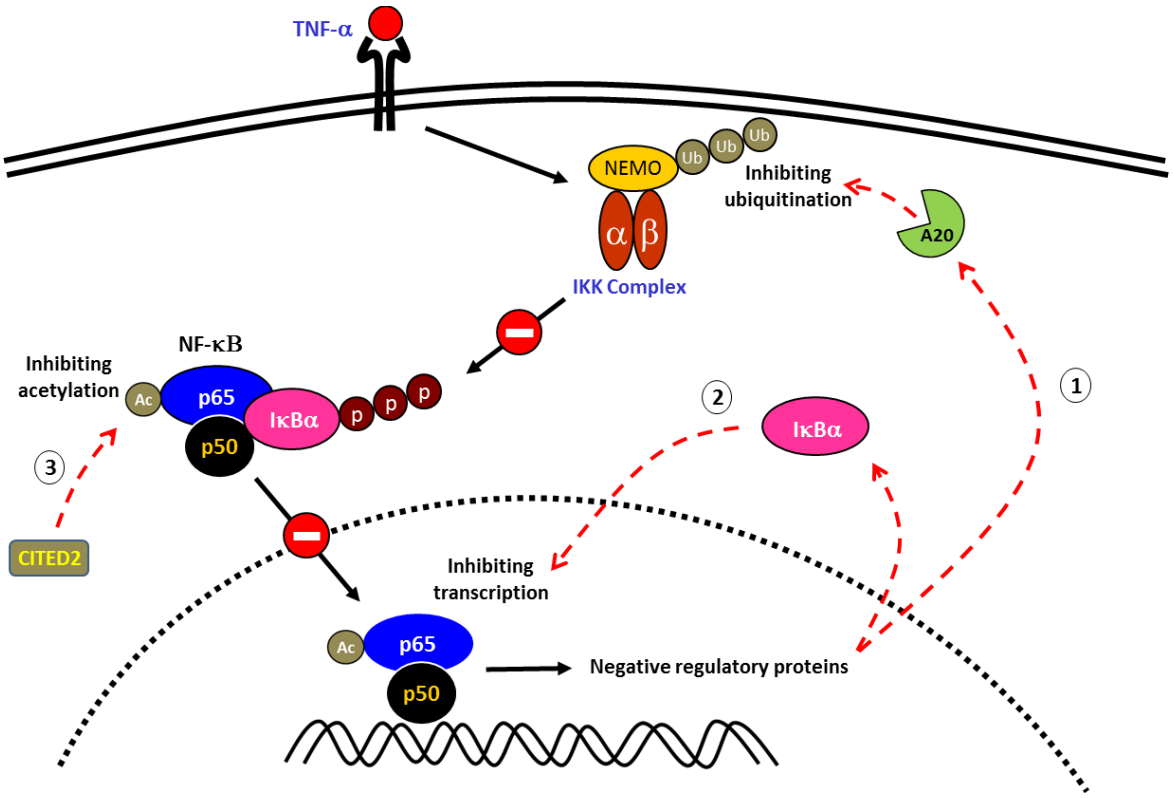


Figure 1.17: The negative feedback mechanisms in the NF-κB pathway. Following activation, NF-κB also transcribes negative regulatory proteins such as IκBα and A20. **(1)** The A20 enzyme ubiquitinates IKK complex, causing disintegration of the complex and prevents it from phosphorylating IκBα protein. **(2)** The newly synthesised IκBα translocates into nucleus, where it binds to the NF-κB transcription factor. This inhibits its transcription function and shuttles p65/p50 complex back into cytoplasm. **(3)** Acetylation of p65 is inhibited by CITED2 protein, affecting its DNA binding activity (Ruland 2011).

1.3.4 The suppression of NF-κB by glucocorticoids

Glucocorticoids (also called glucocorticosteroids: GC) are the most effective anti-inflammatory drugs used in the treatment of various inflammatory disorders. They exert many of their anti-inflammatory effects through inhibition of NF-κB (trans-suppression) and also by induction of anti-inflammatory genes (trans-activation) (Barnes and Adcock 2009). GCs diffuse across the cell membrane and bind to the glucocorticoid receptor (GR) localised within the cytosol (Barnes 2006). GR is usually found in an inactive form associated with molecular chaperone proteins such as heat shock protein 90/HSP90. Once GC is bound to GR, the GC-GR complex dissociates from its chaperon proteins and translocates into the nucleus (Fig. 1.18) (Barnes and Adcock 2009). The activated nuclear GR can bind as a homodimer to the glucocorticoid response elements (GREs) which often reside in the promoter regions of GC-responsive anti-inflammatory genes but can also occur very distal to the transcription start site (TSS), inducing their transcription (So, Cooper et al. 2008; John, Sabo et al. 2011). Anti-inflammatory genes that are induced by GR includes mitogen activated kinase phosphatase- (MPK-1) and IL-10 (Durham, Adcock et al. 2011). Corticosteroids also suppress inflammatory genes by binding to pro-inflammatory transcription factors and reversing their effect on the local chromatin structure mediated by NF-κB and recruited HAT-containing coactivator proteins such as CBP (Ito, Yamamura et al. 2006). For a subset of genes this involves recruitment of histone deacetylase 2 (HDAC2), resulting in the deacetylation of histone at specific promoter regions, thereby, restricting the access of transcription machinery to the TSS (Barnes and Adcock 2003; Barnes 2006; Barnes and Adcock 2009).

Classically, the binding of GCs to GR lead to ligand-induced remodelling of DNA-histone interaction in conjunction with chromatin remodelling proteins. This also allows the recruitment of transcription apparatus which was previously occluded (Barnes 2006; Barnes 2011). However, recently an alternative model for GR binding to GREs has been proposed. It involves targeting of GC-GR complex to pre-opened chromatin structure (John, Sabo et al. 2011). For example, transcription factors such as activator protein (AP)-1 can make the GRE accessible to GR by maintaining open chromatin

structure (Biddie, John et al. 2011; Voss, Schiltz et al. 2011). NF-κB may be facilitating the accessibility of GC-GR and enabling the immediate inhibition of its response genes.

Although, GCs are widely used in the treatment of numerous inflammatory disorders, there are a number of inflammatory conditions including severe asthma and COPD that respond poorly to GCs, even with high doses (Barnes 2012). This lack of response is partially explained by impaired translocation of GR into the nucleus and reduced HDAC2 activity (Barnes 2011). Oxidative stress is associated with impairment of most aspects of GR function (Adcock and Ito 2005).

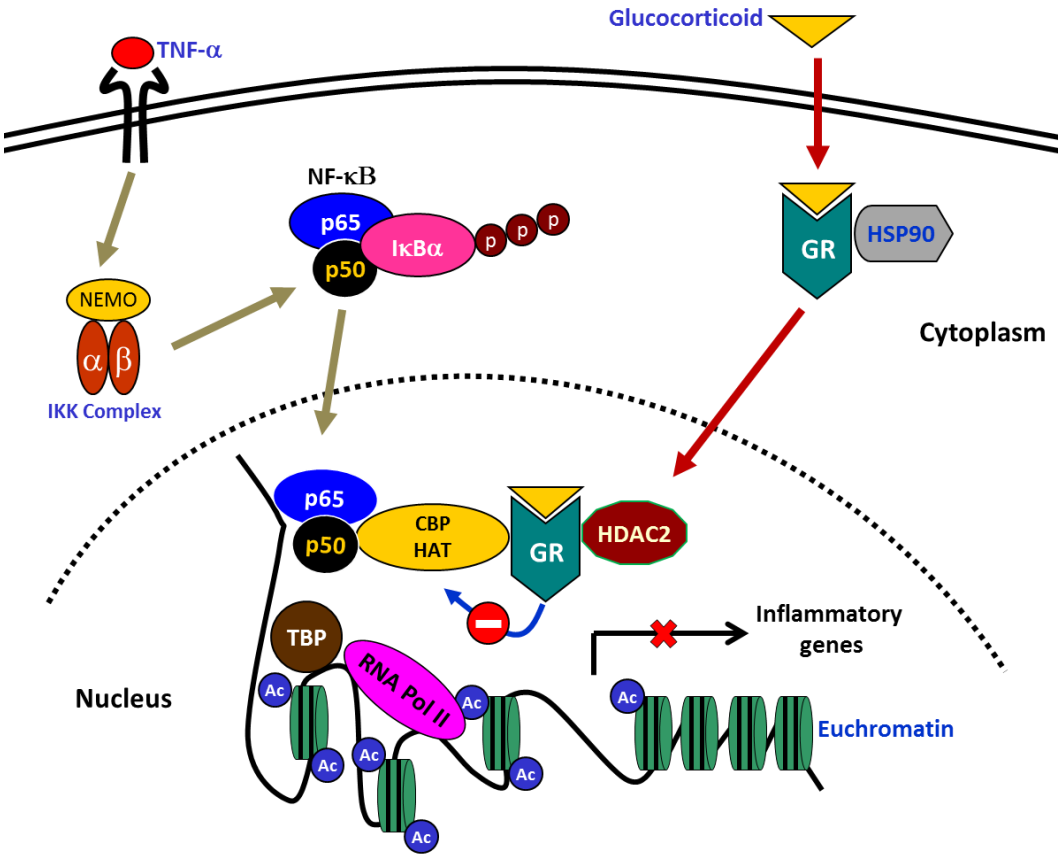


Figure 1.18: The suppression of inflammatory genes by glucocorticoids. The binding of inflammatory (TNF-α/IL-1β) cytokines to their respective receptors, induces activation of a phosphorylation cascade leading to activation of the IKK complex which subsequently phosphorylates the NF-κB IκBα subunit. This triggers its degradation by the proteasome, allowing the translocation of the p65/p50 heterodimer into the nucleus and activating inflammatory genes. This can be reversed by glucocorticoids acting via the GR. Activated GR seeks out and binds to the activated NF-κB complex and inhibits the associated-CBP/HAT activity via recruitment of repressor molecules including HDAC2 to reversed localised histone acetylation (Barnes and Adcock 2009).

1.5 Oxidative and carbonyl stress

The majority of COPD patients are either current or ex-smokers. Cigarrete smoke (CS) is a complex combination of noxious particles, chemicals and reactive oxygen species (ROS) (Rahman and Adcock 2006; Min, Bodas et al. 2011; Biswas, Hwang et al. 2012). ROS are important in cell signalling and are regulated by antioxidant defence mechanisms. However, excessive ROS production or defective antioxidant systems can lead to an imbalance between oxidants and antioxidants, favouring oxidants, leading to oxidative stress (Finkel and Holbrook 2000). Oxidative stress is elevated in COPD and known to be involved in the pathophysiology of the disease. In addition, redox-sensitive transcription factors such as NF-κB and AP-1 are regulated by ROS (Kirkham and Rahman 2006). Beside CS, there are other environmental sources of ROS, including, pollution, UV and xenobiotics. ROS are also produced endogenously by various inflammatory cells such as macrophages and neutrophils though the "respiratory burst" which involves mitochondrial respiration (Pettersen and Adler 2002; Merry 2004). ROS include free radicals such as superoxide anion radical ($O_2\cdot^-$), singlet oxygen ($^1O_2$), hydroxyl radical ($\cdot OH$) and perhydroxyl radical ($HO_2\cdot$) and non-radicals such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Pryor and Stone 1993; Negre-Salvayre, Coatrieux et al. 2008). The unpaired free electron radicals are highly unstable and can cause oxidation of proteins, DNA and lipids (Fig. 1.19). It can also generate a highly reactive metabolic species such as reactive aldehydes through a process of lipid peroxidation (Ahsan, Ali et al. 2003; Rahman and Adcock 2006).

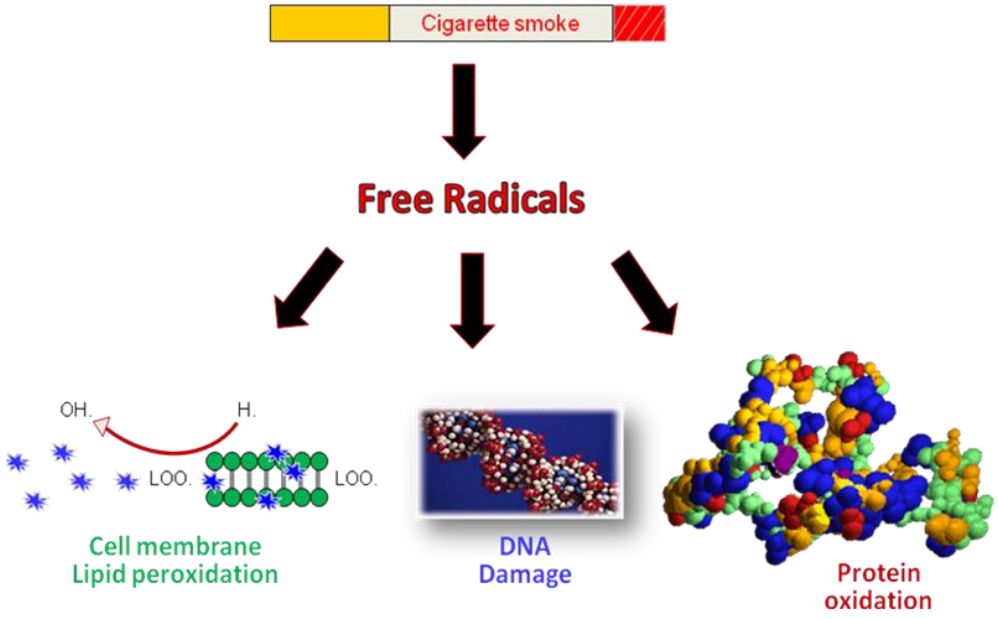


Figure 1.19: ROS induced molecular damage. ROS in cigarette smoke can inflict oxidative damage to cell lipid membranes, DNA and proteins.

The accumulation of these reactive aldehydes is known as carbonyl stress. Elevated hydroxynonenal (4-HNE) modified-proteins have been observed in airways cells and neurodegenerative diseases (Agusti, MacNee et al. 2003). 4-HNE has been reported in the lung of smokers and COPD patients. It can stimulate the release of CXCL8 and transforming growth factor (TGF)-β in the lung (MacNee 2005). Oxidative and carbonyl stress can potentially target any cellular protein including cytoplasmic (e.g. NF-κB) and nuclear (e.g. histone) proteins. Proteins such as histones, which are rich in lysine, cysteine, histidine, arginine and proline amino acids content, are susceptible to oxidation (Daly, Mirsky et al. 1951). Recently, it has been shown that histone carbonylation occurs during cell proliferation and may represent a specific post-translational modification (PTM) during cell cycle (Garcia-Gimenez, Ledesma et al. 2012). HDAC2 carbonylation and its reduced activity have been documented after CS exposure in cells, indicating that carbonylation can affect protein function (Marwick, Ito et al. 2007; Barnes 2008; Meja, Rajendrasozhan et al. 2008). Reactive aldehydes can also react with 2, 4-dinitorphenylhdrazine (DNPH) to form hydrazones (DNP), enabling their detection through the DNP tag with suitable antibodies. DNA damage is also reported under

oxidative stress following several months of cell exposure to cigarette smoke extract *in vitro* (Liu, Killian et al.). Deoxyguanosine residues in DNA produce 8-hydroxy-deoxyguanosine (8-OHdG), a DNA oxidation marker, when subjected to hydroxyl free radicals. Elevated levels of 8-OHdG in lung tissue is associated with exposure to diesel exhaust particles (Tokiwa, Sera et al. 1999). Therefore, genomic and proteomic changes under oxidative stress reinforces the evidence that epigenetic modifications may be involved in the underlying mechanisms of chronic inflammatory diseases including COPD.

Oxidative stress is thought to be contributing to the development of many other diseases including heart, immune, neurodegenerative and cancer (Dalle-Donne, Aldini et al. 2006; Kurien and Scofield 2008; Kirkham, Caramori et al. 2011). As a result, a number of animals and cell/tissue culture models have been developed to study the impact of oxidative stress (Yatin, Varadarajan et al. 1999; Aksenova, Aksenov et al. 2005; Wijeratne, Cuppett et al. 2005). However, most of these models are based on acute ROS exposures which fail to reproduce the disease pathology including that of COPD where chronic oxidative stress is central (Yatin, Varadarajan et al. 1999; Aksenova, Aksenov et al. 2005; Wijeratne, Cuppett et al. 2005).

1.6 Epigenetics and gene regulation

DNA is tightly packed together with histones into structural units called nucleosomes. Each nucleosome is an octomer of four core histone proteins; H2A, H2B, H3 and H4 proteins with ~146-base pair of DNA wrapped around and linked to H1 protein (Adcock, Cosio et al. 2005). Nucleosomes are folded into compact chromatin structure. In transcriptionally active chromosomal regions, the chromatin unwinds to form euchromatin allowing accessibility of transcription machinery. In contrast, the condense chromatin state (heterochromtin) promotes gene suppression (Fig. 1.20). Such transitions are achieved through reversible or possibly permanent post-translational or epigenetic changes (Adcock, Ford et al. 2006).

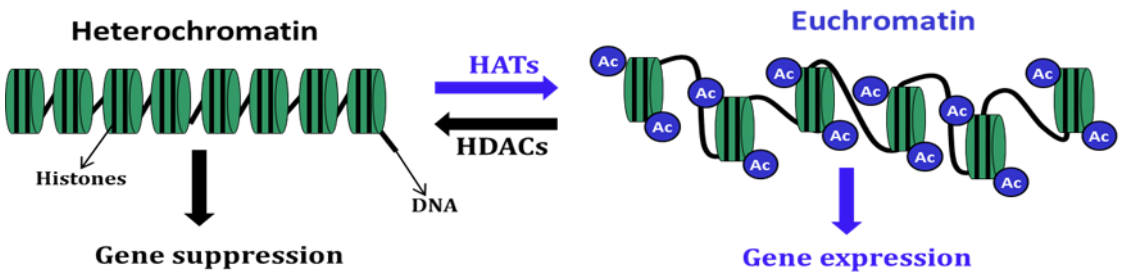


Figure 1.20: Chromatin structure. In heterochromatin histones and DNA are packed together and gene expression is suppressed. Post translational modifications (PTMs) such as acetylation of histones result in activation (euchromatin) of genes. The acetylation and deacetylation of histones are carried out by HAT and HDAC enzymes, respectively. Other PTMs such as methylation and phosphorylation along with DNA methylation also play a similar role (Barnes and Adcock 2009).

Epigenetics is referred as alterations in the DNA and histone status without the actual change in DNA nucleotide sequence (Waddinton 1942). Epigenetics is important for cell diversity and differentiation during development but also critical for multiple cellular functions including genome integrity, proliferation and gene expression. Epigenetic modifications are heritable and although originally believed to be stable it is now clear that they are also reversible under various environmental conditions. Epigenetic mechanisms involved PTMs of histones, DNA methylation either directly or indirectly through the actions of enzymes and non-coding microRNAs (Li 2008). Environmental factors (CS, allergies and infection) may lead to permanent epigenetic changes and thus contributing to disease phenotype (Kabesch and Adcock 2012; Lovinsky-Desir and Miller 2012). Parental exposure to CS may increase the risk of diseases in child`s later life as a result of epigenetic changes in utero (Breton, Byun et al. 2009). This may be true for some smokers who are predisposed to COPD (Kalsheker and Chappell 2008). PTMs of histones such as ubiquitination, sumoylation, acetylation, methylation and phosphorylation generally occur on histone tails which protrude from the nucleosome although some modifications occur within the nucleosomal nexus. In contrast, DNA methylation is associated with CpG islands founded in the proximity of promoter regions of genes (Adcock, Tsaprouni et al. 2007). Generally, these islands are unmethylated and methylation is linked to loss of gene function (Jones and Baylin 2002) although this is not seen in all cases (Guilleret and

Benhattar 2003). In addition, the advent of deep sequencing of bisulphite modified DNA emphasises that DNA methylation can occur in regions away from CpG islands known as CpG shores (Ji, Ehrlich et al.).

The cell-type specific induction of inflammatory genes is regulated, at least in part, by epigenetics. This suggests that this differential activation may be attributed to cell surface receptors, intracellular signalling and local chromatin changes within the nucleus that allow the biding of transcription factors to the promoters of specific genes (Kabesch and Adcock 2012). NF-κB and glucocorticoids work, at least in part, through epigenetic changes in acetylation and deacetylation of histones as elucidated previously (section 1.3.4). DNA methylation and chromatin remodelling are interlinked mechanisms in which DNA methyltransferases (DNMTs) bring together methylation machinery to the promoter region by recruiting HDACs and other chromatin proteins (Franco, Schoneveld et al. 2008). Therefore, a deficit in either DNA methylatransferases or HDACs could result in elevated inflammatory gene expression. Pro-inflammatory mediators can also play important role in the epigenetic regulation of genes. It has been shown that the pro-inflammatory mediator such as IL-6 induce the expression of DNMT1 which is upregulated in inflammatory bowel disease (Foran, Garrity-Park et al. ; Hodge, Xiao et al. 2001). Although, epigenetics is extensively studied in a variety of cancers, little is known about its role in respiratory diseases such as COPD.

1.7 The bromodomain proteins and gene transcription

Bromodomains are a conserved 120 residue peptide motif which are found in chromatin-associated proteins and HATs, included PCAF, p300, CBP, TAF2 and in remodelling proteins such as SW1/SNF (Sanchez and Zhou 2009).The human genome encodes 46 bromodomain proteins that contain a total of 61 domains and are classified into 9 groups. The bromodomain and extra terminal domain (BET) proteins are sub-group of bromodomain proteins that contain two bromodomains at the N-terminal and the extra terminal domain within the C-terminal domain (CTD). The group consists of

four proteins, Brd2, Brd3, Brd4 and Brdt (Fig. 1.20) (Filippakopoulos and Knapp 2012). The acetylation of lysine (K) residues on histone tails is associated with open structure and central to gene regulation and transcription as discussed above (section 1.6) (Adcock, Ford et al. 2006). The bromodomain region of the protein binds to acetylated lysine (AcK) on histone whereas extra-terminal domain recruits chromatin regulatory proteins. Regulatory proteins, transcription factors and histone modifying enzymes (HATs and HDACs), are recruited to the targeted promoters and regulate gene expression (Belkina and Denis 2012). The BET proteins exhibit high binding affinity to acetylated H3 and H4 (Dey, Chitsaz et al. 2003). The acetylation of H3 and H4 is highly specific and encodes for a diverse sets of cellular instructions, for example, cell differentiation, proliferation, growth and gene regulation (Turner 2000; Agalioti, Chen et al. 2002; An, Palhan et al. 2002).

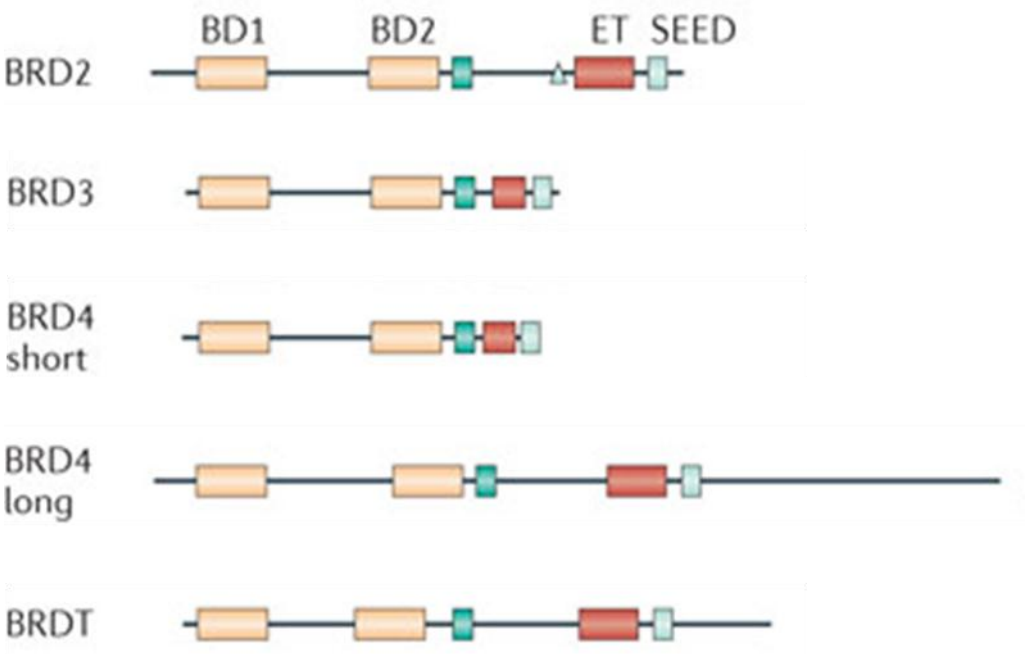


Figure 1.21: The BET family proteins. The BET protein contains two bromodomains (BDs) at N-terminal region, which recognise lysine-acetylated histone and extra terminal region at C-terminal which interacts with chromatin modifying proteins such transcription factors, regulatory proteins and enzymes. The Brd4 protein has two isomers the short and long. Adapted and modified from Belkina and Denis (2012).

The most notable member of the BET family is Brd4. It has two isomers, the short and long forms. The CTD of Brd4 is identical to that of RNA polymerase II (RNA pol II) and interacts with the positive transcription elongation factor b (p-TEFb). p-TEFb is recruited to the promoters of targeted genes and phosphorylates the CTD of RNA pol II, resulting in transcription elongation (Jang, Mochizuki et al. 2005; Yang, Yik et al. 2005). p-TEFb is associated with NF-κB and induces RNA pol II dependent elongation of *IL-8* gene (Barboric, Nissen et al. 2001; Amir-Zilberstein, Ainbinder et al. 2007). Recently, it has been shown that Brd4 forms a complex with acetylated RelA/p65 and enhances NF-κB-responsive inflammatory genes (Huang, Yang et al. 2009). Therefore, Brd4 plays a critical role in gene regulation and forming a complex with key transcriptional activator proteins (Fig. 1.22). Brd4 has been extensively studied in different types of cancers where it is limited to c-Myc driven cell proliferation (Filippakopoulos, Qi et al. 2010; Delmore, Issa et al. 2011; Mertz, Conery et al. 2011; Rodriguez, Huidobro et al. 2012). It is implicated in a rare but lethal form of squamous carcinoma (epithelial cell), known as NUT midline carcinoma. The NUT (Nuclear protein in testis) midline carcinoma (NMC) arises from fusion between *Brd4* and *NUT* genes. This chimeric *Brd4-NUT* gene encodes Brd4-NUT protein which promotes uncontrollable cell proliferation (French 2010; Yan, Diaz et al. 2011; Thompson-Wicking, Francis et al. 2012). HAT proteins were an obvious target for anti-proliferative and anti-proinflammatory drugs but selective agents proved difficult to produce the desire effects (Stimson, Rowlands et al. 2005). However, a number of groups have recently generated small-molecules, such as JQ-1 and I-BET, that inhibits Brd4 by acting as binding site inhibitors (Filippakopoulos, Qi et al. 2010; Nicodeme, Jeffrey et al. 2010). Nicodeme and colleagues reported that LPS-induced inflammation in macrophages is inhibited by the bromodomain mimic (I-BET) and that therapeutic intervention could lead to prevention of septic shock death by down regulating inflammatory gene expression (Nicodeme, Jeffrey et al. 2010). Fillippakopoulos *et al*. demonstrated that another bromodomain inhibitor JQ-1 inhibited tumour growth in an NMC murine model and could transformed malignant cells into non-cancerous cells (Filippakopoulos, Qi et al. 2010). JQ-1 and related drugs are now in the late stage development for acute myeloid leukaemia

(Delmore, Issa et al. 2011; Zuber, Shi et al. 2011). However, there is very little known about the role of Brd4 in the oxidative stress driven inflammation. In addition, to our knowledge, the effect of JQ-1 in oxidative stress induced inflammation is yet to be reported.

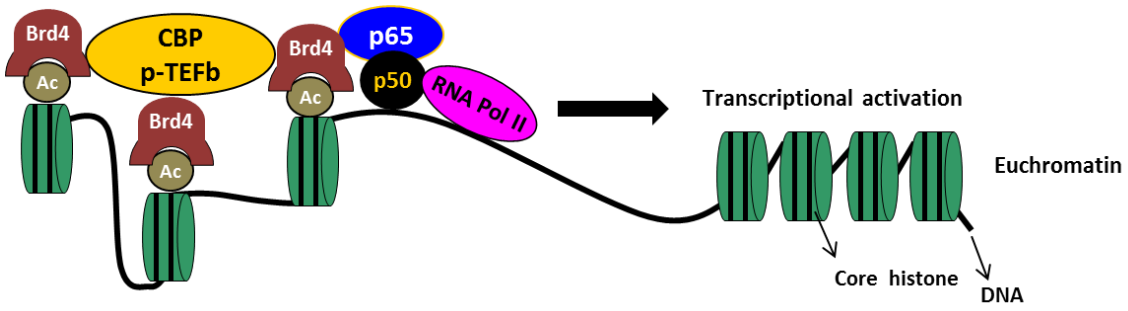


Figure 1.22: The role of Brd4 in gene transcription. Acetylated histones provide a docking site for Brd4 proteins that form a complex with transcription regulatory proteins in order to facilitate transcription. Brd4: Bromodomain protein 4; Ac: Acetylation; CBP; CREB binding protein, p-TEFb: positive transcription elongation factor b; RNA pol II; RNA polymerase II; p65/p50: NF-κB transcription factor.

1.8. Hypothesis

Oxidative stress, a key component of COPD inflammation, is linked to enhanced inflammatory gene expression. Oxidative stress can induce post-translational modifications (PTMs) of intracellular signalling molecules modulating their function and altering the cellular phonotype. It was hypothesised, therefore, that oxidative and carbonyl stress can modulate PTM of p65 and histones enabling enhanced inflammatory gene expression. These changes are generally transient but prolong exposure to ROS will result in fixed PTM which will modify inflammatory responses

1.9. Aims

1) Establish acute and chronic oxidative stress models *in vitro* using hydrogen peroxide
2) Investigate the effect of acute versus chronic exposure on PTMs including acetylation, and carbonylation of histones.
3) Assess the effect of ROS-induced histone acetylation in *IL-6* and *IL-8* promoter regions
4) Explore the role of Brd4 in oxidative stress-driven inflammation
5) Study the recruitment of Brd4 to *IL-6* and *IL-8* promoters
6) Investigate the effect of Brd4 inhibitors such as JQ-1 and PFI-1 on oxidative stress induced inflammation.

Chapter 2

Materials and Methods

2.1 Materials

Materials and kits were purchased from the following companies. All other chemicals and reagents were purchased from Sigma unless otherwise stated.

Companies:	Product:
AbD Serotec, Oxford, UK	Anti-Ace-Histone 4
Active motif, Carlsbad, CA, USA	TransAM p65 activity assay, HAT activity assay, Nuclear Extract kit
Amersham Biosciences, Bukingham, UK	Enhanced chemiluminescent (ECL) developing solution
Bio-Rad Laboratories, Hempstead,UK	Bradford assay kit
Cell Biolabs, San Diago, USA	OxiSelect Protein Carbonyl Kit
DakoCytomation, Cambridge, UK	Secondary HRP-conjugated anti-mouse, rabbit
Invitrogen Ltd, Paisley, UK	MES-running buffer, MOPS running buffer, dry iBlot transfer, Keratinocyte-serum free media, rEGF, BPE, Phenol:Chloroform:Isoamyl Alcohol, SDS, Tris-HCl pH 8
Millipore Ltd, Watford, UK	Anti-Acetylated-Histone 3, Oxyblot Protein oxidation kit, Protein A agarose/Salmon Sperm DNA
Nunc, Roskilde, Norway	ELISA 96-well plate
Promega, Southampton, UK	RNase-free water, Reverse Transcriptase, random hexamer, RNase inhibitor, EDTA
Qiagen, Crawley, UK	QuantiTech SYBR Green, RNeasy Kit, Primers
R&D Systems, Abingdon, UK	CXCL8, IL6 Duo Set ELISA, TNF-α, IL-1β
Roche, Basel, Switzerland	Mini complete protease inhibitors
Santa Cruze Biotech Ltd, CA, USA	Anti-Brd4, p65, SUV39H1/2, JMJD3, TBP, CBP Protein A/G beads
Sarstedt, Nurnbrecht, Germany	150cm^3 flask, 6-well plate, Cell culture 96-well plate

Sigma Ltd, Poole, UK	Dulbecco's Modified Eagle's Medium (DMEM), Glycerol, Sodium deoxycholate, NP40, Bovine serum albumin (BSA), LiCl, NaCl, Formaldehyde, hydrogen peroxide, Proteinase K, 2',7'-Dichlorofluorescin diacetate
Thermo Scientific, Chicago, USA	Brd2, Brd4 and Control siRNA

Table 2: Materials and kits purchased from commercial sources

2.2 Cell Culture

2.2.1 BEAS-2B Cells

SV-40 transformed human bronchial epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (ATCC, VA, USA). Cells were cultured until 80% confluent at 37°C and 5% CO_2 in keratinocyte-serum free media supplemented with recombinant epithelial growth factor (rEGF) 0.2ng/ml and 20g/ml bovine pituitary extract (BPE, all Gibco, Paisley, UK) in 150 cm^3 flasks (Sarstedt, Nurnbrecht, Germany). Cells were left in basal media (without rEGF and BPE) for 24hours prior to experiments in order to achieve cell cycle synchronisation (Jee, Gilmour et al. 2005).

2.2.2 Cell counting and seeding

BEAS-2B cells are adherent cells and were resuspended using 0.25% trypsin. Cells were counted by removing 10µl of cell suspension and placing it on haemocytometer with cover slip on the top. Trypan blue solution (Sigma-Aldrich) was used to exclude dead cells from viable cells. The haemocytometer was viewed at 10x magnification Olympus CH30 microscopes (Olympus UK Ltd., Watford, UK). All the cells in the haemocytometer grid were counted and average numbers of cells were determined. Cells were seeded at $9x10^6$ cells/flask (75 cm^3) in 9ml basal media. Alternatively, $3x10^4$ cells in 200µl of media/well for 96-well plate or $1x10^6$ cells in 1ml of media in 6-well plate (Nunc, Roskilde, Norway) were also used.

2.3 Cell viability

2.3.1 MTT assay

Cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MMT) assay. Cells were incubated with 1 mg/ml MMT solution at 37°C and 5% CO_2 for 30 minutes. MTT solution was then removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan product to produce a purple solution. The absorbance was measured at 550 nm. The colour intensity was correlated with cell viability.

2.3.2 Fluorescent activated cell sorting (FACS)

The Aqua LIVE/DEAD® Fixable dead cell stain kit was used to assess cell viability following treatments. The assay is based on the reaction of fluorescent reactive dye with cellular amines. In viable cells, the dye reacts with outer cell surface amines resulting in relatively dim fluorescence. In contrast, the dye can permeate the compromised cell membrane of necrotic cells and reacts with both inner and outer amines resulting in intense fluorescent staining (Fig. 2.1) (Invitrogen 2012). Cells were collected by trypsinisation and neutralized with medium, washed and resuspended in 1ml PBS. 1µl of Aqua LIVE/DEAD® Fixable dead cell stain was added per sample and left on ice for 30mins in dark. Cells were washed twice with cold PBS and resuspended in 1% BSA and PBS followed by flow cytometric analysis. Parameters were set as such to distinguish between positive cells (heat-treated dead cells) and negative cells (alive), which gave us distinct population of cells (Fig. 2.2). The effect of compounds such as JQ1 and PFI-1 on cell toxicity was subsequently examined using these parameters.

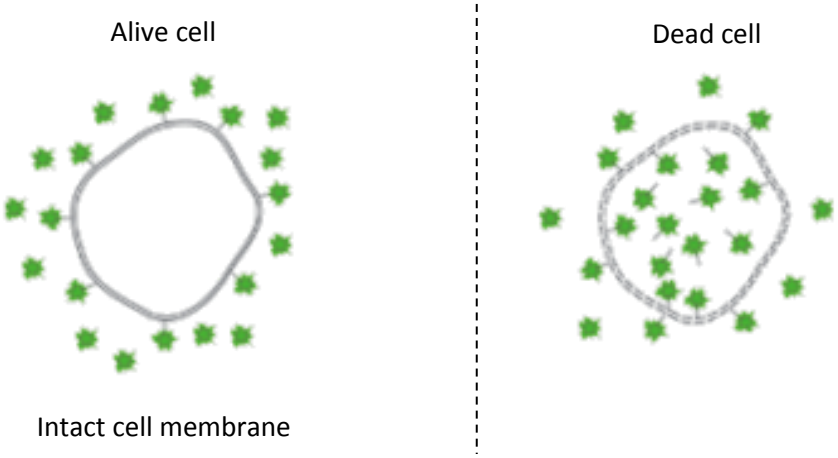


Figure 2.1: Principle of the LIVE/DEAD® Fixable staining. Live cells (left) react with the fluorescent reactive dye only on their surface to yield weakly fluorescent cells. Necrotic Cells show greater intensity as dye reacts with both inner and outer membrane amines (right). Adapted from Invitrogen (Invitrogen 2012).

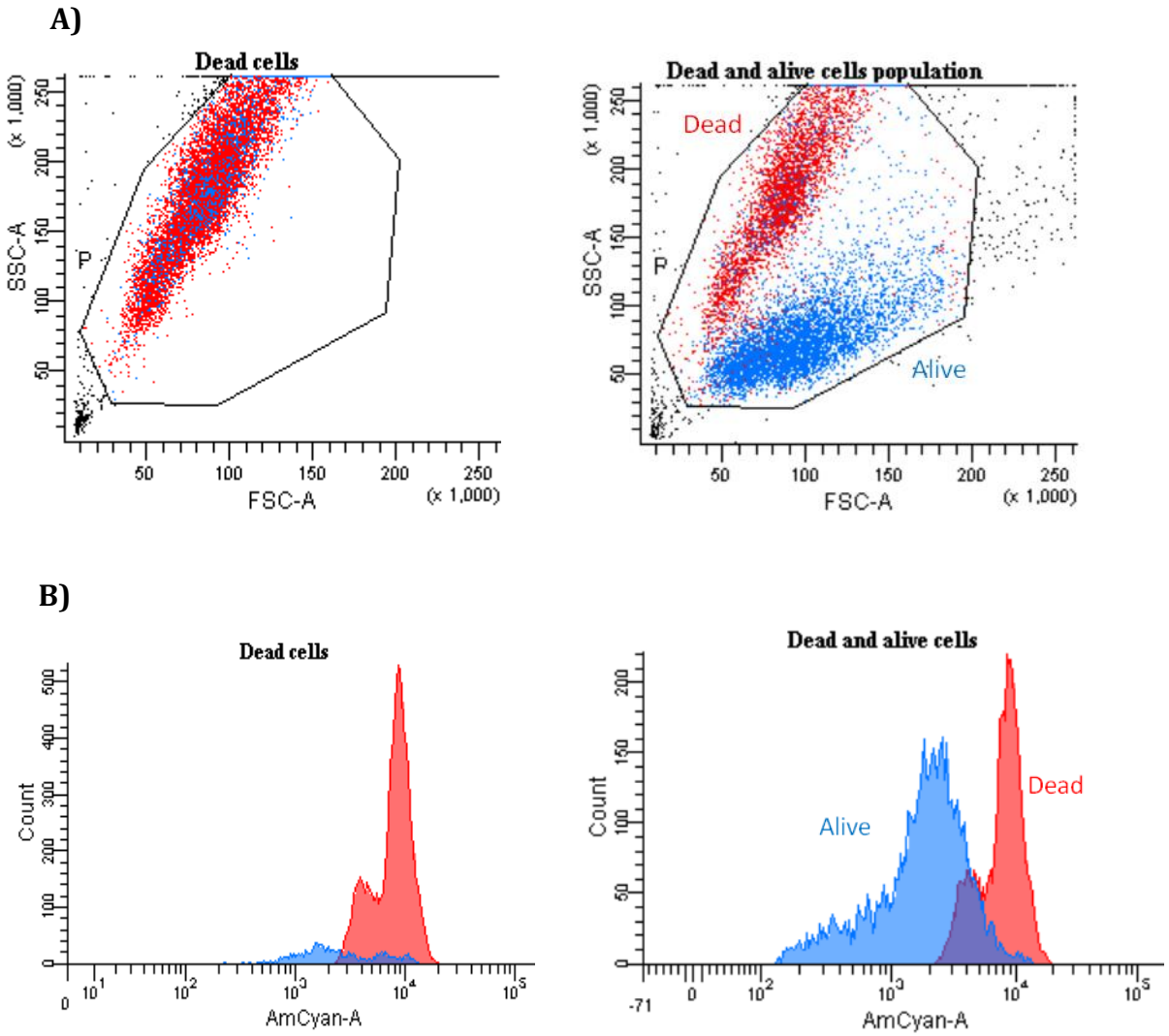


Figure 2.2: Flow cytometric analysis of BEAS-2B cells labelled with LIVE/DEAD® Fixable Aqua stain. Cells were heat treated at 90°C or left untreated, mixed together and stained with LIVE/DEAD® Fixable Aqua stain then analysed by flow cytometry. **(A)** Cells were checked with forward scatter detector (FSC) and side scatter detector (SSC) and analysed by density graph to check cell size and granularity. Fragmented cells were excluded from the study. **(B)** Histogram shows separation of live cells (left) and dead cells (right). These parameters were used to assess cell viability following treatment with chemical compounds (JQ1 and PFI-1).

2.4 CXCL-8 and IL-6 ELISA

BEAS-2B cells were stimulated for 4 hours (acute exposure) and 2 hours/day for 5, 10 and 15 days (chronic exposure) with 100 µM and 50 µM of H_2O_2, respectively, followed by stimulation with 1ng/ml TNF-α. In some experiments cells were also treated with ammonium iron sulphate (100µM) with H_2O_2 followed by IL-1β stimulation. In addition, BEAS-2B cells were also exposed to 100µM H_2O_2

for 2 hours in the presence or absence of 1 ng/ml IL-1β. Culture supernatants were assayed for CXCL8 and IL-6 using commercially available enzyme-linked immunosorbent assay (ELISA), following manufacturer`s instructions (Human IL-8 and IL-6 Duoset, R&D Systems, Abingdon, UK). Absorbance (Optical density, OD) was measured at 450nm and 550nm on microtitre plate reader (Synergy HT, Biotek, Winooski, VT, USA). Final concentrations in each sample were calculated as the mean of the results at the proper sample dilution yielding ODs in the linear parts of the calibration curves.

2.5 Dichlorofluorescein (DCF) assay

Intracellular ROS was detected using 2`,7`-Dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich). Viable cells were plated onto 96-well culture plates for 24 hours in basal media prior to experiments. On the day of the experiment, after removing the media, the cells in the plates were washed with Krebs-Ringer-Hepes-glucose-glutamine buffer (KRH buffer) and then incubated with 100µM of DCF-DA in the loading media in 5% CO_2 at 37°C for 30 minutes. After DCF-DA was removed, the cells were washed and incubated in KRH buffer with H_2O_2 or ammonium Iron sulphate at different concentrations and the fluorescence of the cells from each well was measured and recorded using microtitre plate reader. The excitation filter was set at 485 nm and the emission filter was set at 530nm.

2.6 Whole cell protein extraction

Cell pellets were lysed with RIPA lysis buffer (Santa Cruz, CA, USA) including complete mini protease inhibitors [1 tablet/10ml] (Roche, IN, USA). Samples were agitated and vortexed (10 seconds) twice before incubating on ice for 30 minutes followed by centrifugation at 19,000g for 20 minutes at 4°C. The supernatant was transferred to a fresh eppendorf (1.5 ml) and cell debris was discarded. The protein concentration was measured by Bradford Bio-Rad Protein Assay® kit using bovine serum albumin as a standard (Biorad) [section: 2.15.1].

2.7 Nuclear protein extraction

After stimulation, cells ($9X10^6$ per treatment) were washed with 5ml ice-cold PBS/phosphatise inhibitors and scrapped with cell lifter. Cell pellets were collected and nuclear proteins were extracted according to instruction manual provided by manufacturers (Nuclear Extract kit, Active motif, Carlsbad, CA, USA). Briefly, cell pellets were gently re-suspended in 500µl of hypotonic buffer and incubate on ice for 15mins. Cytoplasmic fraction was obtained by addition of 25µl of detergent followed by centrifugation at 14,000 g for 30 secs. The remaining nuclear pellets were re-suspended in 50µl of complete lysis buffer containing 1mM DTT, lysis buffer AM1 and cocktail of protease inhibitors. The suspension was incubated for 30mins on ice and scraped at intervals to lyse nuclei completely. The nuclear fraction was collected after centrifugation at 14000g for 10mins. The protein concentration was determined using the Bradford assay [section 2.15.1]

2.8 Immunoprecipitation (IP)

The protein of interest (p65) was immunoprecipitated out of whole protein extracts from BEAS-2B cells by adding 5µg of rabbit polyclonal NF-κB p65 antibodies (Santa Cruz, CA, USA). The immune complex was incubated overnight on a rotator at 4°C. Protein A/G-plus agarose beads (20µl 50% slurry; Santa Cruz , CA, USA) were added to the complex and left on the rotator at 4°C for 2 hours. The immune complex was then washed using RIPA solution (50 mM Tris-HCl pH 7.4, 2 % (v/v) NP40, 150 mM NaCl) three times. Proteins were eluted from complex using 35 µl of elution buffer (50 mM Glycine, HCl pH 2.4) for 20 seconds immediately followed by 3.5 µl of neutralising solution (2 M Tris pH 8, 1.5 M NaCl, 1mM EDTA). Samples were stored at -20°C for subsequent analysis.

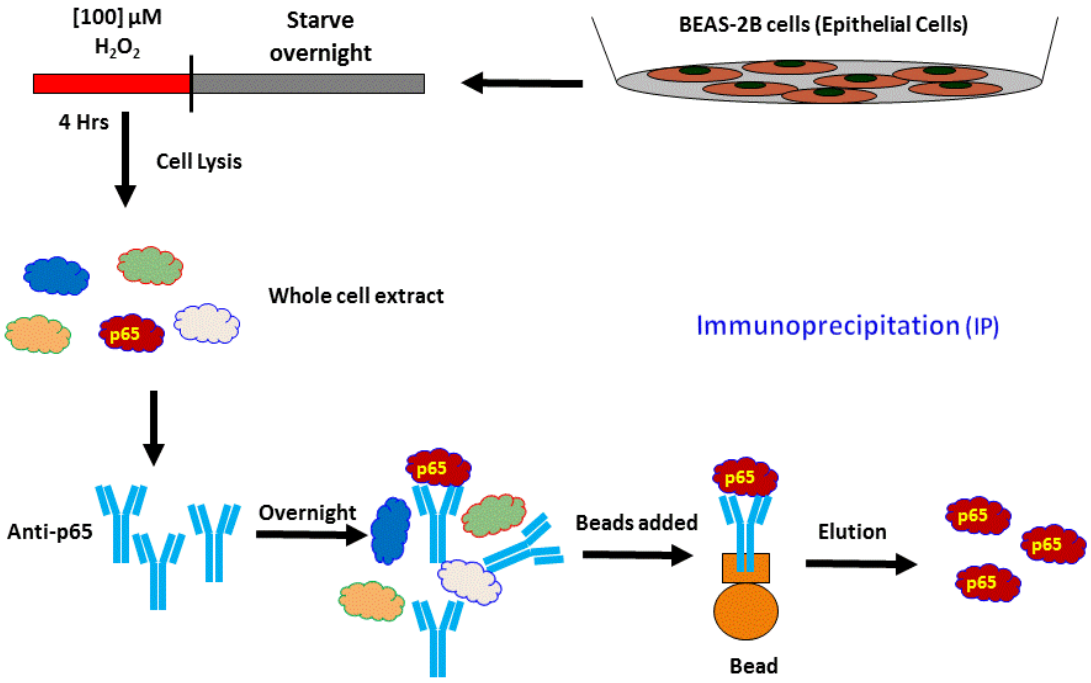


Figure 2.3: Diagrammatic illustration of immunoprecipitation. Whole cell lysates were incubated with anti-p65 antibody overnight followed by the addition of beads to pull out the protein of interest p65.

2.9 Co-Immunoprecipitation (Co-IP)

Whole cell lysates were prepared by incubating cells with IP buffer (150mM NaCl, 50mM Tris-HCl pH 8, 0.5% Sodium deoxycholate, 0.5% NP40, protease inhibitors cocktail/Roche Applied Science) for 30mins on ice and centrifuged at 14000rpm for 10mins. 500µg of protein extract was incubated with 5µg of antibody (Table 3). The immune complex was incubated overnight on rotator at 4°C. Protein A/G-plus agrose beads (20µl 50% slurry; Santa Cruz, CA, USA) were added to the complex and left on the rotator at 4°C for 2 hours. The complex was then washed using IP wash buffer (50mM Tris-HCl pH 7.4, 0.5% (v/v) NP40, 150mM NaCl) three times. Proteins were eluted from complex using 35µl of elution buffer (50mM Glycine, HCl pH2.4) for 20 seconds immediately followed by 3.5µl of neutralising solution (2M Tris pH 8, 1.5M NaCl, 1mM EDTA). Samples were stored at -20°C for subsequent analysis.

Primary antibody (Dilution/Concentration)	Company (Catalogue number)	Molecular weight (kDa)
Rabbit p65 (5μg/sample)	Santa Cruz (sc-8008)	65
Rabbit BRD4 (5μg/sample)	Sant Cruz (sc-48772)	152/80

Table 3: Antibodies used for Co-IP

2.10 Chromatin Immunoprecipitation (ChIP)

Beas-2Bs cells (2 million/sample) were treated with H_2O_2 (100μM) alone or in the presence of IL-1β (1μg/ml) for 2 hours. Some cells were treated with IL-1β alone. Proteins were cross-linked to DNA with formaldehyde (1% final of concentration) for 10 mins at 37°C. Formaldehyde was neutralized with 1M glycine (0.125M final concentration) for 5 mins at 37°C. Cells were scraped on ice cold phosphate buffered saline (PBS) containing protease inhibitors (1 tablet of mini complete protease inhibitor in 7ml PBS). Samples were microcentrifuged at 4,000 rpm for 5 mins. Cells were re-suspended in 250μl SDS lysis buffer (50mM Tris pH 8.1, 1% SDS, 5mM EDTA, complete protease inhibitor) and subjected to six cycles of sonication (Figure 2.5) on ice with 15 seconds pulses and microcentrifuged at 13,000rpm for 10 mins. Cell debris was discarded and soluble chromatin was immunoprecipitated by using 1μg of rabbit anti-NF-κB p65, Brd4 (Santa Cruz CA, USA), Acetylated Histone 3 (Milipore Watford UK) or Acetylated Histone 4 (Abd Serotec, Oxford, UK) antibody. The protein bound immunocomplex was washed with NaCl buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH8.1, 150mM NaCl), LiCl buffer (0.25M LiCl, 1% Igepal (NP40), 1% sodium deoxycholate 1mM EDTA, 10mM Tris pH 8.1) and TE buffer (10mM Tris, 1mM EDTA, pH 8). Complexes were then eluted by adding 250μl fresh elution buffer (1% SDS, 0.1M NaHCO3) twice followed by incubation for 4 hours at 65°C in 200mM NaCl to reverse cross-links between proteins and DNA. The solution was subsequently incubated with 70μg/ml proteinase K (Sigma-Aldrich) for 1 hour at 45°C to digest proteins. DNA was extracted with an equal volume (500μl) of phenol/chloroform/isoamyl alcohol (25:24:1; Invitrogen), precipitated with 1ml ethanol, 0.3M

CH_3COONa and 20µg glycogen followed by re-suspension in 50µl of nuclease free water. QPCR was performed with 5µl of DNA sample and the primer sets listed in Table 4 for quantification. Examples of DNA sonication and qPCR of ChIP DNA are shown in figures 2.5 and 2.6 respectively. Data were normalized to input control samples. A rabbit IgG antibody control was also used as negative control.

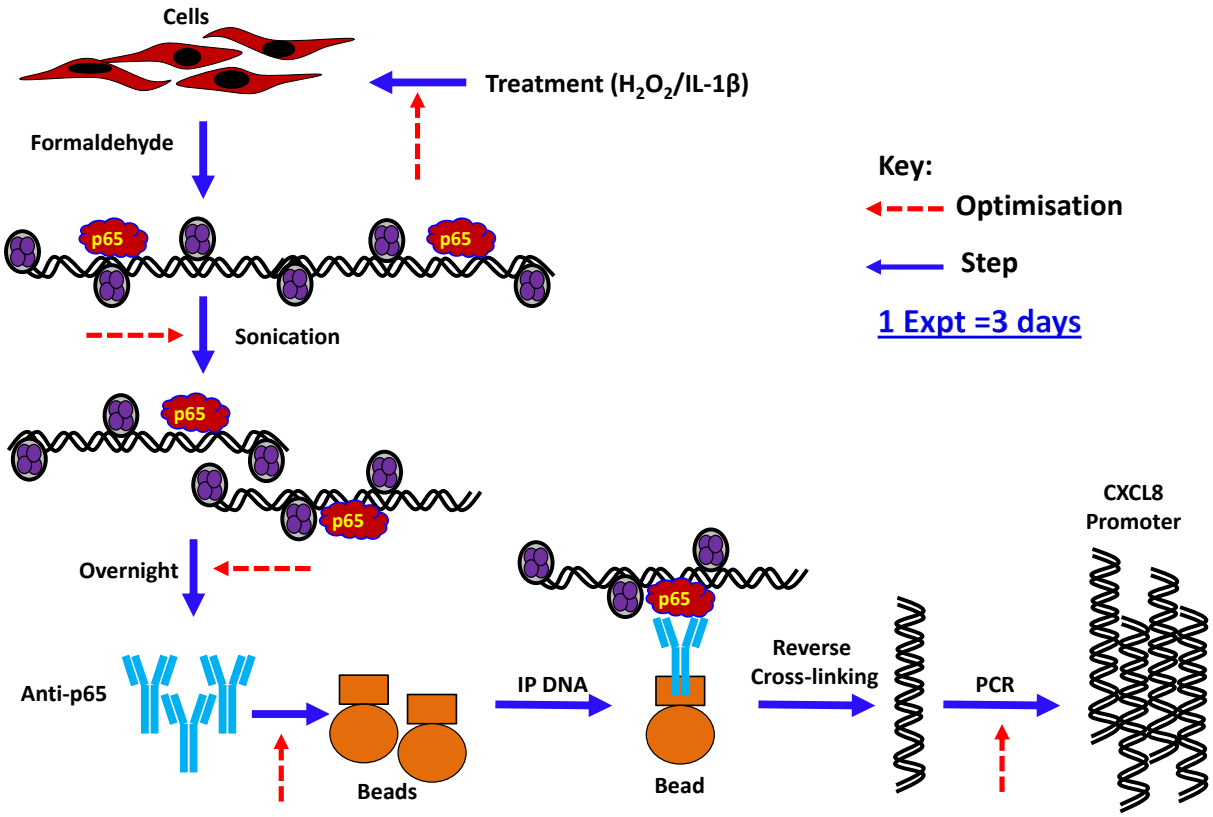


Figure 2.4: Schematic diagram of ChIP. Cells were treated with formaldehyde to cross-link protein with DNA followed by sonication. Antibody of interest (p65) was added and incubated overnight. Beads were added to pull-out the DNA/protein-antibody complex. Cross-linking was reversed and QPCR was performed.

Gene promoter Primers	Primers Sequence
ChIP: IL8 Forward	5'-GGG CCA TCA GTT GCA AAT C-3'
ChIP: IL-8 Reverse	5'-TTC CTT CCG GTG GTT TCT TC-3'
ChIP: IL6 Forward	5'-AGC ACT GGC AGC ACA AGG CAA AC-3'
ChIP: IL6 Reverse	5'-CAA GCC TGG GAT TAT GAA GAA GG-3'

Note: *IL-8* ChIP primers (Ho, Lee et al. 2009; Tsuchiya, Imai et al. 2010) and *IL-6* ChIP primers are previously published (Goransson, Elias et al. 2005; Hollingshead, Beischlag et al. 2008; Nettles, Gil et al. 2008)

Table 4: Primers used for ChIP

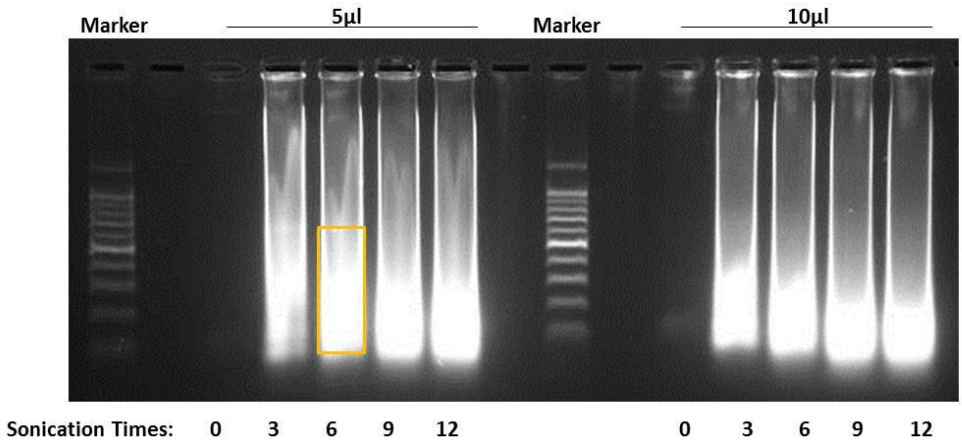


Figure 2.5: Optimisation of ChIP sonication. BEAS-2B cells were fixed with 1% formaldehyde. DNA was sheared between 600-200bp using sonication cycles (3, 6, 9 and 12) at 40% power for 15 seconds each cycle. DNA was purified and separated by 2% agrose gel electrophoresis. Sonication cycle 6 was chosen in subsequent experiments.

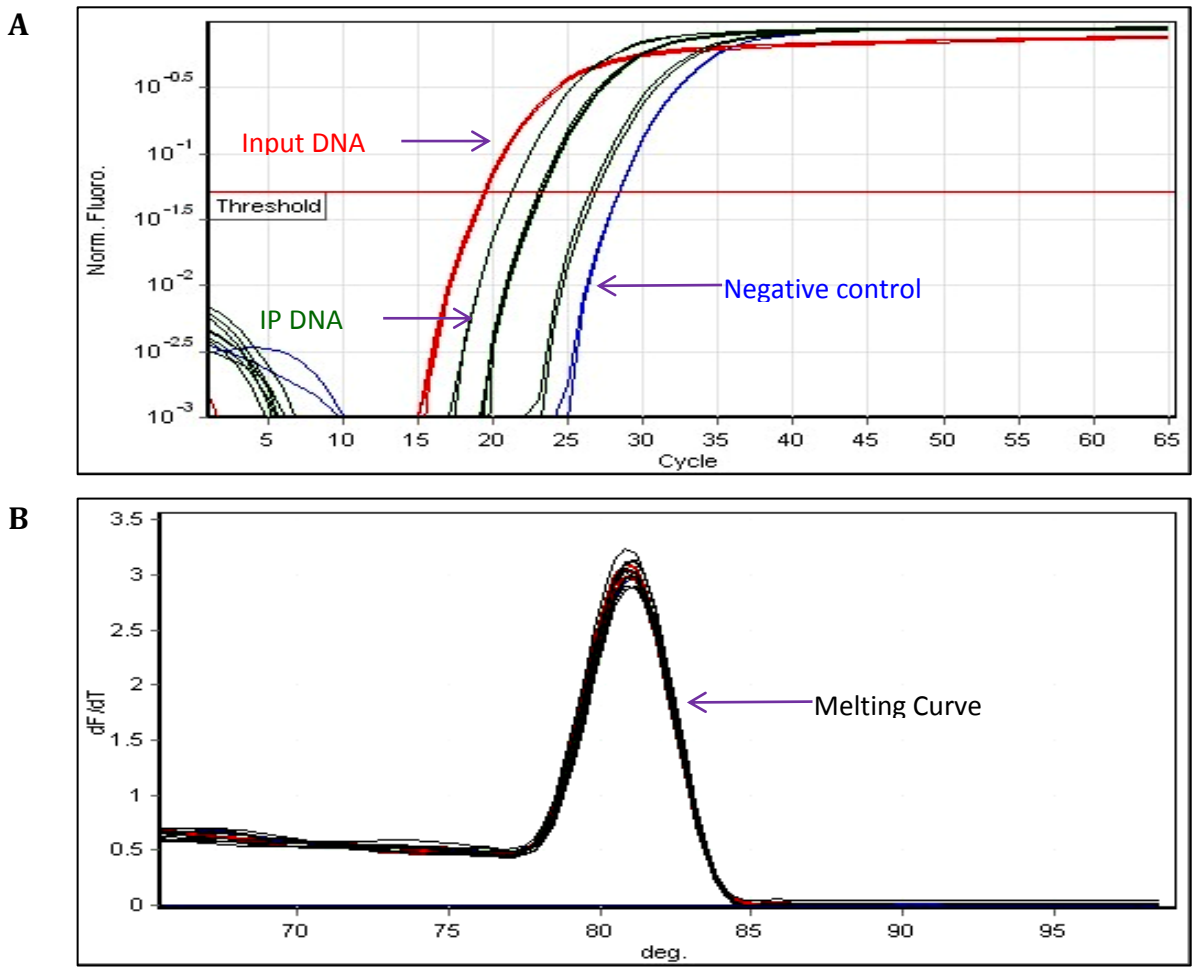


Figure 2.6: Specificity and quantification of QPCR in ChIP. **(A)** Raw channel data for QPCR shows input (Red) IP (Green) and IgG negative DNA (blue). The CT values were used to calculate fold change in treated samples relative to IgG. **(B)** Single melting curve demonstrates good specificity of final product.

2.11 RNA extraction, cDNA preparation and real-time PCR

After cell treatment, total RNA was isolated using RNeasy mini kit (Qiagen, Crawley, UK). A NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, DE, USA) was used to determine the concentration of RNA using absorbance ratio of 260nm/280nm. A ratio of ~2.0 is considered pure RNA. Single stranded cDNA was synthesized by incubating the RNA with reverse transcription mixture (AMV-reverese transcriptase, RT buffer, dNTP mix, random hexamer primers and ribonuclease inhibitor, all Promega, Southampton, UK) at 42°C for 1 hour, followed by termination of the reaction at 90°C for 4min. QPCR was performed in a Rotor-Gene 3000TM PCR machine (Corbett,

Research, Cambridge, UK) using a QuantiTect SYBR Green PCR Kit (Qiagen) to quantify the gene of interest (Table 5). The PCR data for each gene of interest was normalized to *GNB2L1* mRNA. The thermocycle parameters were 95°C for 10mins in order to activate HotStarTaq DNA® Polymerase in master mix, followed by annealing and extension for 55 cycles at 94°C for 15s, 60°C for 25s and 72°C for 25s. DNA was quantified by using delta-delta CT method.

$$\Delta\Delta CT = (2_{\text{target gene}})^{\Delta CT\,(\text{Control} - \text{Sample})} / (2_{\text{Reference gene}})^{\Delta CT\,(\text{Control} - \text{Sample})}$$

RT gene Primers	Primers Sequence
Brd2 Forward (NCBI: NM_005104.3)	5`-GGG GTG GCA GTG CTG CTT TA-3`
Brd2 Reverse	5'- GCT CAG CTG CCG CTT CTC AT -3'
Brd4 Forward (NCBI: NM_014299.2)	5'- CCA CAC TGC GTG AGC TGG AG -3'
Brd4 Reverse	5'- ATC TTG GAG GAG CCG GCA AT -3'
GNB2L1 Forward (NCBI: 006098.4)	5'-CTC CGC CTC TCG AGA TAA GAC C-3'
GNB2L1 Reverse	5'-GCA AAC TGG CCA TCT GAG GA-3'
IL6 Forward (NCBI: NM_000600.3)	5'-AGG AGA CTT GCC TGG TGA AA-3'
IL6 Reverse	5'-GCT CTG GCT TGT TCC TCA CT-3'
IL8 Forward (NCBI: NM_000584.3)	5'-AGA CAG CAG AGC ACA CAA GC-3'
IL8 Reverse	5'-ATG GTT CCT TCC GGT GGT-3'

Table 5: PCR primers for real-time RT-PCR

2.12 TransAM NF-κB p65 activity assay

Nuclear proteins were extracted as previously described using Nuclear Extraction Kit (Active Motif). p65 activity was measured using a TranAM® Kit (Active Motif). Each well of a 96-well plate is immoblised with an olignonucleotide containing an NF-κB p65 consensus site (5-GGGACTTTCC-3') to

which an activated p65 binds. Following manufacturer's instructions, 30µl of complete binding buffer (2µM DTT, 10ng/µl Salmon sperm DNA in binding buffer AM3) was added to each well followed by 20µl of nuclear proteins (15µg) diluted in complete lysis buffer (0.2µl protease inhibitor cocktail in lysis buffer AM1, 1mM DTT) and incubated for an hour with mild agitation. The plate was then incubated with 100µl/well anti-p65 primary antibody and HRP-conjugated secondary antibody for an hour at RT. The plate was washed with washing buffer after each incubation. Developing solution was added and left until a blue colour developed. Stop solution was then added to each well to stop the reaction. Chemiluminescence absorbance was read at 450nm and with the reference wavelength of 655 within 5min.

2.13 Histone Isolation and Purification.

Histones were isolated and purified using a commercially available kit, following manufacturer's guidelines (Histone Purification kit. Active motif, Rixensart, Belgium). Briefly, 10-15 million cells were incubated overnight at 4°C on a rotating platform in 500µl of acid extraction buffer, followed by microcentrifugation at 19,000 RCF for 4°C. Crude histones (supernatant) were saved and pellets were discarded. The supernatant was neutralised and equilibrated using the specific neutralising solution. Histones were purified from crude histones using a fractionating column and precipitated with 70% perchloric acid. Histone pellets were resuspended in sterile water and the protein concentration was determined using the Bradford assay.

2.14 Protein Carbonylation detection

2.14.1 OxyBlot™ Protein Oxidation (Western blot)

Protein oxidation was quantified using a commercially available kit OxyBlot™ from Millipore/Chemicon (Chandlers Ford, Southampton, UK). Briefly, samples were denatured with 12% sodium dodecyl sulphate (SDS) followed by derivatisation with 2,4-dinitrophenylhdrazine (DNPH) for

15 minutes at room temperature. The DNP-derivatised protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting (section: 2.15). A nitrocellulose membrane was blocked in 1% BSA in Tween-20 1% v/v PBS (PBST) and incubated with primary antibodies specific to DNP moiety of the proteins. Alternatively, proteins were derivatised with 2,4-DNPH for 5 minutes at room temperature on the nitrocellulose membrane (post-Western blot) followed by blocking with 1% BSA /PBST and incubated with primary antibodies against specific DNP-proteins. This step was followed by incubation with a horseradish peroxidase-antibody conjugate directed against the primary antibody (HRP-2° antibody --> 1° antibody: DNP). Chemiluminescent reagent (ECL; Amersham Biosciences, Amersham, UK) was added to the membrane and exposed to X-Ray films. Finally, quantitative assessment of band density was performed.

2.14.2 OxiSelect™ Protein Carbonyl ELISA

Protein carbonylation was examined using an ELISA-based assay kit from Cell Biiolabs (San Diago, USA). Briefly, standards and samples (10μg/ml) were added to 96-well plate (100μl/well) and incubated for 2 hours at 37°C. Plate was wahed and 100μl/well of DNPH solution was added to each and left in dark for 45mins. Plate was washed with ethanol/PBS (1:1 v/v) followed by blocking reagent and anti-DNP antibodies for 1 hour each. An HRP-conjugated secondary antibody was added for 1 hour with a washing step after each incubation period. The ECL substrate solution was added and allowed the colour to develop. The stop solution was then added and the plate was finally read at 450nm. The protein carbonyl content in unknown samples was determined by comparing with a standard curve (Figure 2.7).

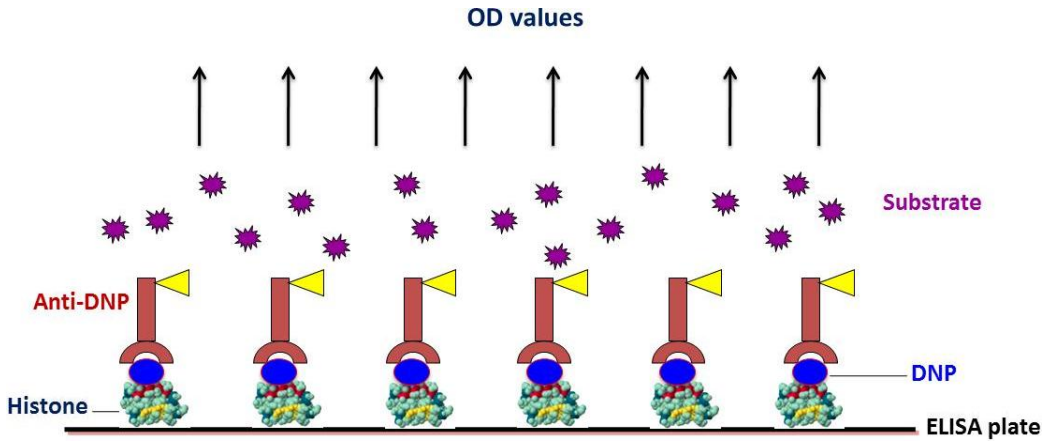


Figure 2.7: Diagram depicting protein-carbonyl assay. Proteins (Histone) 10µg/ml was coated on ELISA plate (2hrs at 37°C) followed by addition of DNP. Primary antibodies (Anti-DNP) were added. After washing, HRP-conjugated secondary antibodies were added and the plate was developed with substrate solution. The OD values were then measured.

2.15 Western Blot

2.15.1 Bradford assay

Histones and protein concentrations were calculated using the Bradford Bio-Rad protein Assay® kit (Bio-Rad Laboratories, Hemel Hempstead, UK). Bovine serum albumin (BSA) was used as a standard. Serial dilution of BSA (0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0µg/ml) were used in duplicates on 96-well plate. Bradford Bio-Rad solution was diluted to 1:5 and 200µl was added/well. The plate was read at 600nm wavelength using microtitre plate reader. A graph of the optical densities (ODs) values of the standards against the BSA concentration was plotted and the concentrations of the unknown values were calculated.

2.15.2 Polyacrylamide gel electrophoresis

Protein samples were size-fractionated electrophoretically using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Precast NuPage® 4-12% Bis-Tris gel (Invitrogen, Paisley, UK) were rinsed with deionised water and set up in the Xcell SuperLock Mini-Cell tank (Invitrogen, CA, USA). Reservoirs in the tank were filled with NuPage® MES SDS running buffer

(Invitrogen, Paisley, UK). Protein samples (10-20µg) were loaded onto the gels and run at 200 Volts, 120mA, 25 Watts for 35 and 60 minutes for p65 and histones, respectively.

2.15.3 Immunodetection of specific proteins

Proteins were transferred and immobilised on to nitrocellulose membrane using an iBlot® system per manufacturing instructions. The nitrocellulose membrane was blocked in either 1% BSA in Tween-20 1% v/v PBS (PBST) or 5% semi skimmed milk/PBST for 1 hour at room temperature on the rocking platform. The membrane was then incubated with primary antibodies diluted in 10ml blocking solution. The details of antibodies used for Western blotting are shown in Table 6. Membranes were rinsed and washed three times with PBS/Tween 20 for 10 minutes. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies raised against the host primary antibodies, diluted in blocking solution for 1 hour at room temperature on rocking platform. The washing step was again repeated. Finally, immunoreactive bands were detected using a chemiluminescence detection kit (ECL; Amersham Biosciences, Amersham, UK) followed by quantification of band density.

Primary antibody (Dilution/Concentration)	Company (Catalogue number)	Secondary antibody (Dilution)	Molecular weight (kDa)
p65 (1:1000)	Santa Cruz (sc-8008)	Goat anti-mouse (1:2000)	65
p65 (1:1000)	Santa Cruz (sc-372)	Goat anti-rabbit (1:2000)	65
Histone core (1:500)	Abcam (7832)	Rabbit anti-sheep (1:1000)	14-17
Anti-acetyl-Histone 3 (0.05 µg/ml)	Millipore (06-599)	Goat-anti-rabbit (1:2000)	17
Anti-acetyl-Histone 4 (1:800)	AbD serotec (AHP418)	Goat-anti-rabbit (1:2000)	10
BRD4 (1:500)	Sant Cruz (sc-48772)	Goat-anti-rabbit (1:2000)	152/80 isomers
Histone H4 (1µg/ml)	Abcam (ab10158)	Goat-anti-rabbit (1:2000)	14
Anti-acetyl K310 p65 (2.5µg/ml)	Abcam (ab19870)	Goat-anti-rabbit (1:2000)	65
Tata binding Protein (1:1000)	Santa Cruz (sc-273)	Goat-anti-rabbit (1:2000)	38
Brd2 1:1000	Bethyl Lab (A302-582A)	Rabbit anti-sheep (1:1000)	110

Table 6: Antibodies used for Western blotting

2.16 siRNA transfection

BEAS-2Bs cells were seeded in 6-well plates at 0.3 x 10^6 cells/well and transfected with ON-Target plus SMART pool Brd2 (L-004935-00-0005) or Brd4 (L-004937-00-0010) or control (D-001810-10-05) small interfering RNAs (siRNA) at 12nM concentration (All from Dharmacon Thermo Scientific, Chicago, USA) using HyperFect transfection reagent (Qiagen, Crawly, UK) as described by the manufacturer. After 72 hours, cells were harvested and nuclear extracts were examined for Brd2 and Brd4 protein expression levels using Western blotting with anti-Brd2 and -Brd4 antibody. Total mRNA was extracted and converted to cDNA and assessed for Brd2 and Brd4 expression using Q-PCR.

2.3 Statistical analysis

All data are presented as means ± standard error of the mean (SEM) with at least *n* = 3 independent observations per experiment. In experiments that contained only two means (Unstimulated versus Stimulated), statistical differences were determined by an unpaired Student's *t*-test. When comparing more than two means (Control, H_2O_2 and H_2O_2+IL-1β) One-way ANOVA was performed followed by Bonferroni's Multiple Comparison Test. Two-way ANOVA followed by post hoc analysis was performed using the Newman-Keuls test for experiments contain more than two means in each set of data (comparison between drugs with various stimulations). GraphPad Prism (La Jolla, CA) was used to evaluate the data. Differences were considered significant for *P* values of <0.05.

Chapter 3

The effect of Acute and Chronic Oxidative stress on pro-inflammatory mediators, p65 and Histone modifications.

3.1 Rationale

There is accumulating evidence that oxidative stress is an important feature of COPD and involved in the pathophysiology of the disease (Barnes 2007). Oxidtive stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants, favouring ROS, which results in detrimental effects including damage to proteins, DNA and Lipids (Ahsan, Ali et al. 2003; Rahman and Adcock 2006). Oxidative stress is also linked to protein oxidation that leads to carbonylation. Carbonyl groups (C=O) can be introduced into proteins by oxidation of side chains of amino acids or by addition of reactive aldehydes generated from lipid peroxidations (Dalle-Donne, Rossi et al. 2003). A specific carbonyl marker, 4-hydroxy-2-nonenal, has been detected in the lung of COPD patients using immunocytochemistry (Rahman, van Schadewijk et al. 2002). There is increasing evidence for autoantibodies against carbonyl-modified proteins in sera of COPD patients (Kirkham, Caramori et al. 2011). It has been shown that cigarette smoke (CS) alters iron homeostasis following lung injury and that this accumulation of iron may also result in oxidative stress (Ghio, Hilborn et al. 2008).

Chronic inflammation in COPD is driven by a plethora of pro-inflammatory cytokines (IL1β TNF-α, CXCL8, and IL-6) and oxidative stress (H_2O_2) (Chung 2001). Sputum from COPD patients show increased levels of IL-β, CXCL8 and TNF-α levels which correlates with disease severity. Similarly IL-6 concentrations are increased in plasma, sputum and bronchalveolar lavage from COPD patients (Sinden and Stockley ; Keatings, Collins et al. 1996; Barnes 2004; Daldegan, Teixeira et al. 2005; Galli, Tsai et al. 2008). Bronchial epithelial cells from COPD patients release more IL-1β than healthy individuals suggesting that cells are pre-programmed to release IL-1β (Rusznak, Mills et al. 2000; Barnes 2004; Singh, Arora et al. 2010). Airway epithelial cells are the first line of defence against noxious particles and gases from smoke and pollutants. They are involved in tissue repair as well as secreting antimicrobial peptides such as defensins to further enhance the innate immune system (Menendez and Brett Finlay 2007). Cigarette smoke may compromise the defence function of epithelial cells, thereby making the host more susceptible to infections (van der Vaart, Postma et al.

2004; Yaghi, Zaman et al. 2012). Smokers and COPD patients have high incidences of lower tract respiratory infection (Arcavi and Benowitz 2004; Sapey and Stockley 2006). Elevated H_2O_2 is also reported in the breath condensates of COPD patients, indicative of oxidative stress (Montuschi 2005). Many inflammatory genes are regulated by the NF-κB transcription factor. These genes are activated by oxidative stress and their expression is increased in smokers and COPD patients (Di Stefano, Caramori et al. 2002; Edwards, Bartlett et al. 2009). NF-κB binds to the promoter regions of inflammatory genes upon activation leading to local histones hyper-acetylation. This allows the local unwinding of DNA and binding of other transcription factors and transcriptional regulatory complexes (Gloire, Legrand-Poels et al. 2006; Watters, Kenny et al. 2007; Franco, Schoneveld et al. 2008). Cigarette smoke has been shown to increase histone acetylation and enhances NF-κB activity via reduced IκB protein in COPD and smokers (Szulakowski, Crowther et al. 2006).

In the developed world, COPD is caused by long-term exposure to oxidative stress derived from cigarette smoke and about 90% of COPD patients are either smokers or ex-smokers (Young, Hopkins et al. 2006; Barnes 2007). Evidence for continued oxidative stress even many years after smoking cessation has been reported (Donohue 2006; Louhelainen, Rytila et al. 2009). Therefore, in order to understand the disease on a cellular level we need to explore the role of both acute and chronic oxidative stress and its effect on inflammatory genes. A number of cell-based systems of oxidative stress have been reported (Kirkham, Spooner et al. 2004; Merry 2004; Rahman and Adcock 2006). However, all these models fail to address the accumulative and chronic effect of oxidants resulting in the disease state. Although, numerous oxidative stress models (*in vivo* and *in vitro*) have been reported (Ito, Hanazawa et al. 2004; Marwick, Kirkham et al. 2004; Ito 2007; Churg, Cosio et al. 2008; Wright, Cosio et al. 2008), acute (2 hours) ROS exposure remains the basis of most experimental models. Therefore, it's important to establish both acute and chronic oxidative stress models *in vitro* using H_2O_2 (oxidant) and bronchial epithelial cells. The NF-κB pathway is activated during inflammation (Rahman and Adcock 2006). In addition, epigenetic changes such as changes in histone

acetylation profile have been linked with inflammation (Eberharter and Becker 2002). However, It's not clear whether oxidative stress derived from cellular or/and exogenous sources (H_2O_2) can modify, through oxidisation, histones and NF-κB p65 that results in carbonylation. The effect of such modification on pro-inflammatory mediators (CXCL8 and IL-6) is also not well understood. In a transcriptionally inactive cell, DNA and histones are densely packed together to prevent the binding of transcription factors such as NF-κB to its targeted inflammatory genes (Adcock, Cosio et al. 2005). There is mounting evidence that increased gene transcription is associated with an increase histone acetylation whereas hypo-acetylation is correlated with gene suppression (Adcock, Ford et al. 2006; Ito 2007). The N-terminal tails, protruding from the subunits of core histones such as H3 and H4, are sites for post-transcriptional acetylation that consists of highly conserved lysine residues. These sites play important roles in gene expression and regulation (Ito, Barnes et al. 2000). Therefore, it's imperative to examine the effect of oxidative stress and inflammatory stimulus (TNF-α) on histone acetylation, particularly H3 and H4.

It has been reported that the iron content is increased in COPD patients in comparison with smokers and healthy non-smokers (Ghio, Hilborn et al. 2008). Iron is usually found as complex with other proteins such as haem, ferritin, transferrin and lactoferrin (Ghio, Stonehuerner et al. 2008). In COPD, inflammation is accompanied by an influx of inflammatory cells including macrophages and neutrophils. Neutrophil elastase, released by neutrophils, degrades iron-bound proteins and thereby increases the free iron burden (Vlahos, Wark et al. 2012). Iron can convert (via the Haber-Weiss/Fenton reaction) superoxide ($^{\cdot}O_2^-$) and/or hydrogen peroxide (H_2O_2) into highly toxic hydroxyl radicals ($^{\cdot}OH$) (Barnes 2004; Fischer, Pavlisko et al. 2011). Therefore, it's important to study the effect of both iron and hydrogen peroxide on the expression of inflammatory mediators and intracellular ROS *in vitro*.

3.2 Experimental protocol

3.2.1 Optimisation of BEAS-2Bs cell seeding density

BEAS-2B cells were cultured in complete media (growth factors) and minimum media (1% growth factors). Both media conditions had cells with a seeding density of $3x10^5$ cells/$150cm^2$ in 8 separate culture flasks (150 cm^2). Cells from each flask were split each day using 0.25% trypsin. Viable cells were counted by Trypan blue exclusion. A 10µl of cell suspension was added to 90µl of trypan blue dye (Sigma, Poole, UK) and 10µl of this mixture (trypan blue/cells) was applied to haemocytometer with glass cover slip on the top. The haemocytometer was viewed at 10X magnification Olympus CH30 microscope (Olympus UK Ltd., Watford, UK). All the cells in the haemocytometer grids were counted and average numbers of cells were determined per flask.

Total cells/ml = total cells within all 4 grids x 10^4 cm^3 (volume of groves) x 10 (dilution factor/dye)

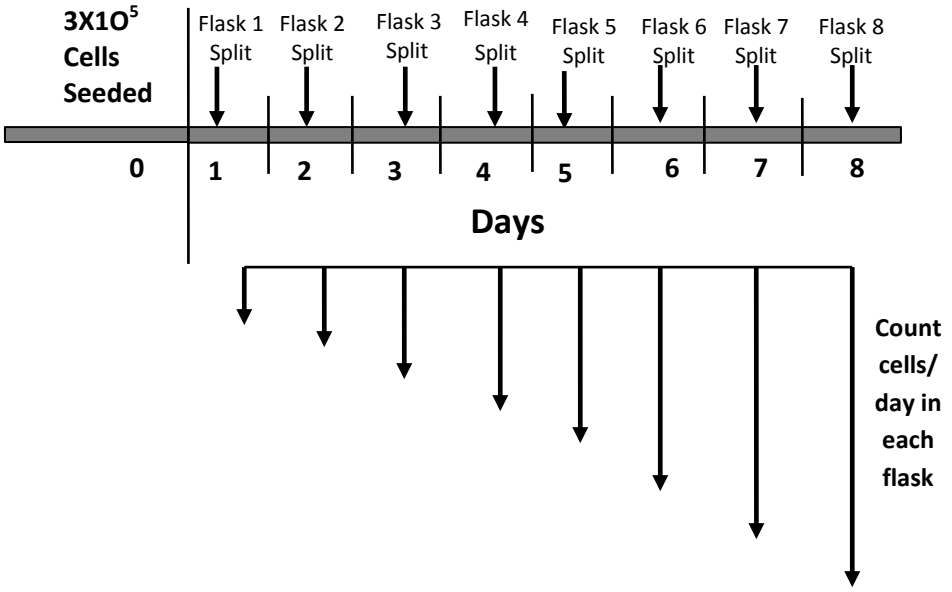


Figure 3.1: Schematic representation of the cells count. 8 flasks were seeded with $3x10^5$ cells and each day a flask was split and cell number was determined by trypan blue exclusion.

3.2.2 Acute and Chronic H_2O_2 treatment

Following overnight starvation, cells (1x10^6 cells/well in 2ml) seeded in 6-well plates were exposed to 100µM of H_2O_2 for 4 hours (acute). Alternatively, cells (seeded at 2x10^6 cells/flask 75 cm^3 in 8ml) were exposed to 50µM of H_2O_2 each day for 5, 10 and 15 days in a chronic model for 2 hours daily. Following exposure, media was removed and minimum media (1% growth factors) was added. Cells were stimulated in the presence or absence of 1ng/ml TNF-α for 20 hours at last day (5th, 10th and 15th). I have also used IL-1β as inflammatory stimulus as alternative to TNF-α in acute model. Supernatants were saved for ELISA and cells were used for proteins extractions (NF-κB p65 and histones).

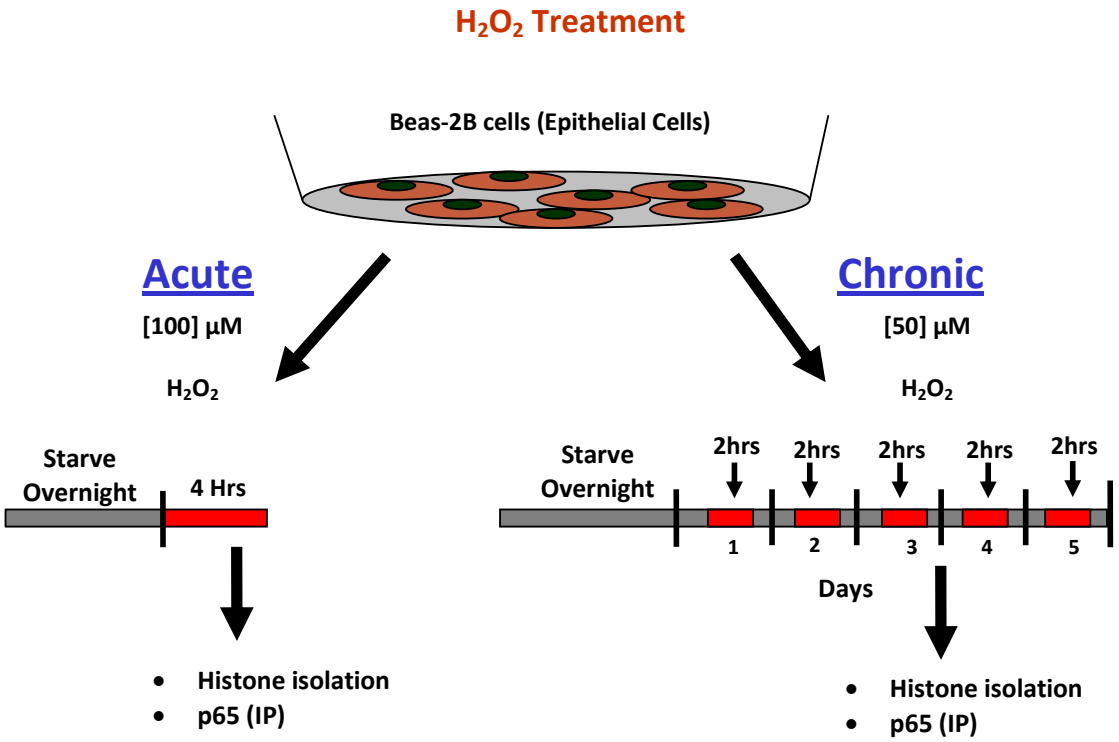


Figure 3.2: A diagrammatic representation of acute and chronic oxidative stress models. BEAS-2B cells were exposed to 100µM of H_2O_2 for 4 hours acutely and 50µM for 2 hours each day for 5 days chronically, followed by p65 and histone isolation.

3.2.3 Effect of H_2O_2 and ammonium iron sulphate on cell viability

After treating cells with H_2O_2 or ammonium iron sulphate, cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MMT) assay. Cells were incubated with 1mg/ml MMT solution at 37°C and 5% CO_2 for 30 minutes. MTT solution was then removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan product to produce a purple solution. The absorbance was measured at 550 nm. The colour intensity was correlated with cell viability.

3.2.4 Measurement of intracellular oxidative stress using Dichlorofluorescein assay

Cells were cultured in serum-free media for 24 hours. Prior to treatments, cells were incubated with dichlorofluorescine diacetate (DCFH-DA) for 30mins in media. After removing the media, cell were washed with KRH buffer followed by treatment with various concentrations of H_2O_2/ammonium iron sulphate or left untreated. Fluorescein from cells was measured using fluorescein plate reader. Fluorescein intensity was correlated to intracellular ROS (See section 2.5 of Chapter 2).

3.2.5 Release of IL-6 and CXCL8 from stimulated cells

BEAS-2B cells, 80-95% confluent, were cultured in serumfree medium for 24 hours. Cells were treated with H_2O_2 or/both ammonium iron sulphate followed by IL-1β stimulation for 16 hours or left untreated. Supernatant was collected and IL-6 and CXCL8 was determined by ELISA (section 2.4 of Chapter 2).

3.2.6 *IL-6* and *IL-8* mRNA expression

BEAS-2B cells were cultured in keratinocyte serum free media for 24hours before treatment. Cells were treated with H_2O_2 (100μM) with/without IL-1β (1ng/ml) for 2 hours. Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was converted to cDNA and subjected to qPCR analysis. The amount of *IL-6* and *IL-8* mRNA was determined using delta delta CT values of gene of interest (*IL-6* and *IL-8*) and a housekeeping gene (Guanine nucleotide binding protein beta polypeptide 2-like 1: *GNB2L1B1*). Refer to section 2.10 Chapter 2.

3.2.7 Immunoprecipitation of p65 (IP p65)

Whole cell protein extract (500µg) was incubated overnight with 5µg rabbit polyclonal anti-p65 antibodies (Santa Cruz, CA, USA) on rotator at 4°C. Protein A/G-plus agrose beads (20µl 50% slurry; Santa Cruz , CA, USA) were added to the complex and left on the rotator at 4°C for 2 hours. The immune complex was then washed using RIPA solution (50mM Tris-HCl pH 7.4, 2% (v/v) NP40, 150mM NaCl) three times. Proteins were eluted from complex using 35µl of elution buffer (50mM Glycine, HCl pH2.4) for 20 seconds immediately followed by 3.5µl of neutralising solution (2M Tris pH 8, 1.5M NaCl, 1mM EDTA). See section 2.8 of Methods and Materials for more details.

3.2.8 Histone isolation and purification

Cells were treated or left untreated with either 50µM or 100µM H_2O_2 in the presence or absence of 1ng/µl of TNF-α. Histones were isolated and purified using a commercially available kit, manufacturer`s instructions (Histone Purification kit. Active motif, Rixensart, Belgium).

3.2.9 Carbonylation assay

Protein oxidation was quantified using a commercially available kit OxyBlot™ from Millipore/Chemicon (Chandlers Ford, Southampton, UK) and ELISA based assay kit from Cell Biiolabs (San Diago, USA). The manufactures' provided instructions were followed (Section 2.14).

3.3 Results

3.3.1 BEAS-2B cells proliferation in a media

In order to quantify BEAS-2B cells proliferation in supplemented medium (containing growth factors) and supplement free medium, cells were allowed to proliferate in media with/without the growth factors for 8 days. BEAS-2B cells were seeded at a density of $3x10^4$ cells/flask ($150cm^2$ Cornning®, NY, USA) in 8 different flasks on day 1 and allowed to proliferate until 8^{th} day. Culture media was changed every two days. Each day a flask was split and cell number was determined by trypan blue exclusion. BEAS-2B cells stopped dividing exponentially after the 6^{th} day, having reaching a density of 15 million cells in a media supplemented with growth factors. The cells number started to decline by 7^{th} and 8^{th} day (Fig. 3.3A). In contrast, 3 million cells were recorded at 6^{th} day in media lacking growth factors (Fig.3.3B). Based on this result, cells were starved 24 hours prior to experiments in order to achieve cell cycle snychronisation and maintain the same number cells in all experiments.

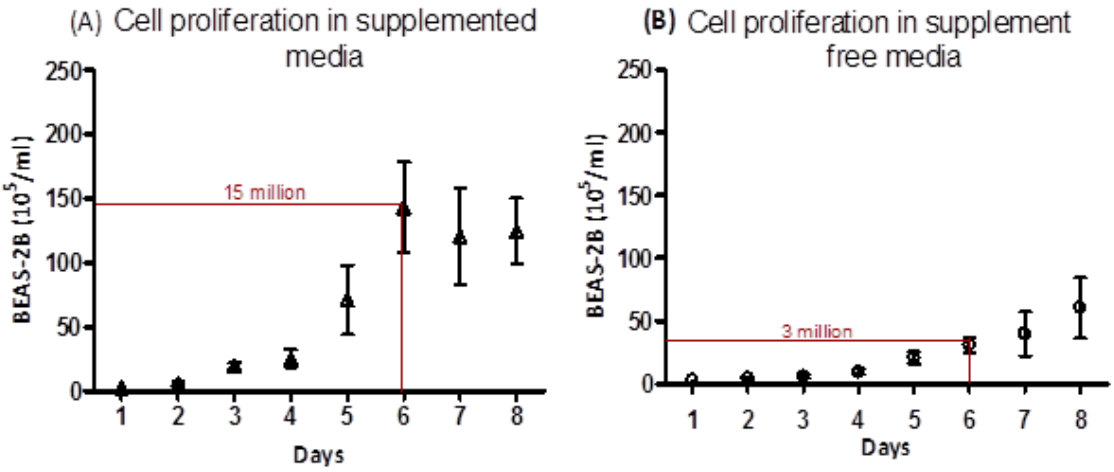


Figure 3.3: BEAS-2B cells proliferation in media. BEAS-2B cells were seeded at $3x10^4$ cells/flask ($150cm^3$) supplemented with or without growth factors at day 1. **(A)** Cells reached 15 million at day 6 followed by decline in cell number. **(B)** Cells grew very slow in media without growth factors reaching 3 million at day 6. Subsequent experiments were done in media without growth factors to maintain the same cell number. Mean ± SEM of 5 independent experiments.

3.3.2 The effect of acute and chronic H_2O_2 on cell viability

Metabolic activity assay (MTT-tetrazolium salt) was used to examine the effect of acute and chronic H_2O_2 treatments on cell viability in order to define appropriate concentration conditions for subsequent studies. Cells were exposed to different concentrations of H_2O_2 ranging from 0-800µM for 4 hours (acute) and 2 hours/day for 5 days (chronic). MTT assay was performed at the end of each experiment. MTT is reduced to purple formazan in living cells. A violet colour solution is developed upon dimethyl sulfoxide (DMSO) addition. The absorbance of this colour corresponds to cell viability. The cell viability was reduced to 70% with 150µM, 50% with 200µM and below 40% in subsequent higher concentrations in acute treatments whereas 50µM and 100µM had no significant effect (Fig. 3.4A). In comparison with acute experiments, cell viability was reduced to 25% in repeated exposure to 100µM of H_2O_2 and below 25% with higher concentrations with chronic treatment (Fig. 3.4B). However, there was no significant change in cell viability with 25µM or 50µM. The chronic treatment was extended to 10 and 15 days using 50µM of H_2O_2. Cells remained viable after 5, 10 and 15 days treatment (Fig. 3.4C-D). Hydrogen peroxide had no obvious effects on cell morphology when examined under the microscope after chronic H_2O_2 treatment (Fig. 3.5). Taking these results into account, 100µM of H_2O_2 was used for acute and 50µM for chronic treatment in subsequent experiments. All the experiments were carried out on the viable cells after exposure to H_2O_2.

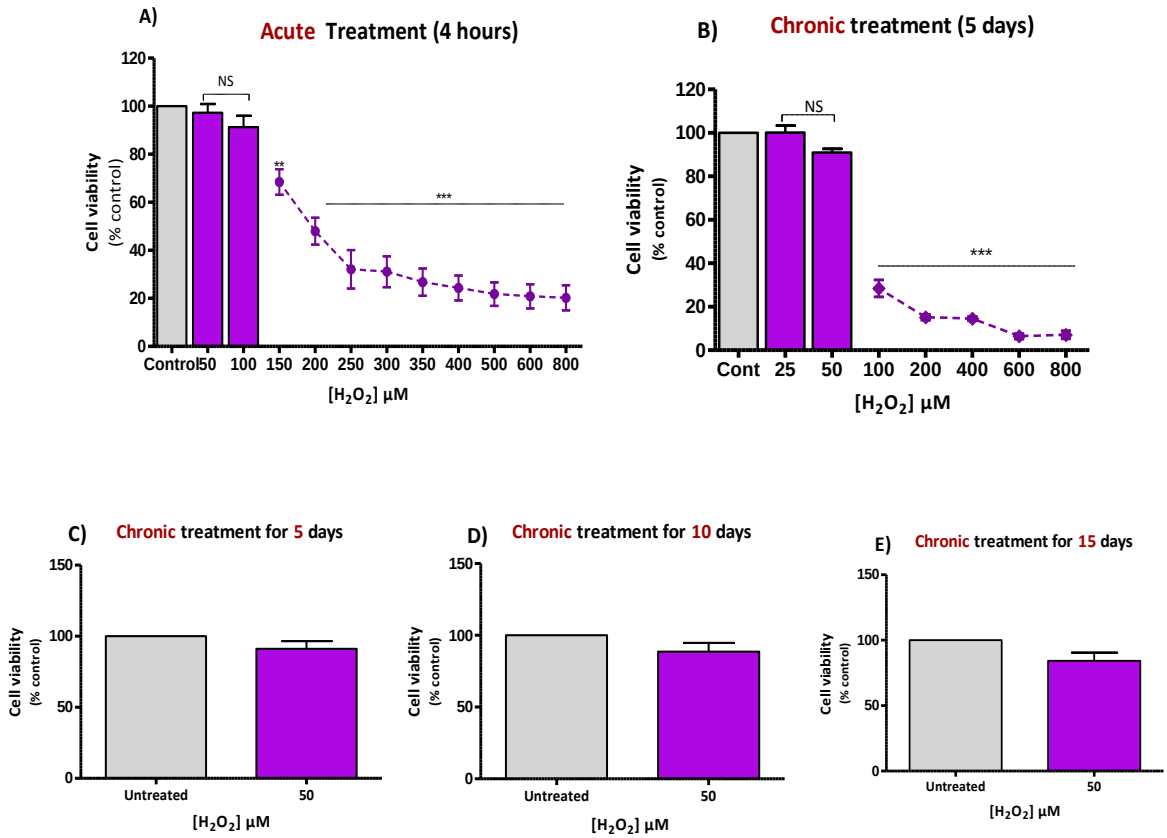


Figure 3.4: The effect of H_2O_2 on cell viability. (A) Cells were exposed to a range of concentrations of H_2O_2 for 4 hours acutely. Cell viability significantly reduced above 100µM concentrations. **(B)** Cells showed significant reduction in cell viability above [50µM] when treated chronically for 2 hours daily for 5 days with range of [H_2O_2]. **(C-E)** Cell remained viable after treatment with [50µM] daily for 2 hours after 5, 10 and 15 days. Results represent the mean ± SEM of 4 independent experiments. **p<0.01, ***p<0.001 (Control vs treated)

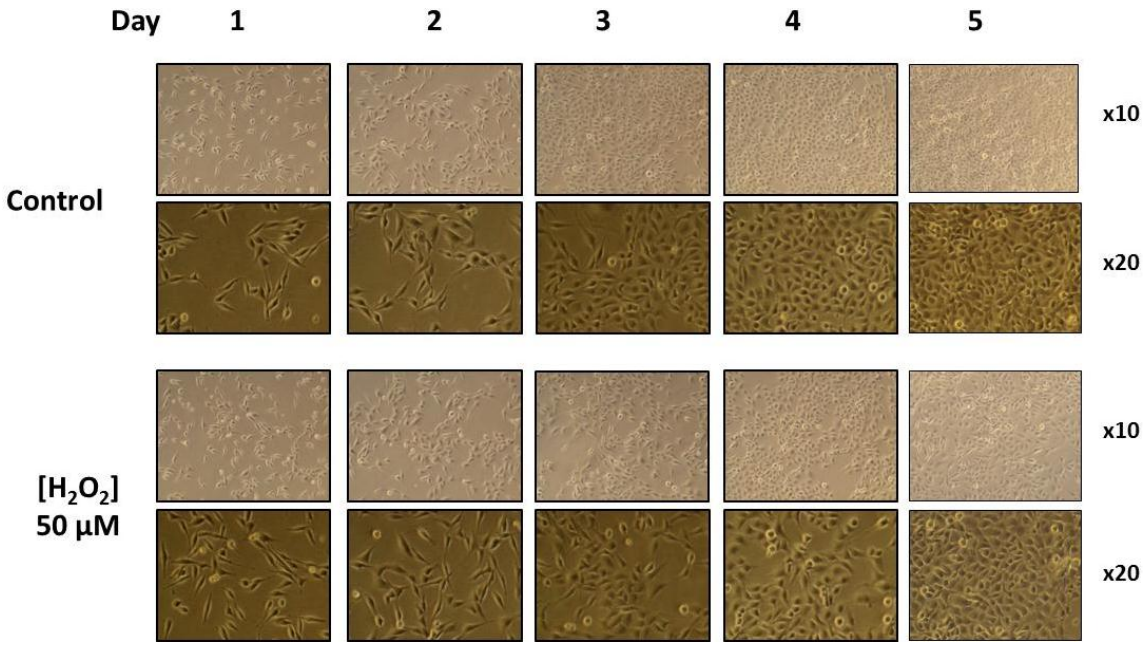


Figure 3.5: Chronically treated cells` morphology. Hydrogen peroxide did not have any obvious effect either on the size or shape of cells when treated chronically for 2 hours daily for 5 days. Cells were examined at x10 and x20 magnifications and photographed. Results are representative of 3 independent experiments.

3.3.3 The effect of H_2O_2 on intracellular ROS

To examine whether H_2O_2 is able to induce intracellular ROS (oxidative stress), cells were seeded ($3x10^4$/well) in 96-well plate and incubated with DCFH-DA fluorescent probe for 30 minutes, followed by treatment with H_2O_2 (0, 25, 50, 100, 200, 400 and 800µM) for 4 hours. Intracellular ROS elevated in concentration-dependant manner (Fig. 3.6). There was a significant increase in intracellular ROS after treating cells with concentrations of H_2O_2 at 100µM or above in comparison with untreated cells. This data in conjunction with cell viability data above, indicate that 100µM and 50µM were optimal H_2O_2 concentrations for use in future chronic and acute exposure experiments, respectively.

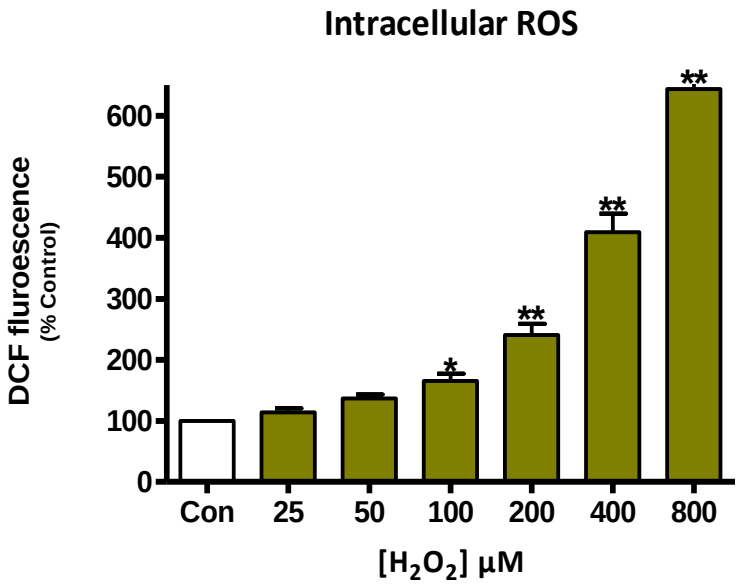


Figure 3.6: The effect of H_2O_2 on intracellular ROS in BEAS-2B cells. Cells were pre-incubated with DCFH-DA for 30 minutes in loading media followed by wash with KRH buffer. Cells were then treated with H_2O_2 in KRH buffer for 4 hours. Mean ± SEM. N=4. *p<0.05; **p<0.01 when compared to basal level (control).

3.3.4 TNF-α induced CXCL8 and IL-6 release in acute and chronic oxidative stress

The effect of H_2O_2 derived oxidative stress on pro-inflammatory mediator (IL-6 and CXCL8) release from BEAS-2B cells, either alone or in combination with TNF-α was investigated. Cells were exposed to acute (100µM for 4hrs) and low chronic (5, 10 and 15 days) H_2O_2 (50µM for 2hrs) followed by 1ng/ml TNF-α challenge at the last day of H_2O_2 exposure. Acute exposure to 50µM of H_2O_2 did not enhanced TNF-α induced IL-6 or CXCL8 expression (Fig. 3.7A and 3.8A). In contrast, acute 100µM H_2O_2 significantly increases in CXCL8 and IL-6 release together with TNF-α (Figs. 3.7B and 3.8B). Furthermore, repeated exposure to low (50µM) of [H_2O_2] for 5 days enhanced TNF-α induced IL-6 and CXCL8 release although there was no effect of a one-off acute exposure with the same H_2O_2 concentration 50µM (Figs. 3.7C and 3.8C). This suggests that chronic exposure affects signalling and/or epigenetics. Interestingly, when chronic treatment of hydrogen peroxide (50µM/day) was extended to 10 and 15 days, it resulted it in the loss of the effect that was seen in 5 days treatment, suggesting that perhaps cells can induce a resistance to hydrogen peroxide and there are no longer

sensitive as reported by several groups (Fig. 3.7D-E and 3.8D-E) (Flattery-O'Brien and Dawes 1998; Bose, Bhaumik et al. 2003; Sen, Mukherjee et al. 2003; Bose Girigoswami, Bhaumik et al. 2005; Ho, Asagiri et al. 2011).

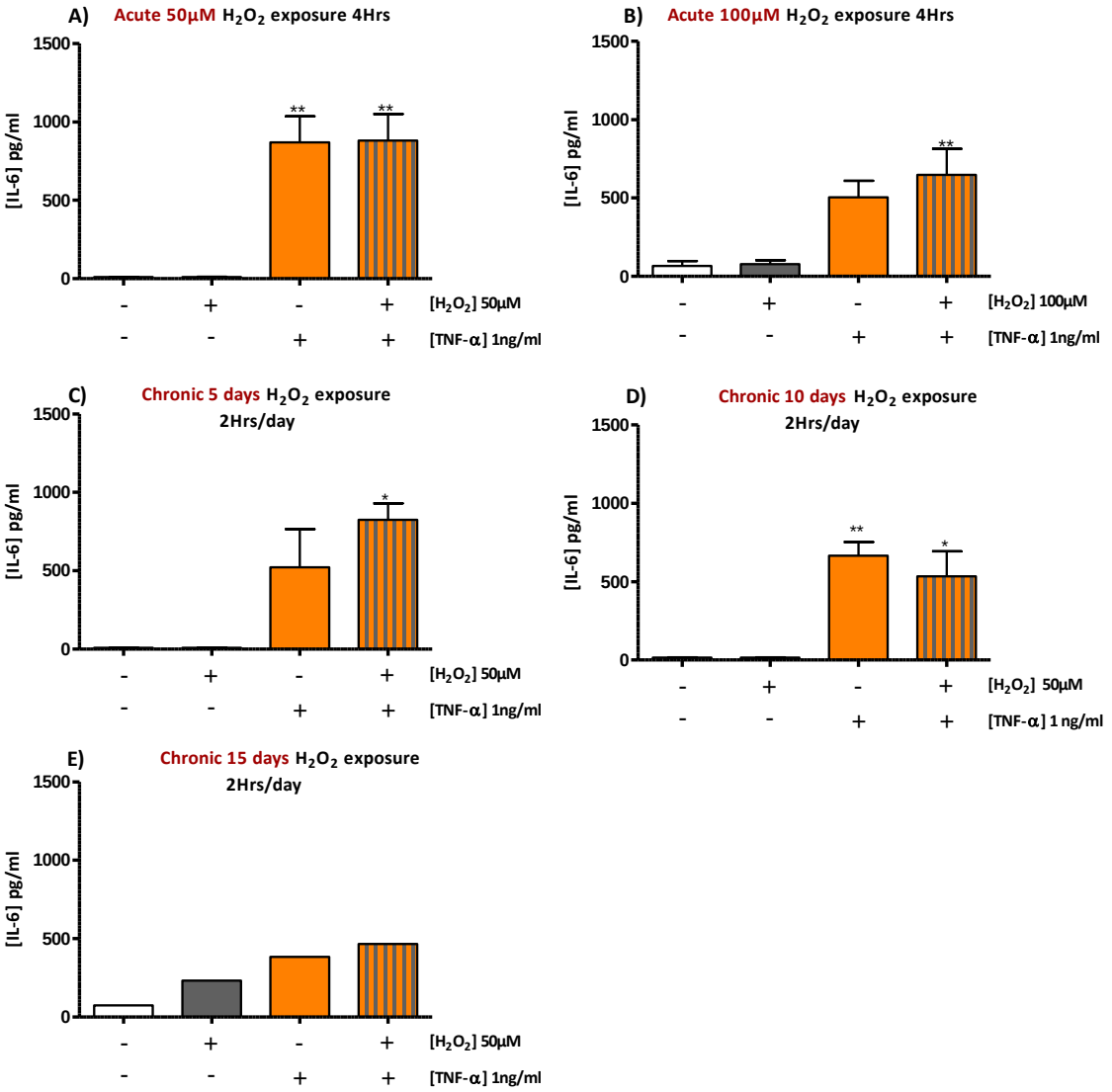


Figure 3.7: TNF-α induced IL-6 release under acute and chronic oxidative stress. The effect of TNF-α induced IL-6 release in acute and chronic oxidative stress *in vitro* models. The bar graphs show quantitative analysis of IL-6 release in **(A-B)** acute and **(C-E)** chronic H_2O_2 treated cells in the absence and presence of TNF-α. Results are expressed as mean ± SEM (n≥3 or n=2). *p<0.05; **p<0.01 when compared to control.

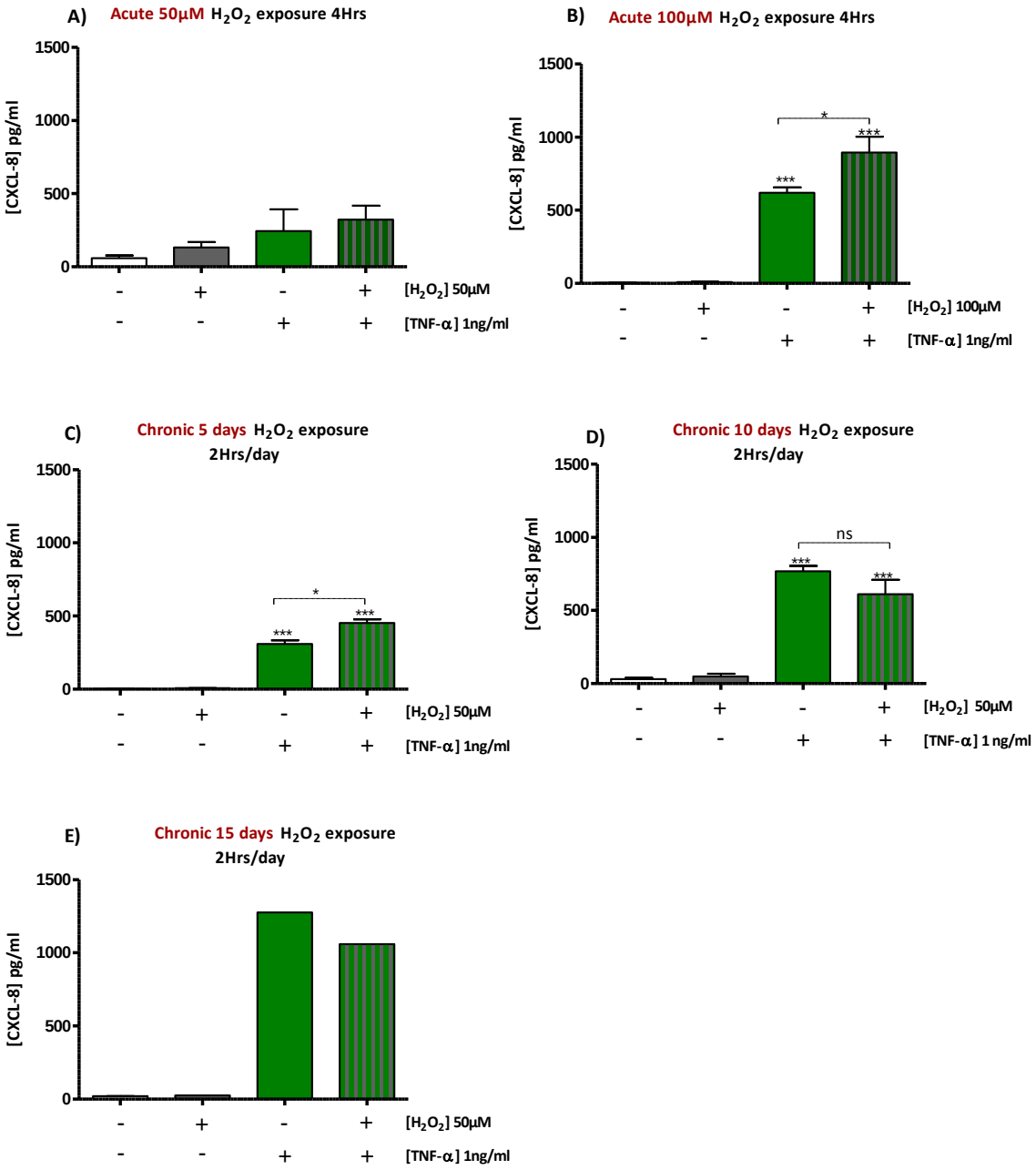


Figure 3.8: TNF-α induced CXCL8 release under acute and chronic oxidative stress. The effect of TNF-α induced CXCL8 release in **(A-B)** acute and **(C-E)** chronic (5-10 days) oxidative stress *in vitro* models. **(C)** Chronic (50µM) exposure has altered the release of CXCL8 release compare with **(A)** acute (50µM) exposure. Similarly, increasing the H_2O_2 concentration from 50µM to 100µM acutely resulted in increased release of CXCL8 **(B)**. However, extending chronic treatment to 10-15 days did not enhance CXCL8 release, indicating that cells have adapted to oxidative stress **(D-E)**. Results are represented as mean ± SEM. (n≥4 or n=2). *p<0.05; **p<0.01; ***p<0.001 (con vs stimulated).

3.3.5 NF-κB p65 and histone carbonylation in acute and chronic oxidative stress

Having established acute and chronic oxidative stress models, I decide to investigate the impact of oxidative stress on intracellular protein oxidation (carbonylation) including NF-κB p65 and histones. A Western blot based protein carbonylation assay (OxyBlot™) was used to assess protein oxidation following p65 immunoprecipitation. The results demonstrated a trend towards increased p65 carbonylation under acute (Fig.3.9A) and chronic oxidative stress (Fig.3.9B) although this did not reach statistical significance. Following similar treatments, total histones were extracts and fractionated by 4-12% SDS-PAGE gel using NuPAGE MES running buffer in order to resolve smaller proteins such as histones. After separation, histones were visualised with Coomassie blue, confirming core histone proteins (Fig. 3.9E). Histone carbonylation was also investigated with ELISA based assay (OxiSelect^{TM} Carbonyl ELISA). There was no change in histone carbonylation profile following exposure to acute or chronic H_2O_2 (Fig. 3.9C-D).

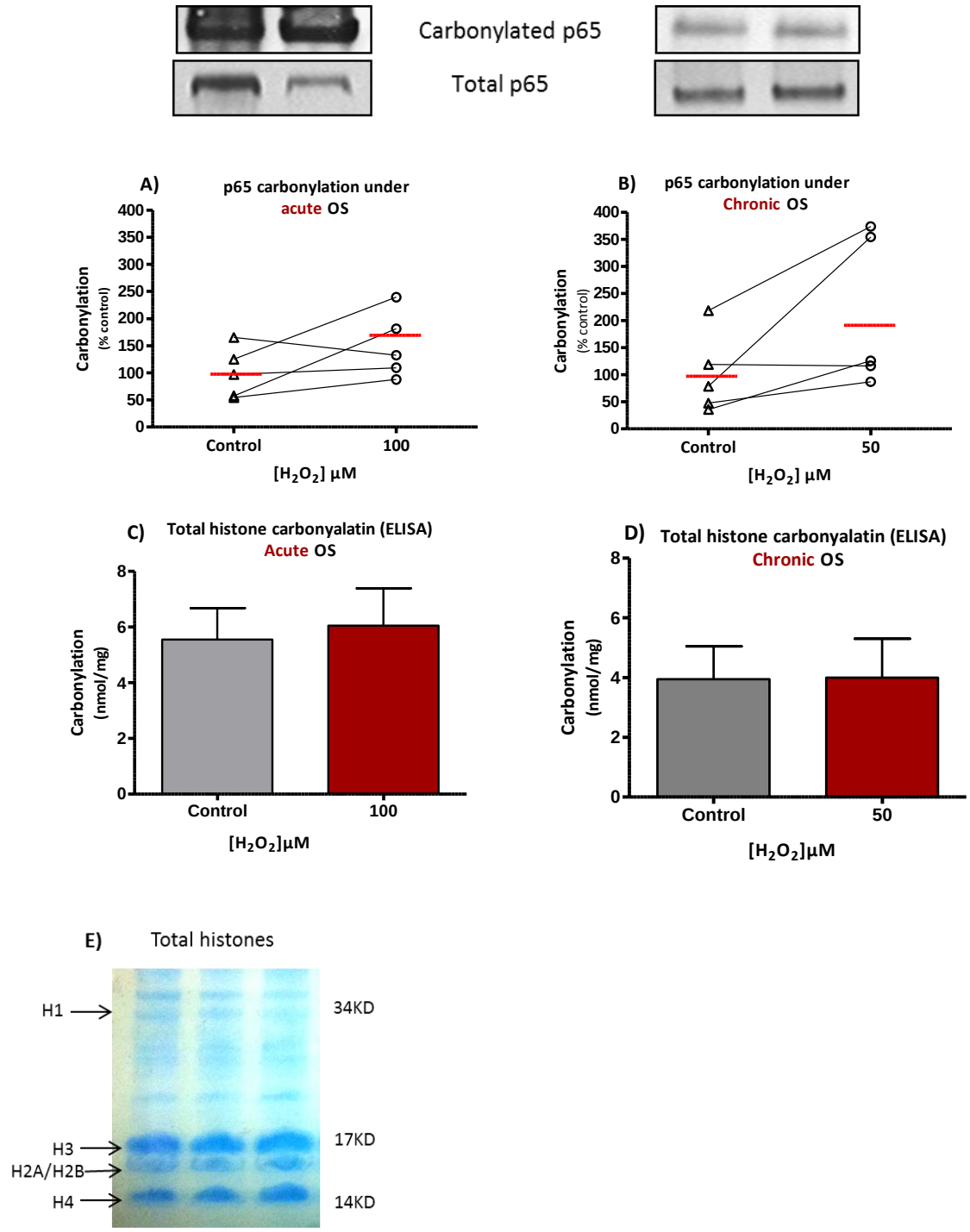


Figure 3.9: p65 and histone carbonylation under acute and chronic oxidative stress. Immunoprecipated p65 proteins were derivatised with DNPH and detected with anti-DNP antibody. The blot was striped and re-blotted with total p65 antibodies. The red line represents average carbonylation in all samples. There was no significant increase in p65 carbonylation following either **(A)** acute or **(B)** chronic H_2O_2 treatment. Histones were purified and 10µg/ml was plated on 96-well plate (100µl/well) followed by DNPH derivatisation. 1° anti-DNP followed by 2° HRP-conjugated antibody was added. Plate was developed and read at 450nm. There was no change detected in carbonylation in either acute or chronic **(C-D)**. Core histone proteins were visualised with Coomassie blue dye (E). Results are expressed as mean ± SEM (n=3).

3.3.6 NF-κB p65 carbonylation under increasing concentrations of H_2O_2

Initial experiments revealed a trend in p65 carbonylation following treatment with 100μM H_2O_2 (above section 3.5). Therefore, I examined p65 carbonylation in response to increasing concentrations of H_2O_2 (100, 150 and 200μM). It was hypothesised that if H_2O_2 does target p65 by inducing carbonylation then use of higher H_2O_2 concentrations that gave a greater increase in intracellular ROS (Fig. 3.4) should give greater p65 carbonylation. Cells were treated for 4 hours with different concentrations of H_2O_2 and p65 was immunoprecipitated and carbonylation was measured. Increasing the concentration of H_2O_2 had no effect on p65 carbonylation after 4 hours treatment (Fig. 3.10A). H_2O_2 has a short half-life and degradation varies in different cell types and models. It has been reported that H_2O_2 disappears in most cell culture medium less than an hour (Song, Driessens et al. 2007). Studies have also shown that oxidised proteins are rapidly eliminated by proteasomal degradation following treatment with H_2O_2 (Davies 2001). Therefore, p65 carbonylation was examined at 30mins and 60mins following treatment with H_2O_2. Cells were also pretreated with the proteasome inhibitor MG-132 in order to inhibit potential degradation of carbonylated p65. Again, there was no significant effect of different concentrations of H_2O_2 on p65 carbonylation after 30mins (Fig. 3.10B) and 60mins (Fig.3.10C). Pre-treatment of cells with MG-132 (10μM for 30mins) significantly increased the p65 carbonylation level at 200μM, suggesting that oxidised p65 might be undergoing proteosomal degradation (Fig. 3.10D).

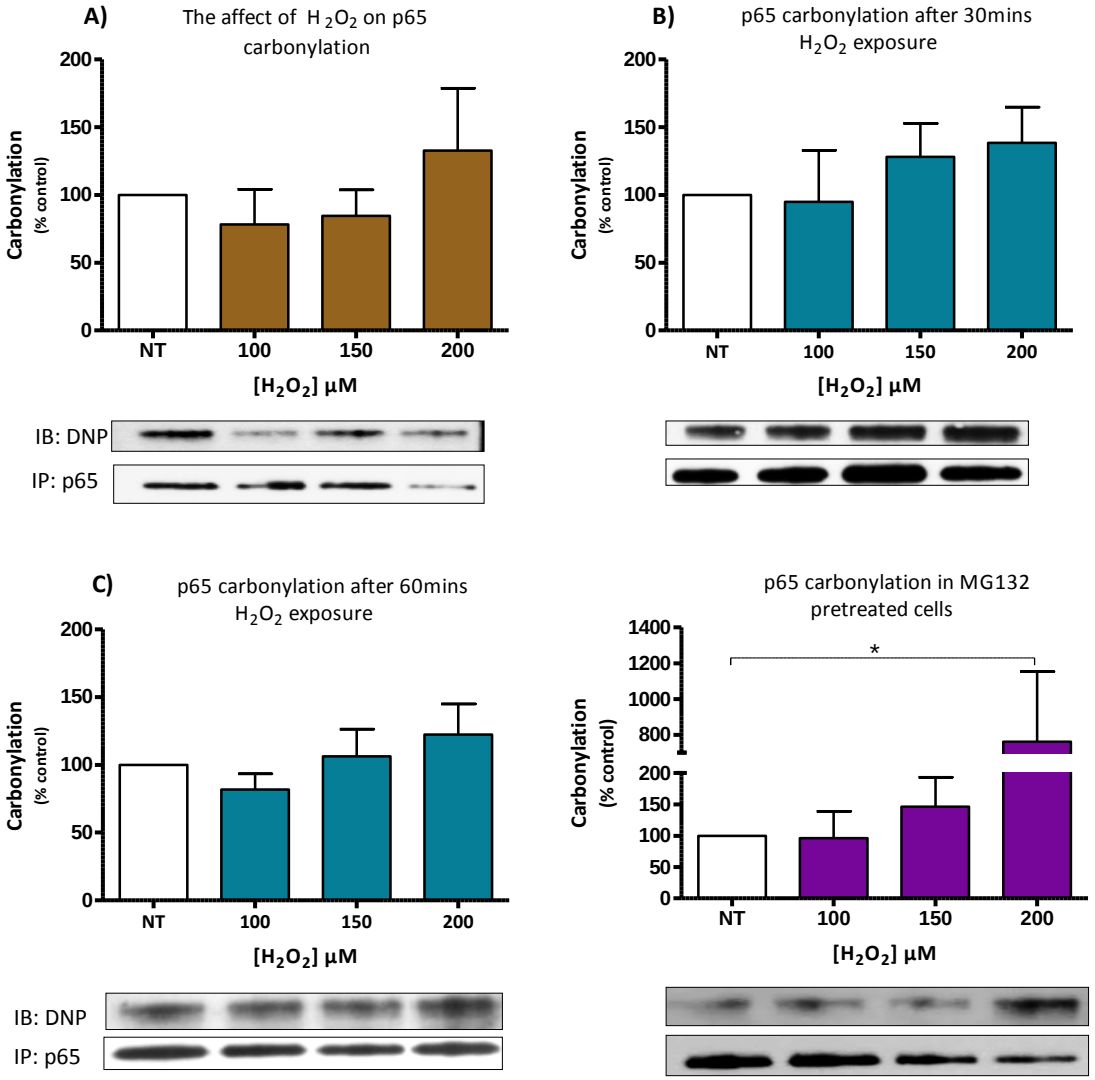


Figure 3.10: The effect of high concentrations of H_2O_2 on p65 carbonylation. (A) BEAS-2B cells were treated with H_2O_2 for 4 hours. p65 was immunoprecipitated (IP) from whole-cell extract (500µg/sample). Equal amounts of IP p65 protein was used for immunoblotting (IB). Carbonylation was detected by derivitising the samples with DNPH and immunoblotting with an anti-DNP antibody. **(B-C)** Similarly, carbonylation was detected after 30mins and 60mins of treatment with H_2O_2. **(D)** BEAS-2B cells were pre-treated with proteasome inhibitor MG-132 (10µM) for 30mins prior to 4 hour H_2O_2 treatment. Blots are representative of n=4 independent experiments. Data is presented as mean ± SEM relative to control. Control samples were set to 100%, * $P < 0.05$.

3.3.7 The effect of H_2O_2 and TNF-α on histone acetylation

Histone acetylation is associated with gene expression. Stimulating human epithelial cell line (A549) with inflammatory stimuli such as IL-1β and TNF-α results in acetylation of specific lysine residues on histone 4 (H4) which correlates with increased inflammatory proteins such as granulocyte-macrophage colony stimulating factor (GM-CSF) (Ito, Barnes et al. 2000). Acetylation of H3 and H4 lysine residues is also thought to be linked directly to gene transcription (Rahman and Adcock 2006). Liu et al have shown that histone acetylation is completely abrogated in epithelial cells following chronic treatments with cigarette smoke condensate (CSC) (Liu, Killian et al. 2010). It is not clear whether oxidative stress or other chemicals within CSC have caused this phenomenon. It is also unknown whether H_2O_2 or TNF-α alone or together has any effect on global acetylation of H3 and H4 histone in BEAS-2B cells. Therefore, in order to examine the effect of H_2O_2 and TNF-α, BEAS-2B cells were co-treated with H_2O_2 (100μM) and TNF-α for 4 hours acutely or left untreated. Cells were also treated chronically for 5, 10 and 15 days daily for 2 hours with H_2O_2 (50μM) and at last day (5^{th}, 10^{th} and 15^{th} day) of each chronic treatment cells were stimulated with TNF-α in combination with H_2O_2. Following treatment, histones were isolated immunoblotted for H3 and H4 pan-acetylation as well as total H3 and H4. There was no effect of H_2O_2 alone or together with TNF-α in acutely or chronically treated cells suggested that local changes associated with specific promoters e.g. *IL-6* and *IL-8* rather than global changes are functionally important (Figure 3.11). Alternatively, acetylation of specific lysine residues linked to gene activation may have been more informative (Bannister and Miska 2000; Fukuda, Sano et al. 2006).

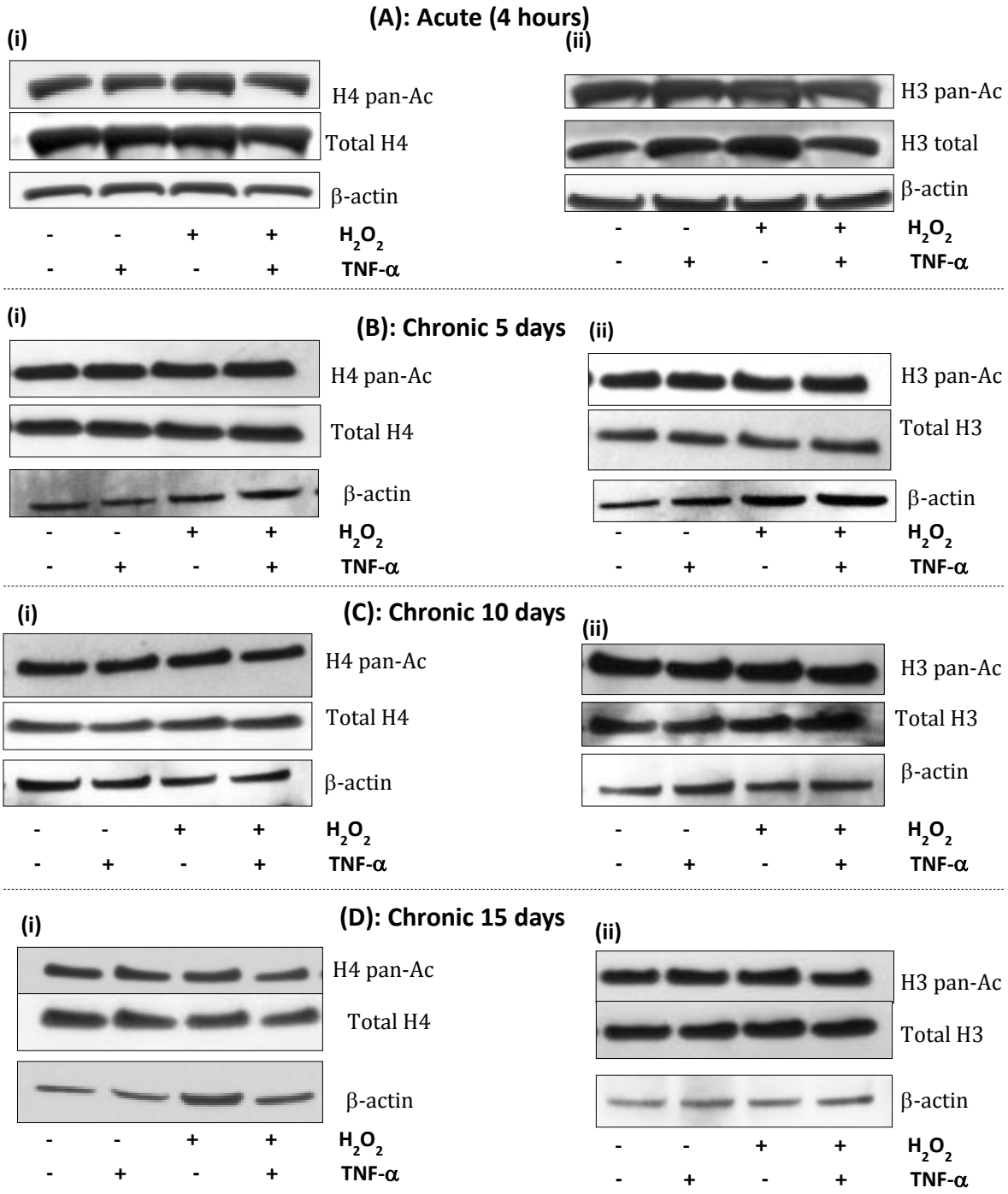


Figure 3.11: The effect of acute and chronic treatments of H_2O_2 on histone acetylation. (A) BEAS-2B cells were treated with H_2O_2 (100μm) for 4 hours together with TNF-α (1ng/ml) or left untreated. Histones (H) were isolated and immnoblotted for global acetylation (Ac) of H3 and H4 preceded by stripping and re-blotting for total H4 and H3. **(B-D)** similarly, the effect of chronic treatment (5, 10, and 15 days) of H_2O_2 was examined in BEAS-2B cells and no noticeable changes were observed in acetylation profile. Western blots are representative of at least 3 independent experiments.

3.3.8 H_2O_2 enhanced *IL-6* and *IL-8* expression in response to IL-1β

To determine the universality of the enhancing effect of H_2O_2 on TNF-α-induced CXCL8 and IL-6 expression in the acute model, it was decided to examin whether IL-1β could replace TNF-α in this model (Fig. 3.7B). The H_2O_2 pre-exposure time was also reduced from 4 hours to 2 hours as H_2O_2 disappears from most systems within an hour (Song, Driessens et al. 2007). H_2O_2 alone had little or no effect on IL-6 or CXCL8 protein production, however, it significantly enhanced the production of IL-6 and CXCL8 release from co-treated with IL-1β (Fig. 3.12A and 3.12B). This effect was parellelled by changes in *IL-6* and *IL-8* mRNA transcripts (Fig. 3.12C and 3.12D). Based on these findings, in all subsequent experiments, H_2O_2 (100µM) and IL-1β (1ng/ml) was used to study molecular mechanism.

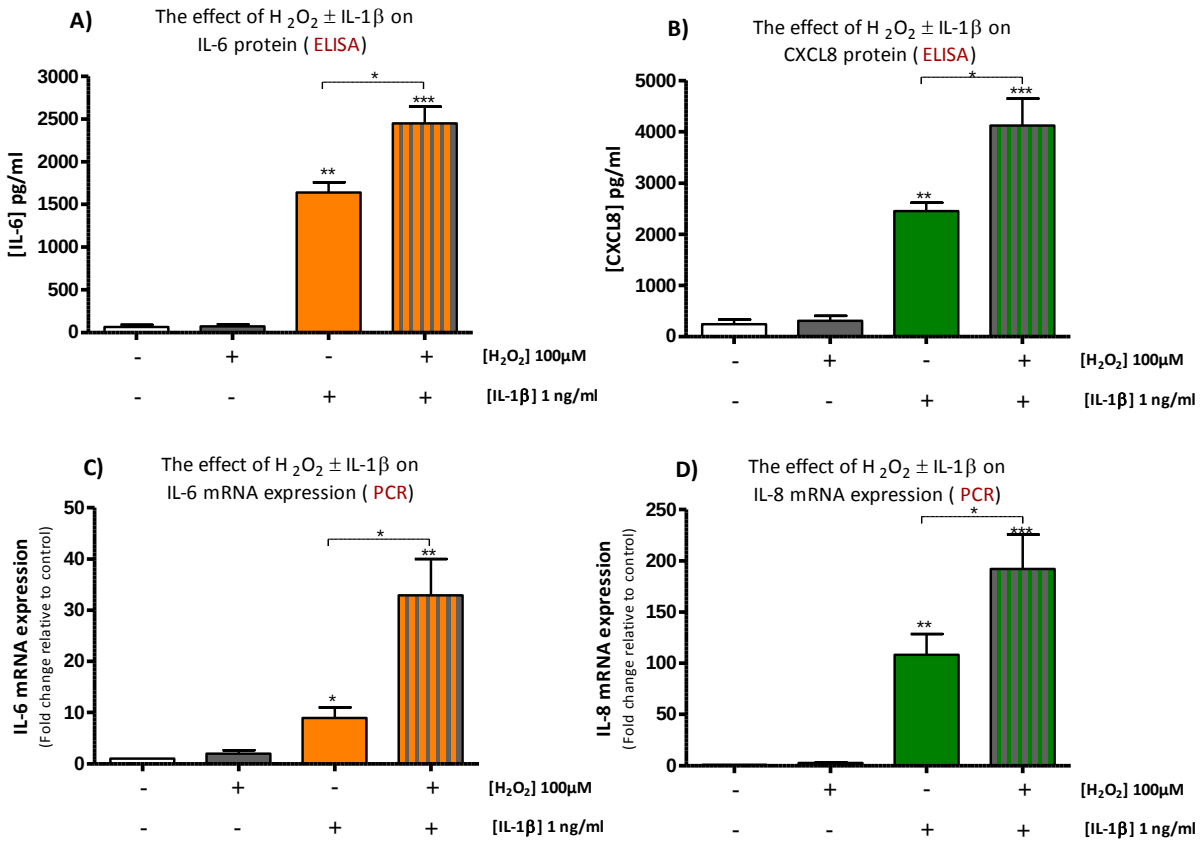


Figure 3.12: H_2O_2 enhanced induction of CXCL8 and IL-6 in response to IL-1β. (A-B) BEAS-2B cells were treated with H_2O_2 for 2 hours in the absence or presence of IL-β stimulation (overnight) or left untreated as a control. CXCL8 and IL-6 protein levels in cell culture supernatants were quantified by ELISA. **(C-D)** *IL-8* and *IL-6* transcript levels were quantified by comparative real-time PCR and were normalised by measuring *GNB2L1* transcript levels. Results are expressed as mean ± sem of at least 4 independent experiments. * P < 0.05, ** P < 0.01, ***P < 0.001 versus controls.

3.3.9 The effect of iron on cell viability, intracellular ROS and inflammation

Free iron is reported to be elevated in COPD patients in compared with smokers and healthy non-smokers (Ghio, Hilborn et al. 2008). Iron produces highly reactive hydroxyl radicals ($^{\cdot}OH$) and superoxide ($^{\cdot}O_2^-$) by reacting with H_2O_2. Free radicals contribute further to oxidative stress as well as instigate lipid peroxidation in cells (Barnes 2004; Fischer, Pavlisko et al. 2011).Thereby, ammonium iron sulphate [$(NH_4)_2Fe\ (SO_4)_2$] was used to investigate the effect of iron on cell viability, intracellular ROS and pro-inflammatory gene expression. Firstly, BEAS-2B cells were exposed to different concentrations of $(NH_4)_2Fe(SO_4)_2$ for 2 hours and MTT assays were performed. There was no reduction in cell viability following any treatment (3.13A). Secondly, the impact of $(NH_4)_2Fe\ (SO_4)_2$ on intracellular was studied by incubated cells with DCFH-DA florescent probe for half an hour followed by treatment with different concentrations of $(NH_4)_2Fe(SO_4)_2$ (0-1600µM). Intracellular ROS was elevated at low concentrations (0-200µM) but reduced at higher concentrations (400-1600µM) of $(NH_4)_2Fe(SO_4)_2$ (Fig. 3.13B). Intracellular ROS was significantly increased at 100µM and 200µM relative to untreated cells and in subsequent experiments 100µM of $(NH_4)_2Fe(SO_4)_2$ was used.

To investigate whether $(NH_4)_2Fe(SO_4)_2$ could further potentiate the effect of H_2O_2 and IL-1β on IL-6 and CXCL8 production, cells were treated with $(NH_4)_2Fe(SO_4)_2$ (100µM) for 2 hours followed by treatment with H_2O_2 and IL-1β either alone or in combination. Cell culture supernatants were analysed for CXCL8 and IL-6 levels by ELISA. The presence or absence of $(NH_4)_2Fe(SO_4)_2$ did not modulate the induction of CXCL8 or IL-6 production by either H_2O_2 or IL-1β alone or when used together (Figure 3.13C and 3.13D).

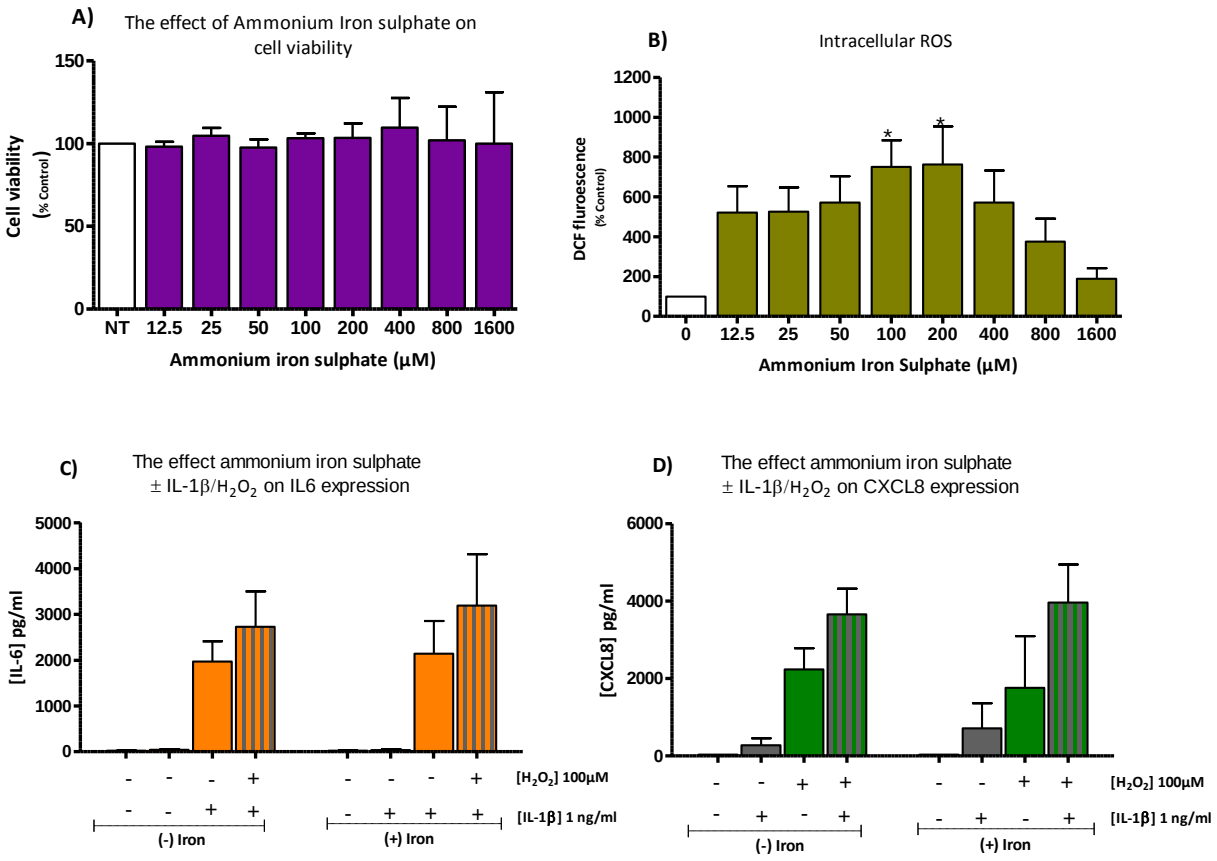


Figure 3.13: The effect of iron on the expression of CXCL8 and IL-6. (A) BEAS-2B cells were treated with a range of ammonium iron sulphate $(NH_4)_2Fe(SO_4)_2$ concentration (0-1600µM) and cell viability was assessed using an MTT assay. **(B)** Intracellular ROS was quantified using DCF-H assay following treatment with different concentration of $(NH_4)_2Fe\ (SO_4)_2$. ROS peaked at 100-200µM. **(C-D)** Cells were pre-incubated for 2 hours with or without $(NH_4)_2Fe(SO_4)_2$ prior to washing with PBS and treatment with H_2O_2 or IL-1β alone or in combination. Results are expressed as mean ± SEM of 3 independent experiments. *p<0.05 vs control.

3.4. Discussion

COPD is caused by long-term exposure to cigarette smoke (CS) and about 90% of COPD patients are either smokers or ex-smokers (Young, Hopkins et al. 2006; Barnes 2007). Cigarette smoke is a complex mixture of over 5000 chemicals, including ROS (10^{17} per puff) (Church and Pryor 1985; Pryor and Stone 1993). It is involved in the activation and recruitment of inflammatory cells, producing not only inflammatory mediators but also generating oxidants (Kirkham, Spooner et al. 2003; Kirkham, Spooner et al. 2004). Elevated oxidants, derived from cells and CS, result in oxidative stress which is frequently found in the lung of COPD patients (Chung and Marwick ; Dekhuijzen, Aben et al. 1996; Mortaz, Rad et al. 2008). Oxidative stress and inflammation are inseparably intertwined processes. Chronic inflammation is associated with elevated ROS and diminished or compromised antioxidant systems (Terlecky, Terlecky et al. 2012). There is also a considerable evidence of oxidative stress entailed in the pathology of many disorders, including aging, cancer, neurodegenerative and cardiovascular diseases (Dalle-Donne, Aldini et al. 2006; Kurien and Scofield 2008). Therefore, it's vitally imperative to understand the effect of oxidative stress in a cell-base system and its subsequent effect on modulating inflammatory pathways before extending it to more complex systems.

Thus, numerous cell-based systems of oxidative stress have been devised (Yatin, Varadarajan et al. 1999; Aksenova, Aksenov et al. 2005; Wijeratne, Cuppett et al. 2005) to study the impact of oxidative stress on cell-signalling, mitochondrial function, cell differentiation and apoptosis (Kirkham, Spooner et al. 2004; Merry 2004; Rahman and Adcock 2006). However, all these models fail to address the accumulative and chronic effect of oxidants resulting in the disease state. Despite a number of oxidative stress models (*in vivo* and *in vitro*) (Ito, Hanazawa et al. 2004; Marwick, Kirkham et al. 2004; Ito 2007; Churg, Cosio et al. 2008; Wright, Cosio et al. 2008), acute ROS exposure remains the basis of most experimental models. Therefore, our initial aim was to establish both acute and chronic *in vitro* oxidative stress models using H_2O_2 (oxidant) and epithelial cells. H_2O_2 is one of many ROS reported in the breath condensates of COPD patients (Nowak, Kasielski et al.

1998; Montuschi 2005). Epithelial cells constitute the inner surface of the airways lumen and are part of the first line of defence against xenobiotics and irritants such as CS (Kirkham, Spooner et al. 2004; Galli, Tsai et al. 2008). We have used H_2O_2 in our models with the knowledge that it does not reflect the complex mixture of stimulants seen in tobacco smoke (Young, Hopkins et al. 2006; Barnes 2007) but it was hypothesised that the major effect of CS are due to ROS. This made the model simpler and allowed us to focus on the ROS.

In our preliminary work, we determined the optimal cell density of human bronchial epithelial (BEAS-2B) cells and correct concentrations of H_2O_2 for acute and chronic treatments. It showed that BEAS-2B cells have the capacity to survive in minimal media (1% growth factor) and can also withstand the continuous insult of low concentration of H_2O_2 (50µM) for 15 days. However, increasing concentrations (>100µM) of H_2O_2 resulted in cell death. Both models of oxidative stress were validated by confirming the release of inflammatory mediators CXCL8 and IL-6. Initially, I used 50µM of H_2O_2 for acute exposure but did not observe any significant change in either IL-6 or CXCL8 release nor did this concentration modify the expression of TNFα-induced IL-6 or CXCL8. These findings may be attributable to the induction of antioxidants genes under low oxidative stress (Gloire, Legrand-Poels et al. 2006). Subsequently, H_2O_2 concentration was increased to 100µM for acute exposure and the 50µM concentration was maintained for the chronic exposures. Interestingly, 50µM of H_2O_2 in chronic system did not have any effect alone but together with TNF-α amplified the production of CXCL8 and IL-6 protein, suggesting that chronic low exposure alters the cell sensitivity to oxidative stress and subsequent inflammatory stress resulting in a different pattern of inflammatory/immune gene expression.

However, when the chronic model was extended to 10 and 15 days exposure to ROS this effect was diminished. This may be explained by the resistance developed by the cells over continuous exposure to H_2O_2. Bose and colleagues have shown that lung fibroblasts (Chinese hamster V79 cells) exhibit stable resistance to high doses of H_2O_2 (200-1000µM) after challenged with low doses of

H_2O_2 (30μM) for 7 days. This enhanced survival was due to increased glutathione content (GSH) and enzymatic activities of antioxidants catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Bose Girigoswami, Bhaumik et al. 2005). Similarly, yeast exposed to low concentrations of H_2O_2 subsequently become more resistant to high concentrations of H_2O_2 (Collinson and Dawes 1992). Furthermore, cells can acquire resistance to the ROS-inducing drug (2-methoxyestradiol: a metabolite of estrogen) when exposed to the same drug over 3 months of time by enhancing the expression of antioxidant enzyme SOD2 (Zhou and Du 2012). H_2O_2 also has a short-life and degrades less than within an hour in all type of cell-culture media (Song, Driessens et al. 2007). Therefore, using H_2O_2 may not be an ideal for chronic treatment though it has been used as a chronic treatment in other studies (Bose, Bhaumik et al. 2003). Alternatively, continuously generated H_2O_2 agents such as 6-formylpterin, menadione, KO_2 or glucose oxidase could be used to supply constant but moderate levels of H_2O_2 (For future work see the end of thesis) (Arai, Endo et al. 2001; Loor, Kondapalli et al. 2010; Ho, Asagiri et al. 2011; Choe, Yu et al. 2012).

The acute model was extended with slight modifications by using 100μM H_2O_2 and IL-1β instead of TNF-α. Interestingly, it was observed by ELISA and PCR analysis that the application of H_2O_2 had little or no effect on IL-6 or CXCL8 protein production. As with the TNF-α model, H_2O_2 significantly enhanced the production of IL-6 and CXCL8 protein in cells that were co-treated with IL-1β confirming that ROS plays a vital role in the induction of inflammatory mediators. We also used ammonium iron sulphate [$(NH_4)_2Fe(SO_4)_2$] to enhance intracellular ROS and mimic the effect of free iron, reported to be elevated in COPD (Barnes 2004; Ito, Hanazawa et al. 2004). Iron can decompose H_2O_2 through the Fenton reaction leading to the formation of superoxide and hydroxyl radicals which are capable of causing biological damage (Jomova and Valko 2011). However, despite $(NH_4)_2Fe(SO_4)_2$ enhancing intracellular ROS at 200 μM, I did not observe any effect of $(NH_4)_2Fe(SO_4)_2$ alone or together with H_2O_2 on IL-6 or CXCL8 production.

Oxidative stress (H_2O_2) mediated induction of inflammation (IL-6 and CXCL8) by IL-1β or TNF-α is reported to be due to activation of NF-κB pathway (Awane, Andres et al. 1999; Zhang, Johnston et al. 2001; Li and Engelhardt 2006). The NF-κB pathway is one of the important inflammatory pathways involved in chronic inflammatory diseases including COPD. NF-κB p65 transcription factor is activated by oxidative stress and its expression is increased in smokers and COPD patients (Di Stefano, Caramori et al. 2002). The NF-κB is a master regulator which is not only involved in the induction of several inflammatory genes but also immunity, apoptosis and cell proliferation (Ito 2007). It consists of a homo or hetrodimer of five proteins (p50, p52, p65/RelB, p100 and RelB), sequestered by an inhibitory protein called IκBα. Upon cell stimulation, IκBα protein is phosphorylated by IKK complex (IKKα, IKKβ and NEMO) and degraded by proteasome. This enables the translocation of NF-κB into the nucleus and transcription of targeted genes such as *IL-6* and *IL-8*. Both TNF-α and IL-1β activates NF-κB through phosphorylation (serine 32 and 36) and degradation of IκBα (Awane, Andres et al. 1999; Barnes 2004; Gloire, Legrand-Poels et al. 2006; Edwards, Bartlett et al. 2009). However, research has shown that NF-κB activation by H_2O_2 is highly cell-type specific. For example in human myeloid KBM-5 cells, H_2O_2 triggers phosphorylation of tyrosine 42 on IκBα via Syk tyrosine kinase (Takada, Mukhopadhyay et al. 2003). Whereas in Hela cells, H_2O_2 triggers IKK complex activation through protein kinase D (PKD) mediated activation (Storz, Doppler et al. 2004).

However, in our models, H_2O_2 by itself had no effect on the expression of *IL-6* or *IL-8* genes. Its effect was only noticeable in combination with IL-1β or TNF-α. One possible explanation accountable for this difference is that H_2O_2 could "prime" p65 to respond greatly to IL-1β or TNF-α signals, involve post-translational modifications such as phosphorylation or acetylation. It has been shown that H_2O_2 can induce p65 phosphorylation by phosphorylating tyrosine residue on IκB-α kinase. However, Syk protein is essential for H_2O_2 mediated activation of IκB-α kinase (Takada, Mukhopadhyay et al. 2003). Similarly, acetylation of p65 is associated with NF-κB dependant gene expression (Rothgiesser, Fey et al. 2010). Alternatively, H_2O_2 may reduce the HDAC2 (Histone deacetylation 2)

activity, leading to enhanced histone acetylation and increased NF-κB-mediated gene transcription (Ito, Hanazawa et al. 2004).

In addition, histones are critical in epigenetic regulation of inflammatory genes through post-translational modifications ("histone code"), such as phosphorylation, acetylation and methylation. All of which have been extensively recorded in the literature (Margueron and Reinberg ; Ito 2007; Kouzarides 2007). However, little is known about histone modification by carbonylation/oxidation. Therefore, it was investigated whether H_2O_2 can induce either histone or p65 carbonylation and subsequently affect gene expression. There is accumulating evidence associating oxidative stress with the induction of protein carbonylation (England and Cotter 2004; Dalle-Donne, Aldini et al. 2006; Giustarini, Dalle-Donne et al. 2009) either by direct introduction of carbonyl groups (R-C=O-R`) into the side chain of amino acids (Pro, Arg, Lys and Thro) or by addition of reactive aldehydes (MDA, 4HNE) released from lipid peroxidation process (Wong, Marcocci et al. 2010). Marrin-Coral *et al.* have reported carbonylation of actin and myosin proteins from diaphragm of COPD patients (Marin-Corral, Minguella et al. 2009). Our group has also shown cigarette smoke induced carbonyl modification of HDAC2 (Marwick, Kirkham et al. 2004). Similarly, carbonyls are implicated in the pathogenesis of a number diseases including systemic lupus erythematosus (SLE), Type I diabetes mellitus, Bechet`s disease, atherosclerosis and rheumatoid arthritis (Kurien and Scofield 2008).

Our initial data indicated a trend towards carbonyl modification of p65 which was more prevalent in the acutely (100μM for 4 hours) treated cell. However, after further study no effect of H_2O_2 was seen on histone or p65 carbonylation between treated and untreated cells. To clarify the role of H_2O_2 in p65 carbonlylation, we used higher concentrations of H_2O_2 (100, 150 and 200μM) and noticed a small but insignificant increase in p65 carbonylation with increasing concentrations. Studies have shown that H_2O_2 is unstable and disappears from the medium quickly once added (Song, Driessens et al. 2007). It has been reported that various forms of both short and long lived intracellular proteins are degraded followed oxidation (Grune, Reinheckel et al. 1996; Davies 2001). For example,

Osoata *et al.* have shown that nitration of HDAC2 is flagged for proteosomal degradation once nitrated (Osoata, Yamamura et al. 2009). In line with this notion, p65 carbonylation was examined at earlier time points (30mins and 60mins). We also pre-treated cells with MG-132 proteasome inhibitor to block degradation of potential carbonylated p65. Under these conditions, we observed a 4-fold-increased in p65 carbonylation followed by challenge with 200μM of H_2O_2. These results suggest that carbonylation occurs when cells are challenged with high concentrations of H_2O_2 which is quickly degraded by proteasome 20s as it might be detrimental for cells function. Indeed, H_2O_2 induced oxidation of RNase A is selectively recognised and degraded by the proteasome 20s (Lasch, Petras et al. 2001; Pickering, Koop et al. 2010).

In contrast, histones are considered to be highly conserved proteins and may not be subjected to ad hoc enzymatic or proteosomal degradation (Sandman and Reeve 2000). Recently, it has been shown that ROS induce histone carbonylation during cell proliferation and decreases with activated proteasome (Garcia-Gimenez, Ledesma et al. 2012). This also suggests that histone carbonylation may be an important post-translation modification that represents a specific event during cell cycle. Furthermore, the rapid replacement of histones H3 can occur during transcriptional activation and the fate of the replaced histone are unclear (Schwartz and Ahmad 2005; Workman 2006). Besides, histone oxidation (carbonylation) may be time-dependant and thus require longer exposure to oxidative stress i.e. greater than 5 days. In fact, in many age related diseases such as Alzheimer's, Parkinson's and cancer, carbonyl-modified protein aggregates have been reported (Davies 2001; Rahman, van Schadewijk et al. 2002; Dalle-Donne, Rossi et al. 2003; Dalle-Donne, Aldini et al. 2006; Rahman and Adcock 2006). Liu and colleagues have observed epigenetic changes after 5 months cigarette smoke condensate (CSC) exposure. They have shown that H4K16Ac and H4K20Me3 are completely abrogated in human small airway epithelial cells (SAEC) after 5 month exposure to cigarette CSC in comparison with 72 hour (Gloire, Legrand-Poels et al. 2006; Liu, Killian et al. 2010). Similarly, it has been shown that DNA oxidation increases significantly in mice fibroblast exposed to CS for 6 months compare to acute exposure (3 days and 1 month) (Deslee, Adair-Kirk et al. 2010).

Of concern in these studies is a recent report by Wong and Powell who have shown that Oxyblot™ kit (Chemicon/Millipore) does not yield reproducible results due to loss of sensitivity (Wang and Powell. 2010). There is no standard assay to detect carbonylation, which is reflected in the wide-variations in basal levels of protein carbonylation in different tissues (Dalle-Donne, Rossi et al. 2003). Our results also show variations in carbonylation from sample to sample. One possible explanation for this is that nucleic acids also contain carbonyl groups (C=O) will react with DNPH. Any contamination of samples during protein extraction could cause an over estimate of protein-bound carbonyls (Levine, Garland et al. 1990). Alternatively solution may be the deployment of different techniques for different proteins under investigation. For example, spectrophotometer DNPH assay is recommended for large purified proteins. Slot blot, immunoassay, is also recommended for small samples and cell extracts (Dalle-Donne, Rossi et al. 2003).

In addition to histone carbonylation, we also looked at global acetylation of H3 and H4. Acetylation of H3 and H4 lysine residues are associated with gene expression (Rahman and Adcock 2006). However, little has been reported on the impact of acute or chronic H_2O_2 treatment in epithelial cells or on global histone acetylation. There was no change in global histone acetylation in either acute (4 hours) or chronic (5, 10 and 15 days) H_2O_2 treated cells alone or in combination with TNF-α. However, Ito and colleagues have shown that H4 acetylation occurs at specific gene loci. They have shown that treating epithelial cells with H_2O_2 and IL-1β induced H4 acetylation at the NF-κB response element within the *IL-8* promoter (Ito, Hanazawa et al. 2004). Therefore, it is possible that H3 and H4 are acetylated at specific promoter regions. In addition, Western blot analysis is a very crude way of assessing acetylation and more sensitive methods such as immunofluorescence labelling may be a better indicator of histone acetylation.

In summary, epithelial cells (BEAS-2B) were used to establish both acute and chronic oxidative stress models using H_2O_2. It was found that the chronic effect of exposure to H_2O_2 was lost over 10 and 15 days of consecutive treatment with low concentration of H_2O_2. This can be explained by the cells

acquiring resistance to oxidative stress following repeated treatment with H_2O_2 through induction of antioxidant gene expression. Therefore, it was decided to focus and use acute oxidative stress model in our subsequent investigation. We have also shown that p65 can be oxidised/carbonylated by H_2O_2 but its effect is only noticeable at high concentrations following treatment with MG-132 (proteasome inhibitor). We did not observe any change in pan-acetylation of either H3 or H4 or carbonylation of histones. Acetylation has been shown to be more gene specific than global. We have also explored the role of ammonium iron sulphate on *IL-6* and *IL-8* genes in our acute model but did not report any significant effect. In the next chapter, we will address some of the key questions that have risen in this chapter including:

1. Whether NF-κB pathway is activated in our oxidative stress model which leads to the induction of *IL-6* and *IL-8* genes?
2. Is there post-translation modification of p65 such as phosphorylation and acetylation, which are important for NF-κB dependant gene activation?
3. If there is no difference in global acetylation of H3 and H4 following simulation is it gene specific, for example occurs at *IL-6* and *IL-8* promoter sites?
4. Do transcriptional co-regulatory proteins such Brd4 play a role in gene activation?

Chapter 4

The role of NF-κB p65 and Brd4 protein in oxidative stress-induced inflammation

4.1 Rationale

NF-κB is a master regulator of many response genes including immune and inflammatory mediators. NF-κB-mediated inflammation in COPD is believed to drive the pathogenesis of disease resulting in remodelling of small airways and destruction of lung parenchyma (Barnes 2008). This is a feed forward process since inflammatory stimuli induce NF-κB activation, leading to the expression of pro-inflammatory cytokines (IL-6), adhesion molecules and chemokines (CXCL8/IL-8) (Watters, Kenny et al. 2007). NF-κB is usually found as an inactive heterodimer (p65/RelA and p50 subunits) in the cytosol of cells and concealed by the inhibitory IκBα protein. The binding of an inflammatory stimulus such as TNF-α, IL-1β or LPS to its respective receptor activates the IκB kinase 2 (IKK2) complex. The majority of diverse inflammatory signalling pathways that leads to the activation of NF-κB converge on the IKK complex which phosphorylates IκB. This is known as the classical or canonical pathway (Fig 4.8). Oxidative stress (H_2O_2) has also been shown to phosphorylate IκBα directly (Morgan and Liu 2011). Phosphorylation of IκBα (at Ser32 and Ser36) results in it being targeted for ubiquination and subsequent degradation by the proteasome (Edwards, Bartlett et al. 2009). The degradation of IκBα allows the translocation of the NF-κB p65/p50 dimer into the nucleus where it binds to the promoter regions of inflammatory genes (*IL-6*, *IL-8* etc.) and recruits coactivator molecules such as p300/CBP, pCAF and ATF-2.

Coactivator molecules have intrinsic histone acetyltransferase (HAT) activity and subsequently acetylate core histones (Histones H3 and H4) (Barnes and Adcock 2009). This results in disruption of the electro-static attraction between histones and DNA. In addition, these epigenetic markers (acetylated histones) are recognised by transcriptional co-regulatory proteins such as Brd4 and SW1/SNF DNA remodelling factors. The local unwinding of DNA, which results from the actions of the protein complexes, confers access to transcriptional machinery with p-TEFb (positive transcription elongation factor b) and RNA polymerase II at the transcription starting site (Fig. 4.1) (Rahman and Adcock 2006; Huang, Yang et al. 2009).

Additional NF-κB regulatory activity is achieved through post-translation modifications (PTMs) of the core component of NF-κB. Acetylation (Lysine 310) and phosphorylation (serine 276 and 536) of NF-κB are important for its transcriptional activation (Chen, Mu et al. 2002; Sasaki, Barberi et al. 2005; Nowak, Tian et al. 2008; Huang, Yang et al. 2009). In addition, oxidative stress is shown to affect NF-κB activity via several mechanisms including p65 PTMs (Rothgiesser, Fey et al. 2010). Acetylated NF-κB recruits bromodomain and extra-terminal (BET) protein Brd4 (discussed in details in next chapter) which also interacts with acetylated histones H3 and H4 by forming a complex with RNA Pol II and pTEFb at the site of transcription (Chiang 2009). Recently, Zhang and colleagues have confirmed that Brd4 inhibition results in down-regulation of a subset of NF-κB targeted genes in Th2 cells suggesting its role in gene transcription and regulation (Zhang, Liu et al. 2012).

Therefore, we have investigated NF-κB activation, phosphorylation and acetylation in our acute oxidative stress model (H_2O_2). In the previous chapter, It has been shown that IL-1β in combination with H_2O_2 enhanced the expression of *IL-6* and *IL-8* genes (Chapter 3, Figure 3.10), thereby, we sought to determine whether these genes are under transcriptional control of NF-κB. We also have delineated the role of Brd4 in regulation of *IL-6* and *IL-8* transcription which has never been addressed before. Finally, we have looked at H3 and H4 acetylation at the *IL-6* and *IL-8* promoter κB sites to confirm that gene specific induction is dependent on local alteration of histone acetylation profile.

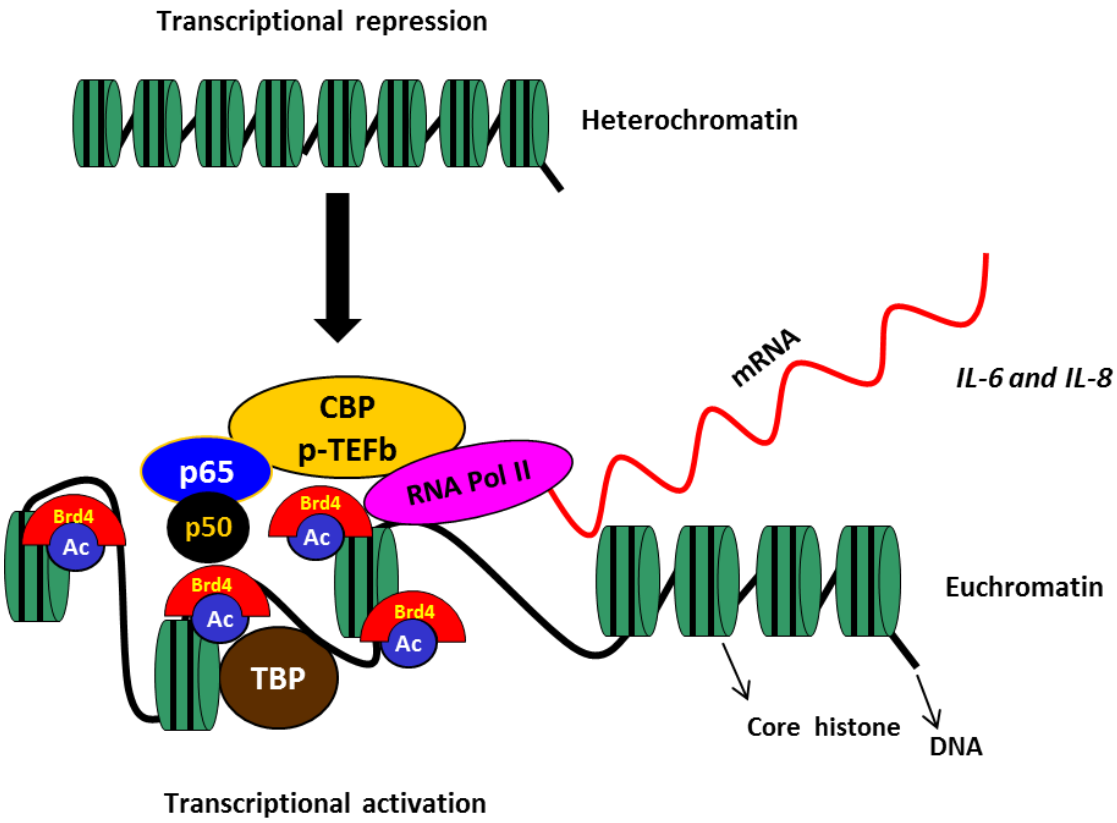


Figure 4.1: Histone acetylation and remodelling of chromatin during transcription. The depiction illustrates that following inflammatory stimuli, heterochromatin is acetylated (Ac) and becomes less condensed, making it accessible to transcription factor NF-κB (p65/p50) and transcription machinery (CBP/p-TEFP and RNA Pol II). Acetylated histones are also recognised by co-regulatory protein Brd4, leading to the transcription of pro-inflammatory genes such as *IL-6* and *IL-8*. Ac: Acetyl group; NF: Nuclear factor; CBP: CREB binding protein; TBP: TATA binding protein; p-TEFb: positive transcription elongation factor b.

4.2 Experimental protocol

4.2.1 Effect of the IKK2 inbibitor (AS602868) on CXCL8 and IL-6 release (ELISA assay)

BEAS-2B cells were pre-treated with AS602868 (5μM, IKK2 inhibitor) for 1 hours, followed by treatment with IL-1β (1ng/ml) and H_2O_2 (100μM) for 16 hours. Supernatants were collected and assayed for CXCL8 and IL-6 using commercially available enzyme-linked immunosorbent assay (ELISA), following manufacturer`s instructions (Human IL-8 and IL-6 Duoset, R&D Systems, Abingdon, UK).

4.2.2 NF-κB p65 DNA binding activity (TransAM assay)

Cells were stimulated with IL-1β (1ng/ml) with or without H_2O_2 (100μM) left untreated for 2 hours. Nuclear proteins were extracted and NF-κB binding activity was determined by TransAM NF-κB p65 activity assay kit (Active motif, Belgium) according to manufacturer `s instructions.

4.2.3 NF-κB p65 phosphorylation, acetylation and translocation into nucleus (Western blot)

Nuclear extracts were fractionated by SDS-PAGE. Proteins were transferred and immobalised on nitrocellulose membranes. Membranes were incubated with specific antibodies directed against NF-κB p65, Acetylated p65 or phosphorylated p65 (All Santa Cruz Biotechnology). Rabbit HRP-conjugated 2° antibody was added and immunoreactive bands were detected with chemiluminescence detection kit.

4.2.4 The association between Brd4 protein and NF-κB p65 (Co-IP assay)

Cells were incubated with medium alone or H_2O_2 (100μM), with or without IL-1β (1ng/ml) for 2 hours. Whole cell lysates were immunoprecipitated with Brd4 specific antibody (section 2.15.3). The associated NF-κB p65 was determined with Western blotting using p65 specific antibodies.

4.2.5 The binding of Brd4 and p65 to *IL-6* and *IL-8* promoter regions (ChIP assay)

BEAS-2B cells were treated with H_2O_2 (100μM) in the presence or absence of IL-1β (1ng/ml) or left untreated for 2 hours. After incubation, protein-DNA complex were fixed by formaldehyde (1% final concentration) and immunoprecipitated with p65 and Brd4 antibody (section 2.15.3). NF-κB p65 and Brd4 binding to the *IL-8* (-121 to +61) and the *IL-6* promoter (-852 to -563) were quantified by real-time QPCR using a QantiTech SYBR green PCR kit (Qiagen) on Rotor gene 3000 (Corbett Research, NSW, Australia).

4.2.6 Acetylation of H3 and H4 at IL-*6* and *IL-8* promoter regions (ChIP assay)

ChIP was performed as previously described. Anti-acetylated H3 and H4 antibodies were used to determine histone acetylation status at the *IL-6* and *IL-8* promoters and amounts were quantified by RT-QPCR.

4.3 Results

4.3.1 *IL-6* and *IL-8* expression is mediated via NF-κB signalling pathway

To determine whether NF-κB mediated the expression of *IL-8* and *IL-6*, an IKK2 selective inhibitor AS602868 (5μM) was added for an hour prior to treatment with H_2O_2 (100μM) and IL-1β (1ng/ml) for 20 hours. IL-6 and CXCL8 proteins were assayed in cell supernatant using ELISA and mRNA transcripts were quantified by real-time RT-QPCR. Co-stimulation with H_2O_2 and IL-1β induced the release of both IL-6 and CXCL8 proteins which were completely suppressed by AS602868 (Fig. 4.2A and 4.2B). Consistent with ELISA data, pre-treatment with AS602868 completely prevented the *IL-6* and *IL-8* mRNA induction by IL-1β and H_2O_2, confirming transcriptional regulation (Fig. 4.1C and 4.1D). The MTT assay showed that cell viability was not compromised at 5μM concentration of AS602868 (Fig. 4.1E). This data confirmed that expression of *IL-6* and *IL-8* genes are mediated by the NF-κB signalling pathway in our oxidative stress model.

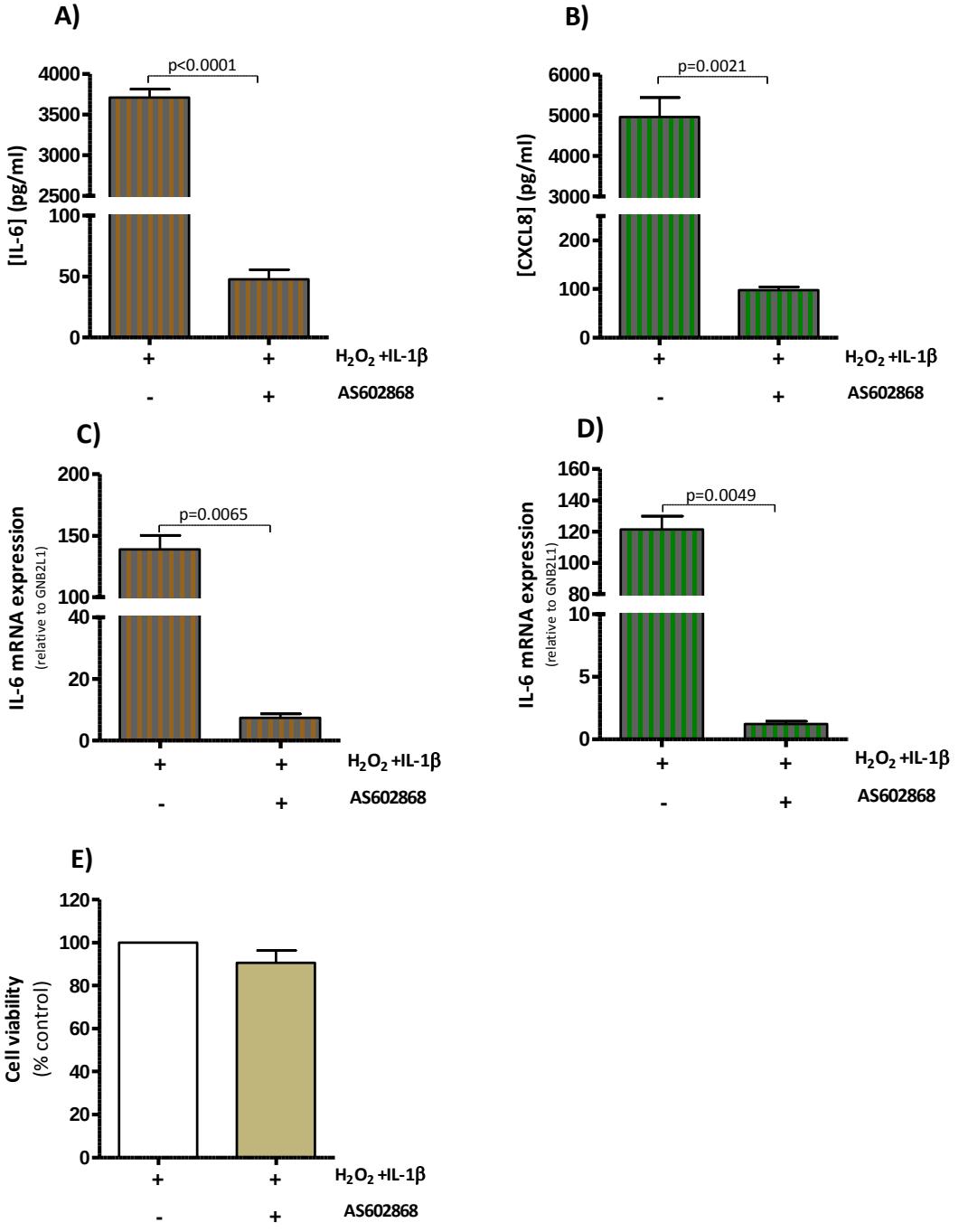


Figure 4.2: The effect of the IKK2 inhibitor AS602868 on IL-6 and CXCL8/IL-8 release. Cells were pre-treated with AS602868 (5μM, IKK2 inhibitor) followed by the treatment with H_2O_2 with or without IL-1β (1ng/ml) for 20 hrs. IL-6 and CXCL8 proteins were assayed by ELISA. **(A-B)** The release of IL-6 and CXCL8 was completely inhibited in cells pre-treated with AS602868. **(C-D)** Levels of *IL-6* and *IL-8* mRNA were quantified by real-time RT-QPCR and were normalised with respective to *GNB2LI* mRNA levels. The RT-QPCR findings are consistent with the ELISA data **(E)** AS602868 (5μM) did not affect cell viability using MTT assay. Results are expressed as mean ± SEM control. n=4. Significant if $p<0.05$

4.3.2 The effect of H_2O_2 and IL-1β on NF-κB p65 activation and nuclear translocation

In unstimulated cells, the NF-κB transcription factor is localized in the cytosol and sequestered by IκB protein. Upon activation, IκB is degraded and NF-κB is translocated into the nucleus (Adcock, Tsaprouni et al. 2007). Thus, the amount of NF-κB p65 protein translocated into the nucleus was examined, following treatments with either IL-1β (1ng/ml), or H_2O_2 (100μM) alone or in combination (IL-1β+H_2O_2) or left untreated. Nuclear proteins were extracted and the amount of NF-κB p65 protein was detected by Western blotting with a specific p65 antibody. H_2O_2 (100μM) alone induced some p65 nuclear translocation but this was minimal compared to the significant increase with IL-1β or in combination with H_2O_2 (Fig. 4.3A). The nuclear translocation of NF-κB p65 does not necessarily mean that the transcription factor will be active. To address this, we used the ELISA based TransAM NF-κB p65 kit to measure the DNA binding activity of NF-κB p65 in these nuclear extracts. Consistent with Western blot data, IL-1β markedly increased NF-κB p65 DNA binding activity by 7-fold compared with untreated cells. H_2O_2 alone didn't have a significant effect on NF-κB p65 binding activity; however, when used together with IL-1β, the binding activity was enhanced 9-fold (Fig. 4.3B). The data demonstrates that although H_2O_2 *per se* cannot enhance p65 nuclear translocation or a functional response (DNA binding), it manifests its importance by amplifying the effect of IL-1β.

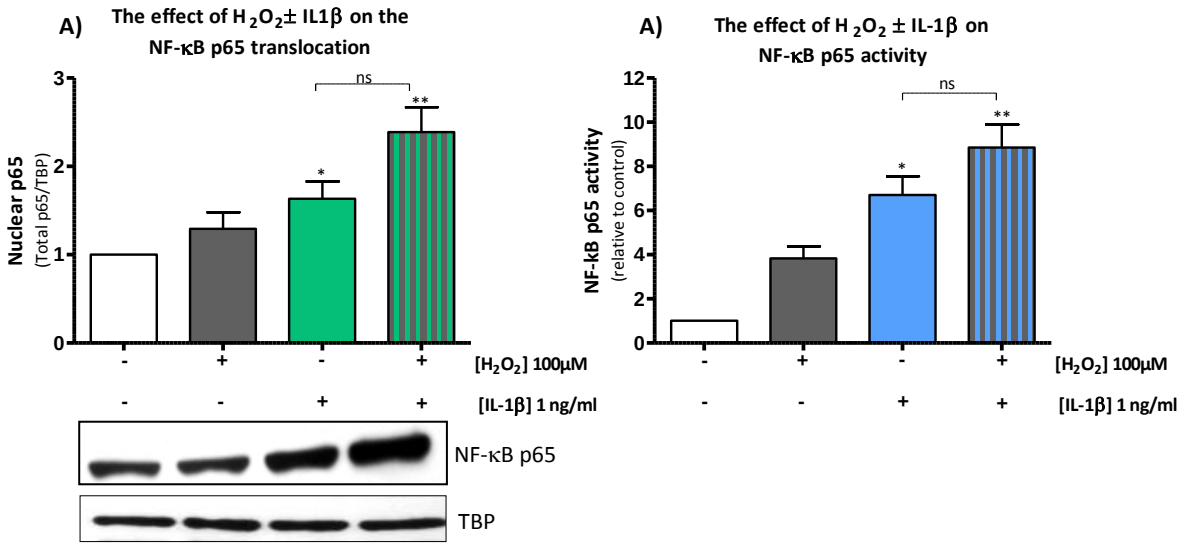


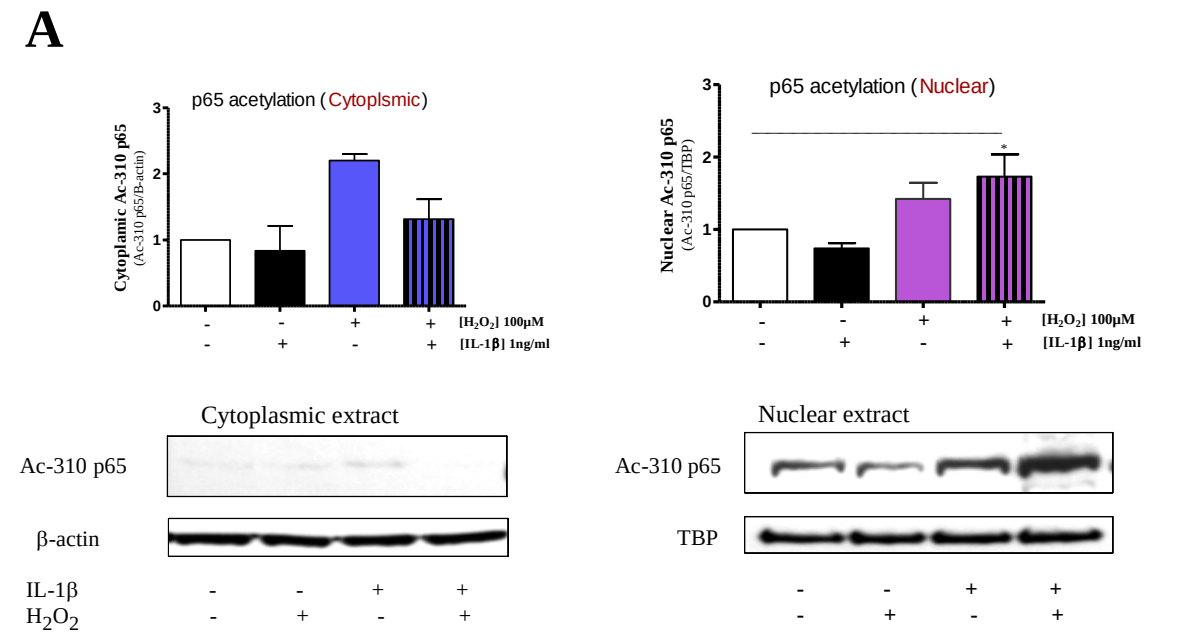
Figure 4.3: NF-κB p65 activation and nuclear translocation. (A) Cells were harvested and nuclear proteins were extracted from BEAS-2B cells after treatment with H_2O_2 (100μM) in the presence (+) or absence (-) of IL-1β (1ng/ml) for 2 hours. Using Western blot analysis, NF-κB p65 nuclear protein was quantified. Densitometric analysis of each band is plotted above. **(B)** NF-κB p65 DNA binding activity was measured using TransAM kit. Results are expressed as means ± SEM as ratio of NF-κB p65/TBP or relative to untreated cells. n=4 independent experiments. .*p<0.05, **p<0.01 compared with control (unstimulated).

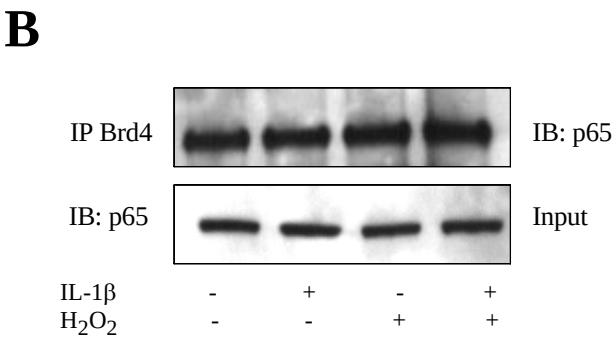
4.3.3 NF-κB p65 phosphorylation, acetylation and association with Brd4 protein

NF-κB p65 is subjected to post-translational modifications (e.g. acetylation, phosphorylation and ubiquitination) that modulate its activity (Rothgiesser, Fey et al. 2010). Acetylation of NF-κB p65 is important for NF-κB-dependent activation of inflammatory genes. NF-κB p65 acetylation has been shown to play an important role in oxidative stress induced inflammation in COPD (Ito, Charron et al. 2007). Therefore, it was investigated whether NF-κB p65 is acetylated in our oxidative stress model. Furthermore, Brd4, a transcriptional cofactor, can be associated with NF-κB p65 by binding to acetylated NF-κB p65 at lysine-310 in a complex with p-TEFb (positive transcription elongation factor b) and RNA polymerase II (Huang, Yang et al. 2009; Zhang, Liu et al. 2012). In order to identify the localisation of acetylated NF-κB p65 in the cell, cytoplasmic and nuclear proteins were extracted from cells treated with either IL-1β (1ng/ml), or H_2O_2 (100μM) alone or in combination (IL-1β+H_2O_2) or left untreated for 2 hours. Proteins were fractionated by SDS-PAGE gel and electro-transferred

onto nitrocellulose membranes. The membranes were probed with an anti-acetylated Lysine-310 NF-κB p65 antibody. Western blot analysis revealed that acetylated NF-κB p65 is predominantly found in the nuclear compartment whereas no acetylated NF-κB p65 was detected in the cytoplasm (Fig. 4.4A). This suggests that acetylation of NF-κB at lysine-310 is associated with activation and/or translocation into the nucleus. Indeed, it has been shown that acetylation of lysine-310 is required for full transcriptional activity of NF-κB p65 (Chen, Mu et al. 2002). Although, H_2O_2 did not induce p65 acetylation when compared with IL-1β, acetylation was enhanced when co-stimulated with IL-1β and H_2O_2 (Fig.4Aii).

A number of groups have reported that Brd4 interacts with NF-κB p65 (Chiang 2009; Huang, Yang et al. 2009; Zhang, Liu et al. 2012). To test the possibility of Brd4 association with NF-κB p65, BEAS-2B cells were stimulated with H_2O_2 in the presence or absence of IL-1β or left untreated for 2 hours. Whole cell extracts from stimulated and unstimulated cells were immunoprecipitated with an anti-Brd4 antibody, and co-immunoprecipitated NF-κB p65 was detected with an anti-NF-κB p65 antibody. Our results show that Brd4 interacts with NF-κB p65. However, the treatment (H_2O_2 or IL-1β) did not affect this association, suggesting that although Brd4 is an integral part of the NF-κB transcriptional complex and both (Brd4 and NF-κB p65) are found at site of transcription. Brd4 association with p65 is not linked to ROS-induced enhancement of NF-κB function. Serine phosphorylation at various sites of the NF-κB p65 subunit has been shown to be important for its transcriptional activity (Sasaki, Barberi et al. 2005; Nowak, Tian et al. 2008). We were unable to show consistent changes in phosphorylation of p65 at serines 276 and 536 (Fig.4.4C) after H_2O_2 alone or in the presence of IL-1β.


A
p65 acetylation (Cytoplsmic)
Cytoplamic Ac-310 p65
(Ac-310 p65/B-actin)
[H2O2] 100μM
[IL-1β] 1ng/ml
p65 acetylation (Nuclear)
Nuclear Ac-310 p65
(Ac-310 p65/TBP)
Cytoplasmic extract
Ac-310 p65
β-actin
IL-1β
H2O2
Nuclear extract
Ac-310 p65
TBP


B
IP Brd4
IB: p65
IB: p65
Input
IL-1β
H2O2

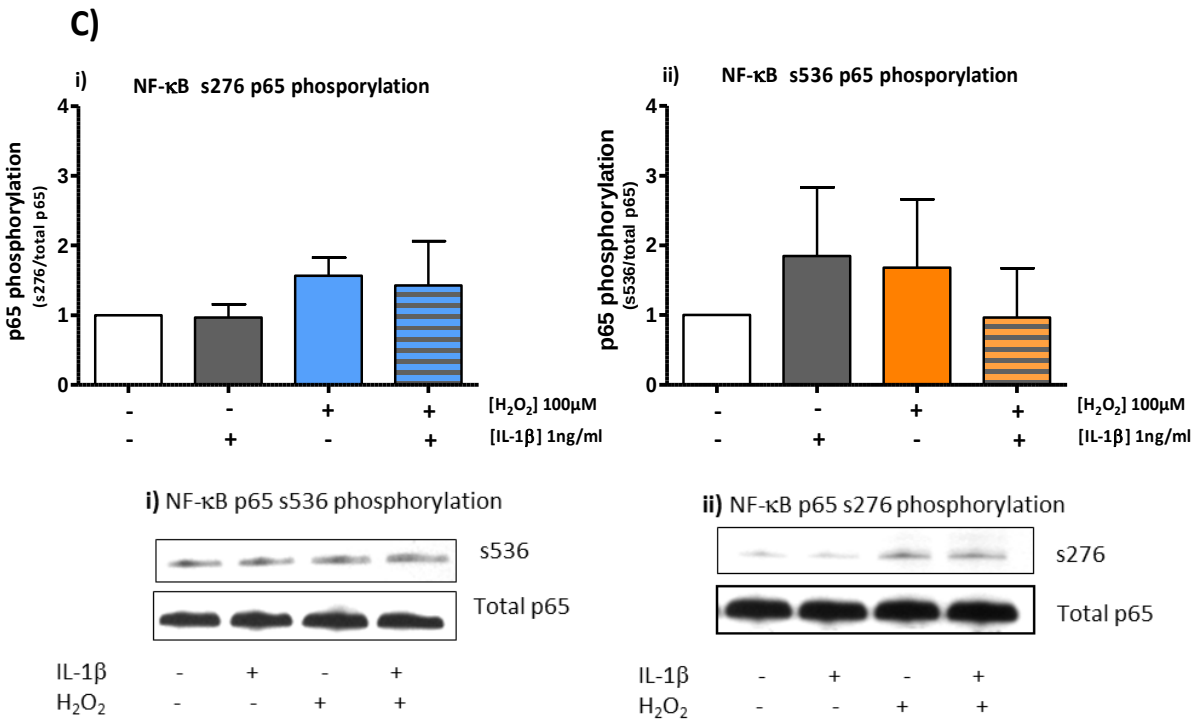

C)
i)
NF-κB s276 p65 phosporylation
p65 phosphorylation
(s276/total p65)
ii)
NF-κB s536 p65 phosporylation
p65 phosphorylation
(s536/total p65)
[H2O2] 100μM
[IL-1β] 1ng/ml
i) NF-κB p65 s536 phosphorylation
s536
Total p65
ii) NF-κB p65 s276 phosphorylation
s276
Total p65
IL-1β
H2O2

Figure 4.4: NF-κB p65 acetylation, phosphorylation and association with Brd4 protein. (A) BEAS-2B cells were stimulated with H_2O_2 with presence (+) or absence (-) of IL-1β (1ng/ml) for 2 hours, cytoplasmic and nuclear extracts were fractioned by Western blot and membranes were probed with anti-acetylated NF-κB p65 antibody. The blots show that acetylated-310 (Ac310) NF-κB p65 is predominantly found in the nucleus when compared with the cytoplasm. **(B)** Brd4 protein was immunoprecipitated from whole cell extracts following treatments and separated by SDS-PAGE and subsequently analyzed by Western blotting using an anti-NF-κB p65 antibody. **(C)** The effect of H_2O_2 and IL-1β on p65 phosphorylation at serine 536 and 276. Each blot is representative of 3 independent experiments and densitometric analysis of each band is plotted as bargraph above it. *p<0.05 compared with control (unstimulated). Ac: acetylation, TBP: TATA-Binding protein, s276/536: Serine phosphorylation at 276/536

4.3.4 The recruitment of NF-κB p65 to *IL-6* and *IL-8* promoters

Previously, we demonstrated that NF-κB p65 activity and translocation into the nucleus is elevated in our oxidative stress model which then leads to increased expression of *IL-6* and *IL-8* cytokines. We used ChIP analysis to investigate the recruitment of the NF-κB p65 to the *IL-6* and *IL-8* promoters κB sites. Following IL-1β treatment, p65 ChIP showed a marked enhancement in binding to the *IL-6* (-852 to -563) and *IL-8* (-121 to +61) promoters which was amplified by co-stimulation with H_2O_2 (7- and 20-fold respectively). H_2O_2 alone had no effect on p65 binding to either the *IL-6* or *IL-8* promoter κB regions (Fig. 4.5A and Fig 4.5B), but it enhanced IL-1β-induced association, implying a priming role for H_2O_2 in NF-κB p65 activation.

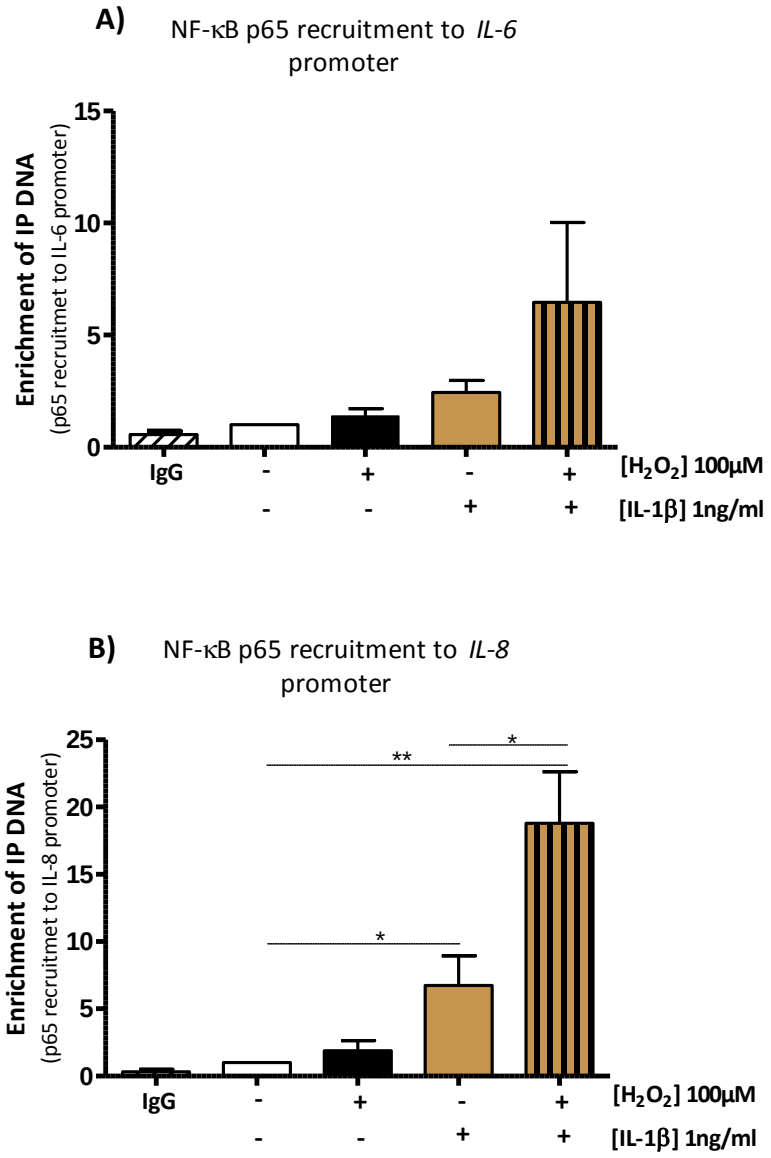


Figure 4.5: The effect of IL-1β and H_2O_2 on p65 binding to *IL-6* and *IL-8* promoter κB sites. Chromatin immunoprecipitation assay shows that IL-1β induces p65 DNA binding to both *IL-6* **(A)** and *IL-8* **(B)** promoter regions. H_2O_2 by itself does not affect p65 DNA binding activity; however, when accompanied by IL-1β, the affinity is enhanced 7-fold at *IL-6* promoter site and 20-fold at *IL-8* promoters. IgG is non-specific antibody used as a negative control. Results are representative of three (*IL-6* promoter) and six (*IL-8* promoter) independent experiments.*p<0.05, **p<0.01 compared with control (unstimulated).

4.3.5 The recruitment of Brd4 to *IL-6* and *IL-8* promoters

Recent studies have shown that Brd4 is necessary for the p65 associated transcriptional complex to recruit RNA polymerase II and p-TEFb to a promoter. In addition, p-TEFb has been shown to interact with NF-κB (Chiang 2009; Huang, Yang et al. 2009; Li, Hu et al. 2010; Zhang, Liu et al. 2012). The

binding of Brd4 to *IL-6* (-852 to -563) and *IL-8* (-121 to +61) promoter κB sites were studied using ChIP. IL-1β significantly increased binding of Brd4 to the *IL-6* and *IL-8* promoters. This effect was augmented by co-stimulation with H_2O_2 and IL-1β at the *IL-6* (6-fold) and *IL-8* (8-fold) promoters compared to unstimulated cells (Fig. 4.6A and Fig. 4.6B).

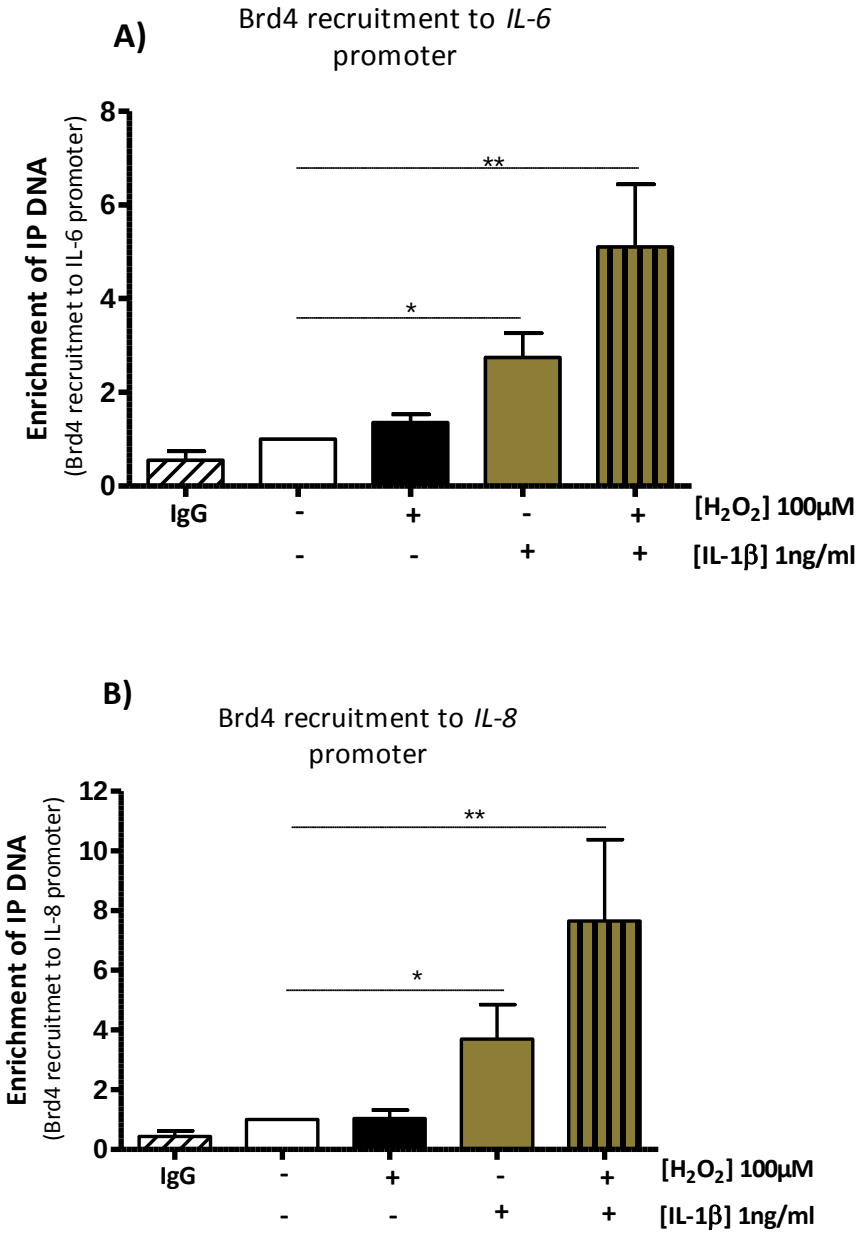


Figure 4.6: Brd4 binding affinity to *IL-6* and *IL-8* promoter sites. ChIP experiments reveal that Brd4 is recruited to *IL-6* promoter **(A)** and the *IL-8* promoter **(B)** upon stimulation with IL-1β (1ng/ml), whereas H_2O_2 (100µM) alone had no effect. H_2O_2 enhanced IL-1β-induced Brd4 binding to *IL-6* and *IL-8* promoter. Results are representative of 5 independent experiments. *p<0.05, **p< 0.01 compared with control.

4.3.6 Histone 3 and 4 acetylation at *IL-6* and *IL-8* promoters

We next sought to determine whether H3 and H4 are acetylated at the *IL-6* and *IL-8* promoters using ChIP analysis. This would clarify the role of histone acetylation and subsequent recruitment of Brd4 and p65 to the respective promoters. Following IL-1β treatment H3 acetylation was induced both at the *IL-6* and *IL-8* promoter κB sites. Similar findings were recorded when cells were co-treated with H_2O_2 and IL-1β (Fig. 4.7A and Fig.4.7B). However, in contrast H_2O_2 did not affect IL-1β-induced acetylation of H4 at either the *IL-6* or *IL-8* promoters at the time point studied (2 hours) (Fig. 4.7C and Fig.4.7D). These results suggest that acetylation of H3 and H4 are gene selective 2 hours after cell stimulation. The lack of H4 acetylation may reflect the use of a single time point to study this dynamic process.

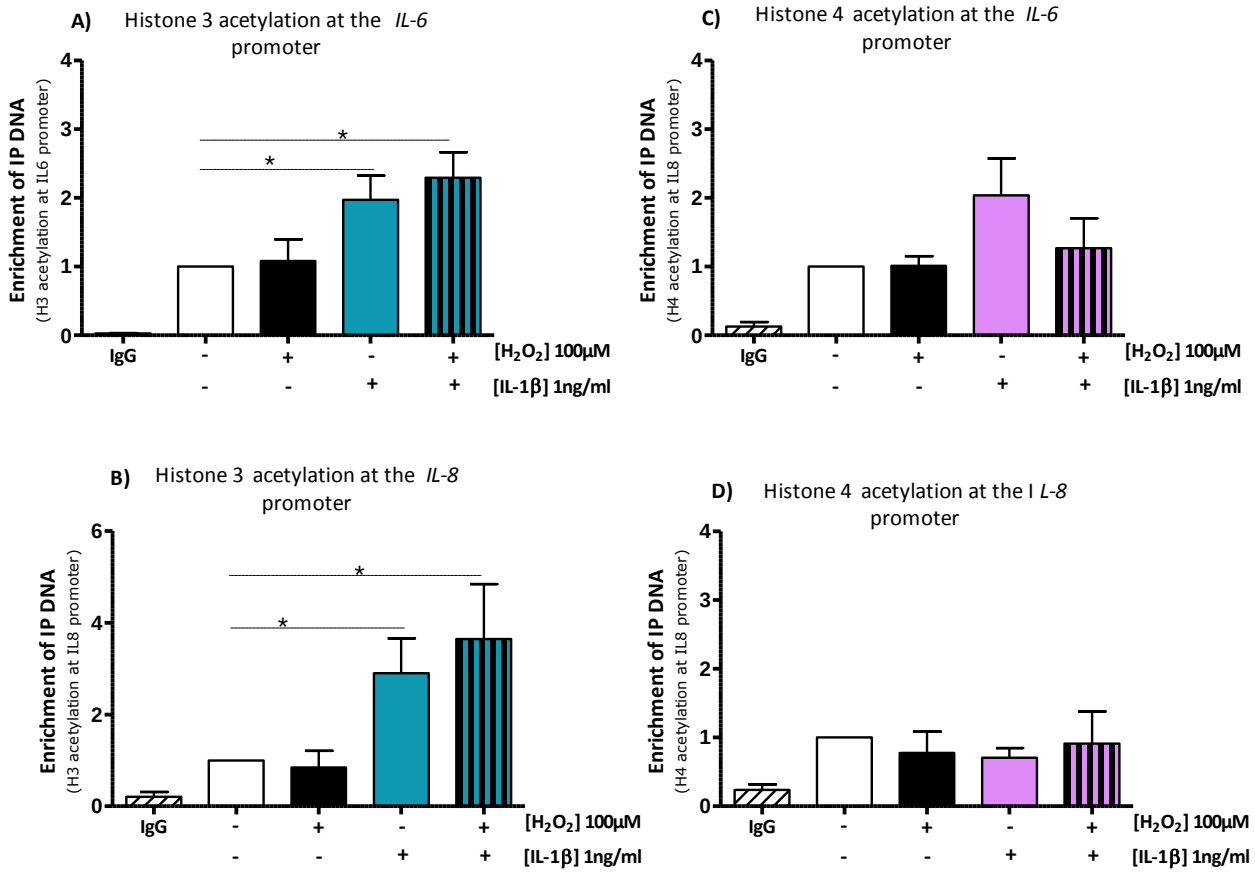


Figure 4.7: H3 and H4 acetylation at *IL-6* and *IL-8* promoter sites. BEAS-2B cells were treated with H_2O_2 (100µM) for 2 hours in the presence (+) and absence (-) of IL-1β (1ng/ml). Proteins and DNA were crossed-linked by formaldehyde treatment and chromatin pellets were extracted. Following sonication, acetylated-H3 and-H4 were immunoprecipitated and the associated DNA amplified by QPCR. **(A-B**) Histone 3 is associated with both *IL-6* and *IL-8* promoter κB sites following treatments. **(C-D)** However, there is no significant acetylation of H4 at either at the *IL-6* or *IL-8* promoters. Results are representative of n > 3 independent experiments.

4.4 Discussion

In this chapter, we have demonstrated that p65 activity is elevated in our oxidative stress model. The Western blot data showed that p65 is acetylated at lysine-310 and translocates into the nucleus upon stimulation with IL-1β alone or in combination with H_2O_2. This confirms that the NF-κB pathway is activated. In addition, both p65 and Brd4 are recruited to the *IL-6* and *IL-8* promoter κB sites. However, we did not see p65 phosphorylation at either serine-276 or -536. ChIP analysis of H3 and H4 revealed that only H3 is acetylated, but not H4, 2 hours after cell stimulation.

The expression of inflammatory genes is regulated through a number of signalling pathways that involves the activation of stress kinases (JNK, MAPK, p38, phosphoinositide 3 (PI-3)-kinase/PI-3K-activated serine-threonine kinase Akt) and redox sensitive transcription factors such as NF-κB and activator protein 1 (AP-1) (Rahman and Adcock 2006). However, Inflammation in COPD appears to be mainly driven through the activation of NF-κB signalling pathway as it is activated by numerous inflammatory stimuli (Barnes 2009; Yao and Rahman 2009). Reactive oxygen species (ROS) and its intermediate (H_2O_2), together known as oxidative stress is found in COPD and contribute to the pathophysiology of the disease as well as driving chronic inflammation in COPD (Ahsan, Ali et al. 2003; Rahman and Adcock 2006; Barnes and Adcock 2009). Therefore, we firstly demonstrated that the NF-κB is activated in our acute oxidative stress model following treatment with IL-1β and H_2O_2. H_2O_2 alone did not have any significant effect either on NF-κB translocation or activation but when used in combination with IL-1β, the effect of IL-1β was potentiated, suggesting that oxidative stress plays a vital role in enhancing inflammation in COPD. In COPD patients, inflammation persists long after smoking cessation (exogenous source of ROS) and its partly due to ROS produced by several structural and immune cells, providing a steady influx of oxidative stress (Agusti, MacNee et al. 2003; Kirkham, Caramori et al. 2011).

H_2O_2 alone does not activate NF-κB directly in many cell types. However, its response is cell-type specific and in part dependent upon the experimental setup (Oliveira-Marques, Marinho et al. 2009).

There is somewhat conflicting data on NF-κB activation and translocation by H_2O_2. NF-κB activation by ROS has been extensively studied in T-cells as they are often exposed to high levels of ROS during inflammation (Antunes and Cadenas 2001; Kraaij, van der Kooij et al. 2011). It has been shown that ROS, in the absence of SHIP-1 kinase, activates NF-κB through the atypical pathway, which is distinct from the classical pathway investigated here, that involves IκBα phosphorylation at serine 32 and 36 following activation of the IKK complex by pro-inflammatory cytokines such as TNF-α and IL-1β (Gloire, Charlier et al. 2006). In the atypical pathway, ROS induced Syk protein kinase activation phosphorylates IκBα at tyrosine 42 (Tyr42). Phosphorylation at Tyr42 allows Calpain (a protease) to digest IκBα in a proteasome-independent manner, releasing NF-κB into the nucleus (Takada, Mukhopadhyay et al. 2003). Others have shown that in Hela cells; H_2O_2 induces NF-κB transcriptional activity via activation of protein kinase D (PKD). H_2O_2-induced mediated phosphorylation of PKD in return phosphorylates IKKβ (a subunit of the IKK complex) thereby activating the classical pathway (Storz and Toker 2003; Chan, Chou et al. 2010). All these studies imply that NF-κB activation by H_2O_2 is highly cell-type specific and involves different activation mechanisms (Fig. 4.8).

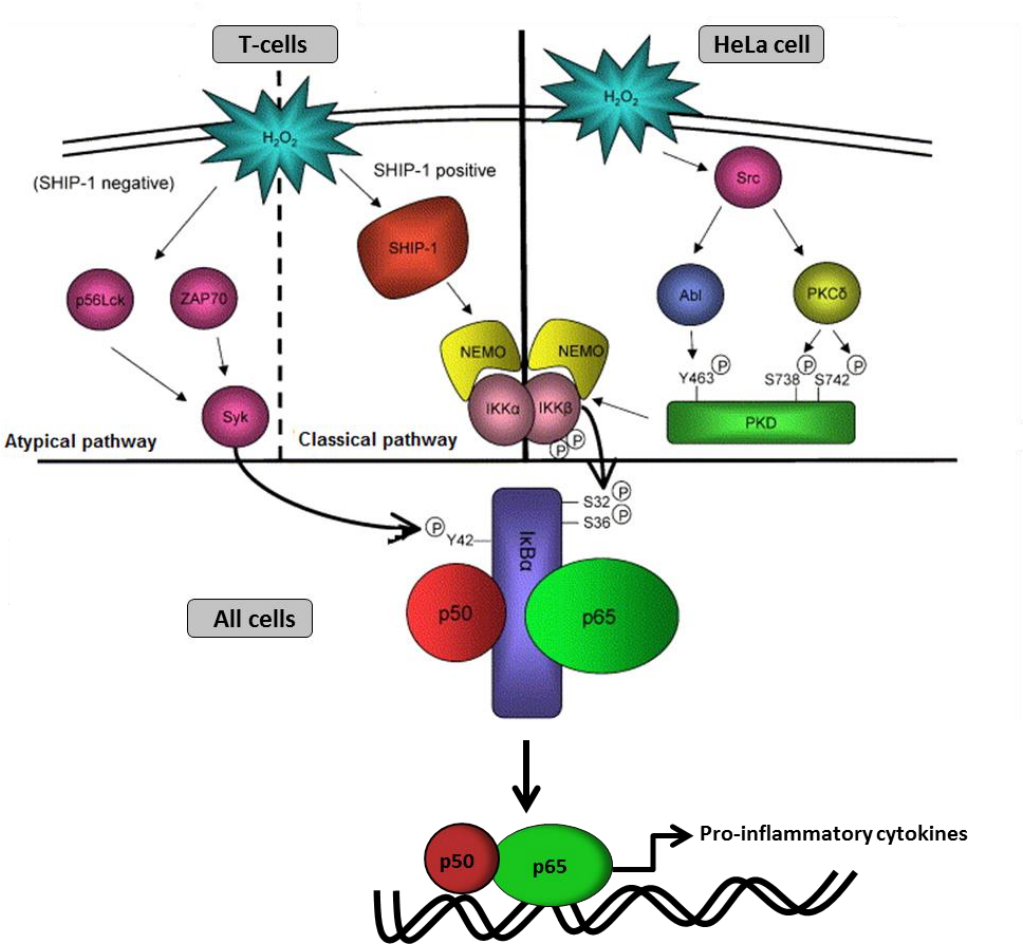


Figure 4.8: Cell-type specific activation of NF-κB by ROS/H_2O_2. H_2O_2 exerts its modulatory effects on NF-κB activation in cell-type specific manner. In T-cells, the classical pathway is activated (via IKK complex) in the presence of SHIP-1 kinase whereas in its absence, activation is switched to the atypical pathway involving phosphorylation of IκBα at tyrosine 42 (Y42). However, in HeLa cells, H_2O_2 induces phosphorylation of IKK complex at serine 32 and 36 via protein kinase D (PKD), leading to the induction of the classical pathway. Modified from Gloire, Legrand-Poels et al. (2006).

Inflammatory stimuli, including IL-1β, generate intracellular ROS which is required for NF-κB activation (Gloire, Legrand-Poels et al. 2006). We propose the following mechanism of NF-κB activation in our oxidative stress model. The initial challenge with H_2O_2 (100μM) most likely evokes a change in the intracellular redox status (chapter 3, Fig. 3.4). This allows enhanced activation of NF-κB by IL-1β in the presence of ROS compared with that seen with IL-1β alone. In addition, it has previously been demonstrated that H_2O_2 (100μM) prolongs NF-κB nuclear retention following inflammatory stimuli (IL-1β and TNF-α) in epithelial cells (A549 and HeLa cells) by inhibiting the

negative regulator of IκBα (Cezanne), leading to poly-ubiquitination and degradation of newly synthesized IκBα molecules (Enesa, Ito et al. 2008). However, the exact signaling pathway that converges on induction of IKK complex-mediated phosphorylation of IκB by H_2O_2 is yet to be fully defined in our model.

Optimal induction of NF-κB activity also requires post-translation modification (phosphorylation, acetylation and ubiquitination) of p65, following activation, which regualtes DNA binding and transcriptional activity (Huang, Yang et al. 2010). Phosphorylation of NF-κB p65 at serines 276 and 536 has been suggested to facilitate the recruitment of p300/CBP and enhance the acetylation of p65 (Chen, Williams et al. 2005; Hoberg, Popko et al. 2006; Nowak, Tian et al. 2008). However, I did not observe consistent phosphorylation at either residue following analysis by Western blot. This may reflect the quality of the antibodies that are commercially available or the time-point studied. There is growing evidence that acetylation of p65/RelA at Lysine-310 (K310) is essential for optimal NF-κB transcriptional activity (Huang, Yang et al. 2009; Huang, Yang et al. 2010; Zhang, Liu et al. 2012). In the present study, I have demonstrated that following stimulation with IL-1β and H_2O_2, p65 is acetylated. Acetylated K310 on p65 was found predominantly in the nucleus rather than in the cytoplasm following stimulation. Seven lysine sites have been identified as being acetylated mainly by p300/CPB acetyltransferase with each modification conferring a specific NF-κB function. For example, acetylation at K221 promotes DNA-binding activity whereas acetylated K218 impedes p65 and IκBa assembly (Oeckinghaus and Ghosh 2009).

Huang and colleagues have reported that p65 acetylation on K310 is recognized by the bromodomain and extra-terminal (BET) protein Brd4 and that this provides a docking site for Brd4 (Huang, Yang et al. 2009). This observation led us to investigate the association between Brd4 and NF-κB p65. Co-immunoprecipitation studies revealed that Brd4 is associated with p65 in the resting cells, although, we did not observe greater binding between Brd4 and p65 with any treatments studied despite H_2O_2 and IL-1β co-stimulation enhancing p65 acetylation. The association between

these proteins also implies that they might be recruited together as a complex to the same site during transcriptional activation. In fact, the same authors have also shown Brd4 recruitment to the promoter regions of the p65 targeted genes TNF-α and E-selectin (Huang, Yang et al. 2009). The binding of Brd4 to the *IL-6* and *IL-8* κB-responsive promoter sites has not been previously reported to our knowledge. We have shown that Brd4 is recruited to the same promoter region of *IL-6* and *IL-8* as p65 using ChIP. In addition to HDACs (Erasers) and HATs (writers), Brd4 (reader) adds another level of transcriptional regulation and has been proposed as a potential therapeutic target for COPD and other inflammatory diseases (Kabesch and Adcock 2012).

Gene activation is marked by histone acetylation whereas de-acetylation causes gene suppression. Acetylated histone results in unwinding of DNA, providing accessibility to the transcription machinery (RNA polymerase II, basal and transcription factors) (Rahman and Adcock 2006). The addition and removal of acetyl groups to histones are carried out by HATs and HDACs, respectively (Eberharter and Becker 2002; Rahman and Adcock 2006). NF-κB-dependent transcriptional regulation of pro-inflammatory cytokines *IL-6* and *IL-8* is directly related to hyper-acetylation of H3 and H4 residues at promoter associated sites (Szulakowski, Crowther et al. 2006; Yang, Valvo et al. 2008; Tsaprouni, Ito et al. 2011; Zhang, Cai et al. 2011). However, we report here that H3 pan-acetylation, but not H4 pan-acetylation, occurs at the *IL-6* and *IL-8* promoters following stimulation with H_2O_2 and IL-1β. It does not mean that H4 is not acetylated during gene expression. We analysed H4 acetylation at 2 hours post-stimulation and it will require further analysis at different time points to understand this dynamic process. Although, H_2O_2 was unable to induce H3 or H4 acetylation directly in BEAS-2B cells, it has been shown by our group that H_2O_2 (100μM) exerts its effect by reducing HDAC2 activity in BEAS-2B cells (Ito, Hanazawa et al. 2004). This causes a shift in the balance towards HATs (CBP/p300) and delaying the removal of attached acetyl-groups on histones (Fig. 4.9). The overall result is greater expression of *IL-6* and *IL-8* in cells co-stimulated with H_2O_2 and IL-1β.

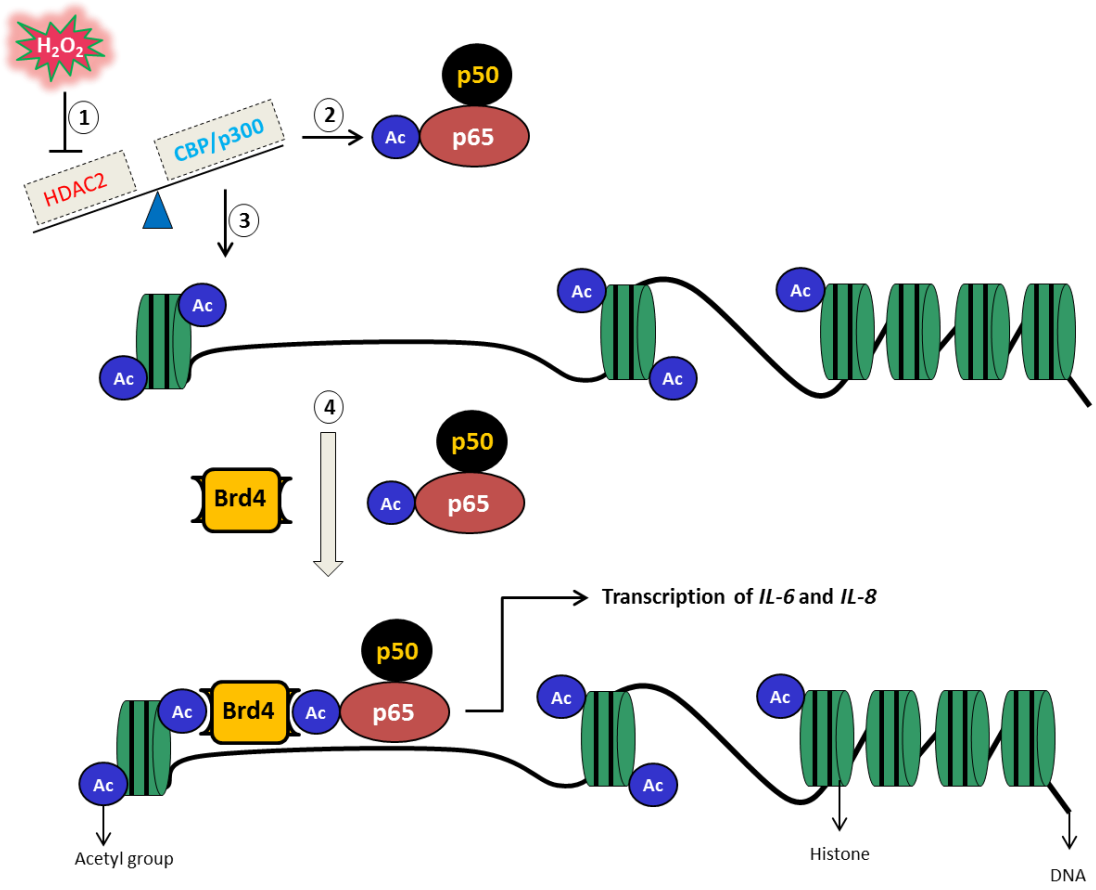


Figure 4.9: The role of H_2O_2 in protein acetylation and gene induction. (1) H_2O_2 (100µM) reduces HDAC2 activity in BEAS-2B cells (Ito, Hanazawa et al. 2004) allowing CPB/p300 (HAT) to acetylate **(2)** NF-κB p65 and **(3)** histones 3. **(4)** These acetylated proteins are recognised by Brd4 protein, forming a complex with NF-κB at site of transcription leading to the expression of *IL-6* and *IL-8* genes.

In summary, we have shown that following treatment of BEAS-2B cells, with H_2O_2 and IL-1β, NF-κB p65 is activated. We have also shown that p65 and H3 are acetylated in our model. These acetylated proteins are recognised by Brd4, forming a complex that is present at the *IL-6* and *IL-8* promoter κB sites leading to expression of *IL-6* and *IL-8*. This makes the Brd4 a potential therapeutic target for attenuating inflammation. Drugs such as JQ1, I-BET and PFI-1 can inhibit the binding of Brd4 to acetylated proteins such as histones. Therefore, in the next chapter we will investigate the effect of these drugs on:

1. Oxidative stress-induced expression of *IL-6* and *IL-8* mRNA and protein,
2. The binding of Brd4 and p65 to *IL-6* and *IL-8* promoters and
3. Compare the effect of these drugs versus knockdown of Brd4 on inflammatory gene expression.

Chapter 5

Bromodomain inhibitors attenuate oxidative stress-induced inflammation

5.1 Rationale

Chronic inflammation in COPD is mediated through the activation of NF-κB pathway (Barnes 2008). Inhaled corticosteroids (ICS) are frequently used in the management of inflammation in COPD patients; however, they provide little or no benefits to COPD patients (Adcock, Chou et al. 2009). Complete suppression of NF-κB activity could lead to adverse side effects as it is involved in the activation of immune response against invading pathogens (Hayden, West et al. 2006; Salminen, Huuskonen et al. 2008). However, a selective and temporal inhibition of its activity may prove to be beneficial in the course of inflammation. During gene expression, NF-κB is incorporated into the basal transcription machinery at the site of transcription that consists of a number of co-regulatory proteins including Brd4 (Reese 2003; Rahman and Adcock 2006). Brd4 is part of bromomodmain and extra-terminal (BET) family proteins that includes Brd2, Brd3 and Brdt proteins (Filippakopoulos, Qi et al. 2010). BET proteins contain two conserved bromo-domains that recognise acetylated chromatin and a carboxyl extra-terminal domain (CTD) which interacts with the positive transcription elongation factor b (p-TEFb). p-TEFb is involved in transcription elongation and phosphorylation of the CTD of RNA polymerase II (Rahman, Sowa et al. 2011).

Once bound to acetylated histones, bromodomain proteins recruit enzymes (HATs, and HDACs) and chromatin remodelling proteins (SW1/SNF) to the targeted promoter sites and function as either co-activators or co-suppressors (Belkina and Denis 2012). We have demonstrated in our previous chapter that acetylated-p65 forms a complex with Brd4. In addition, we have shown that both Brd4 and p65 are recruited to the promoter site of *IL-6* and *IL-8* genes following H3 acetylation, confirming that Brd4 is one of the NF-κB-associated proteins that regulate gene transcription by recruiting proteins to form a complex and initiating gene expression (Chapter 4, Fig. 4.4 and 4.5 to 4.7). Therefore, targeting Brd4 could provide a potential therapeutic strategy for treating inflammation in COPD.

JQ-1 is a small synthetic molecule which exists as 2 enantiomers (+)-JQ1 (active) and (-)-JQ1 (inactive). JQ-1 prevents Brd4 binding to acetylated chromatin by acting as a molecular inhibitor and is contained within many transcriptional regulator proteins and act by binding to bromodomains, recognising acetylated-lysine residues (Fig. 5.1). In contrast, (-)-JQ1 enantiomer does not exhibit significant binding affinity to BET bromodomains (Filippakopoulos, Qi et al. 2010; Filippakopoulos and Knapp 2012). JQ-1 has been studied in NUT (Nuclear protein in testis) midline carcinoma (NMC) mouse model and various cancer cell lines, showing inhibition of cell proliferation and differentiation (Filippakopoulos, Qi et al. 2010; Mertz, Conery et al. 2011). However, its role in inflammation is yet to be reported. Recently, It has been reported that BET protein inhibitor, I-BET, reduces inflammation *in vivo* and *in vitro* following LPS challenge (Nicodeme, Jeffrey et al. 2010).

In an effort to understand the role and function of bromodomain proteins, in particular Brd4, in inflammation, we have investigated the effect of JQ1 and another chemically distinct BET-domain inhibitor PFI-1 in oxidative stress (H_2O_2) and IL-1β-induced inflammation. We have investigated the binding affinity of Brd4 and p65 to these promoters in JQ-1 post-treated cells. Finally, we have looked at the IL-6 and CXCL8 expression in Brd2 and Brd4 knocked down cells following stimulation.

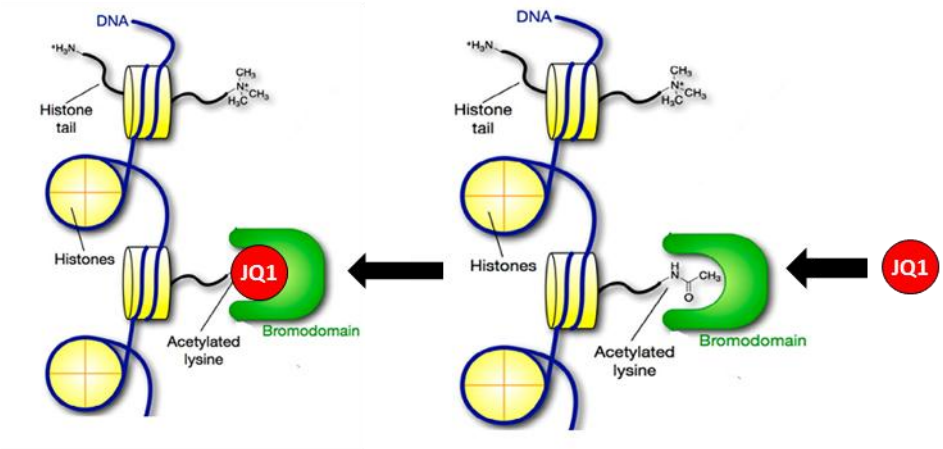


Figure 5.1: Bromodomain binding and inhibition by JQ1. Histone tails are subjected to post-translation modifications including acetylation of lysine residues. These acetylated lysines provide a docking site for bromodomain proteins. Bromodomain inhibitors such as JQ-1 bind competitively to the acetyl-lysine recognition pocket thus disrupting the association between acetylated H3 lysine and bromodomain containing proteins. Adapted from Hewings, Rooney et al. (2012).

5.2 Experimental protocols

5.2.1 The quantification of IL-6 and CXCL8 cytokines (ELISA)

BEAS-2B cells were pre-treated with JQ1 (500nM) or PFI-1 (1μM) followed by stimulation with IL-1β (1ng/ml) and H_2O_2 (100μM) for 16 hours. Supernatants were collected and assayed for CXCL8 and IL-6 using commercially available ELISA kits, according to manufacturer`s instructions (Human CXCL8 and IL-6 Duoset, R&D Systems, Abingdon, UK).

5.2.2: *IL-6* and *IL-8* mRNA expression (RT-QPCR)

After treatments, total RNA was extracted using an RNeasy kit following the manufacturer's instructions (Qiagen, Hilden, Germany). RNA was converted to cDNA and subjected to qPCR analysis. The amount of *IL-6* and *IL-8* mRNA was determined using the delta delta CT values of gene of interest (*IL-6* and *IL-8*) and normalised to a housekeeping gene (*GNB2L1B1*).

5.2.3 The effect of JQ1 and PFI on cell viability using (MTT assay and FACS analysis)

5.2.3.1 MTT

The MMT assay was used to assess cell viability following treatments with different concentrations of JQ-1 (0-1000nM) and PFI-1 (0-4μM) for 16 hours. Cells were incubated with 1mg/ml MMT solution at 37°C and 5% CO_2 for 30 minutes. MTT solution was then removed and DMSO was added to dissolve the formazan product to produce a purple solution. The absorbance was measured at 550 nm. The colour intensity was correlated with cell viability.

5.2.3.2 FACS analysis

Cells were collected by trypsinisation and neutralized with medium, washed and resuspended in in 1ml PBS. Aqua LIVE/DEAD® stain was added (1μl/sample) for 30mins in dark. Cells were washed with cold PBS and resuspended in 1% BSA and PBS followed by flow cytometric analysis. Parameters were set as such to distinguish between positive cells (heat treated-dead cells) and negative cells (alive),

which gave us distinct population of cells. The effect of JQ1 (500nM) and PFI-1 (1μM) on cell toxicity was subsequently examined using these parameters (Refer to Chapter 2 page 64 for more details).

5.2.4 siRNA transfection

BEAS-2Bs cells were seeded in 6-well plates at 0.3 x 10^6 cells/well and transfected with ON-Target plus SMART pool Brd2 or Brd4 or control small interfering RNAs (siRNA) at 12nM concentration (All from Dharmacon Thermo Scientific, Chicago, USA) using HyperFect transfection reagent (Qiagen, Crawly, UK) as described by the manufacturer. After 72 hours, cells were harvested and nuclear extracts were examined for Brd2 and Brd4 protein expression levels using Western blotting. Total mRNA was extracted and converted to cDNA and assessed for Brd2 and Brd4 expression using Q-PCR. Cells were serum starved post-transfection overnight and treated with H_2O_2 (100μM) and stimulated with/out IL-1β (1ng/ml) for 16 hours. Supernatant was analysed for IL-6 and CXCL8 protein using ELISA as described above.

5.2.5 Western blot analysis of Brd2 and Brd4 KD

Cells were harvested at 72 hours post-transfection and nuclear extracts were prepared as described in chapter 2 (page 77). Forty micrograms of nuclear extract was resolved on 10% SDS gels. Proteins were transferred to a nitrocellulose membrane using iblot (Invitrogen) and immunoblotted with anti-Brd2 and Brd4 antibody. TATA-binding protein (TBP) was used as loading control. (Further details are provided in chapter 2 page 68).

5.2.6 The binding of Brd4 and p65 to *IL-6* and *IL-8* promoter regions (ChIP assay)

BEAS-2B cells were pre-treated with either (-)-JQ1 or (+)-JQ1 both at 500nM for 4 hours followed by co-stimulation with H_2O_2 (100μM) and IL-1β (1ng/ml) or left untreated for 2 hours. After incubation, protein-DNA complexes were fixed by formaldehyde (1% final concentration). NF-κB p65 and Brd4

binding to the *IL-8* promoter (-121 to +61) and the *IL-6* promoter (-852 to -563) were quantified by real-time QPCR using a QantiTech SYBR green PCR kit (Qiagen) on Rotor-gene 3000 (Corbett Research, NSW, Australia). These sites were chosen as they have been previously been shown to contain function of κB response elements (Goransson, Elias et al. 2005; Hollingshead, Beischlag et al. 2008; Nettles, Gil et al. 2008; Ho, Lee et al. 2009; Tsuchiya, Imai et al. 2010).

5.3 Results

5.3.1 Comparison of the suppressive effect of JQ1 on IL-6 and CXCL8 release

Glucocorticoids (GCs) are frequently used in the management of several inflammatory conditions. However, they provide little or no benefits to COPD patients (Barnes 2011). Abnormal histone acetylation profiles have been linked to COPD and steroid unresponsive (Barnes and Adcock 2009). Bromodomain and extra terminal domain (BET) proteins such as Brd4 recognise the acetylated histones and recruit transcriptional regulators to specific gene site (Mertz, Conery et al. 2011). Compounds such as I-BET recognise and target acetylated modified histones in macrophages leading to reduction in LPS-induced inflammation (Nicodeme, Jeffrey et al. 2010). Therefore, we assessed the effect of the recently described (Filippakopoulos, Qi et al. 2010) BET inhibitor (+)-JQ1 (a Brd4 inhibitor) on IL-6 and CXCL8 expression in IL-1β-stimulated cells. BEAS-2Bs cells were treated with range of concentrations ($5x10^{-9}$-10^{-6} M) of (+)-JQ1 and its inactive enantiomer (-)-JQ1 (Fig. 5.2), which has no appreciable binding affinity to BET bromodomains (Filippakopoulos, Qi et al. 2010), for 4 hours, followed by IL-1β (1ng/ml) stimulation for 16 hours. IL-6 and CXCL8 were measured by ELISA. (+)-JQ1 inhibited IL-1β-induced IL-6 and CXCL8 release in a concentration-dependent manner with maximal suppression of 80% (IL-6) and 70% (CXCL8) at $5x10^{-7}$ M (Fig. 5.3A and 5.3B). Cell viability was significantly reduced at the highest concentration tested (10^{-6} M, Fig. 5.2C).

In contrast, (-)-JQ1 ($5x10^{-7}$ M) had little effect on suppressing IL-1β-stimulated IL-6 (35 ± 5.9%) and CXCL8 (34 ± 3.4%) release. Therefore, we used $5x10^{-7}$ M of (+)-JQ1 in our subsequent investigations. The results showed a significant difference in the effect of (-)-JQ1 versus (+)-JQ1 at $1x10^{-7}$ M for IL-6 (35.2 ± 3.3%) and CXCL8 (22.5 ± 2.8%) and at $5x10^{-7}$ M for IL-6 (51.7 ± 6.2%) and CXCL8 (38.4 ± 4.6%).

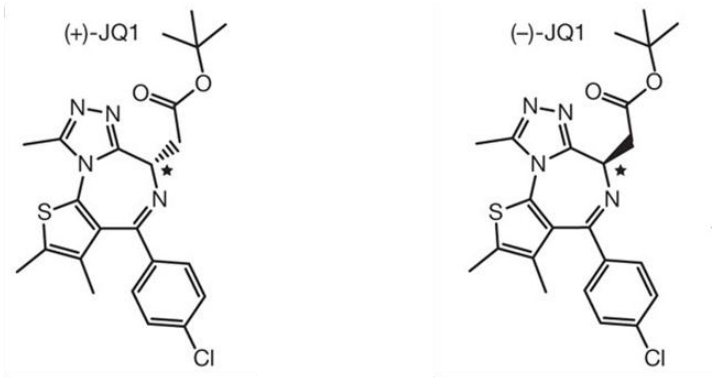


Figure 5.2: JQ1 stereoisomers 'structure. (+)-JQ1 is an active isomer which has a high binding affinity to Brd4, whereas (-)-JQ1 enantiomer has no significant binding affinity (Filippakopoulos, Qi et al. 2010).

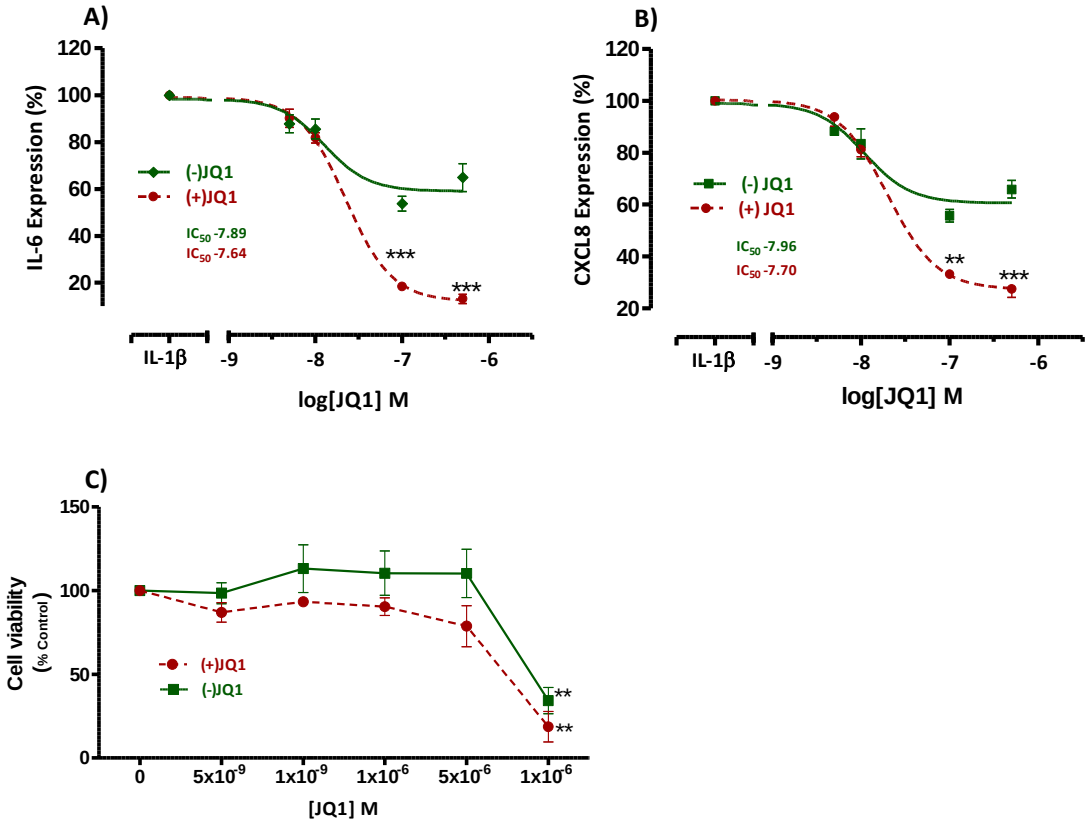
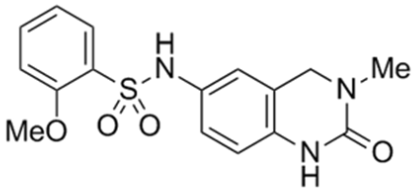


Figure 5.3: The effect of JQ1 on IL-6, CXCL8 release and cell viability. Cells were pre-treated with (+)-JQ1 and (-)-JQ1 enantiomer for 4 hours followed by IL-1β (1ng/ml) for 16hrs. IL-6 and CXCL8 proteins were assayed by ELISA **(A-B)**. Cell viability was assessed by MTT assay release **(C)**. n=3 independent experiments. Points represent mean ± SEM **p<0.01***P<0.001 (-)-JQ1 versus (+)-JQ1.

5.3.2 Comparison of the suppressive effect of PFI-1 on IL-6 and CXCL8 release

PFI-1 is of BET bromodomain probe which is structurally distinct from JQ1 (Fig. 5.4). PFI-1 also inhibits the BET protein from binding to acetylated histones (Structural Genomics Consortium 2012). Therefore, we sought to examine the effect of PFI-1 compound on IL-1β-induced expression of IL-6 and CXCL8. Cells were pre-treated with variable concentrations of PFI-1 ($1x10^{-7}$- $4x10^{-6}$ M) for 4 hours prior to stimulation with IL-1β (1ng/ml) for 16 hours. IL-6 and CXCL8/IL-8 proteins were quantified using ELISA. The results showed that PFI-1 reduced IL-1β-induced IL-6 and CXCL8 proteins levels in concentration-dependent manner (Fig. 5.5A). The IC_{50} (half-maximal inhibition) for PFI-1 inhibition of IL-1β-induced IL-6 protein levels was $7.26x10^{-7}$ M. A similar IC_{50} for PFI-1 was seen for inhibition of IL-1β-induced CXCL8 release ($7.39x10^{-7}$ M). PFI-1 inhibited IL-1β-induced IL-6 and CXCL8 release with maximal suppression of (80.7 ± 2.05%) and (63.6 ± 3.8%), respectively. High concentrations of PFI-1 ($4x10^{-6}$ M) reduced cell viability significantly and were excluded from subsequent experiments (Fig. 5.5B). These results confirmed that PFI-1 is about 10% less potent than the JQ-1. Therefore, we used PFI-1 at $1x10^{-6}$ M in our subsequent experiments.



PFI-1

Figure 5.4: PFI-1 structure. PFI-1 is structurally different than JQ-1 and less potent (Courtesy of Structural Genomics Consortium (SGC) (Structural Genomics Consortium 2012).

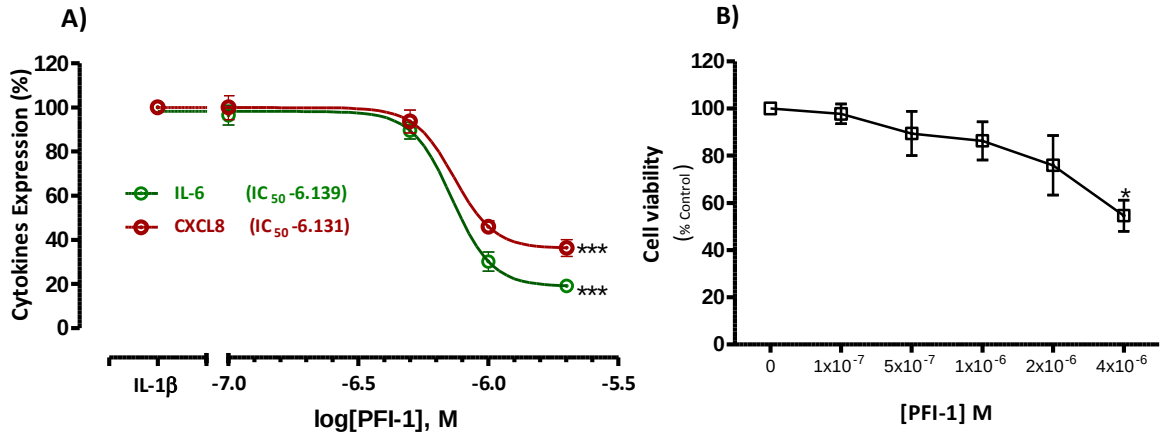


Figure 5.5: The effect of PFI-1 on IL-6, CXCL8 release and cell viability. Cells were pre-treated with different concentrations of PFI-1 for 4hrs before stimulating with IL-1β (1ng/ml) for 16hrs. IL-6 and CXCL8 proteins were assayed by ELISA **(A)**. Cell viability was assessed by MTT assay **(B)**. n=3 independent experiments. Points represent mean ± SEM *p<0.05 ***P<0.001 versus stimulated only (IL-1β).

5.3.3 The effect of JQ-1 and PFI-1 on cell viability using FACS analysis

It has been demonstrated above that JQ1 and PFI-1 significantly suppressed IL-1β-induced expression of IL-6 and CXCL8 at 0.5μM and 1μM concentrations, respectively. We have also shown that neither JQ1 nor PFI-1 compromised cell viability at these concentrations using an MTT assay. However, we wanted to confirm this by flow cytometry analysis which is more stringent. Cells were treated with either (+)-JQ1 or its isomer (-)-JQ1 or PFI-1 or left untreated for 16 hours. Cells were also heat treated or mix with live cells to setup appropriate parameters for analysis. Cells were analysed by flow cytometry following the Live/Dead® Aqua blue staining reaction. Heat treated cells (Dead cells) gave us a single distinct peak shifted towards right and represented by red colour. Heat (80°C) ruptured about 95% of cells` membrane (Fig. 5.6A). In contrast, we observed two peaks with the live and dead cells mixture, with the right peak representing dead cells and the left turquoise peak representing intact cell membranes (Fig. 5.6B). The effect of JQ1 and PFI1 on cell viability was analysed based on these parameters. Cells treated with DMSO (<1%) alone or with PFI-1 (1μM)

resulted in only 5% overall cell death with 95% cells being viable (Fig. 5.6C-D). Similarly, both (-)-JQ1 and (+)-JQ1 at 0.5µM concentration hand no significant effect on cell viability (Fig. 5.6E-F).

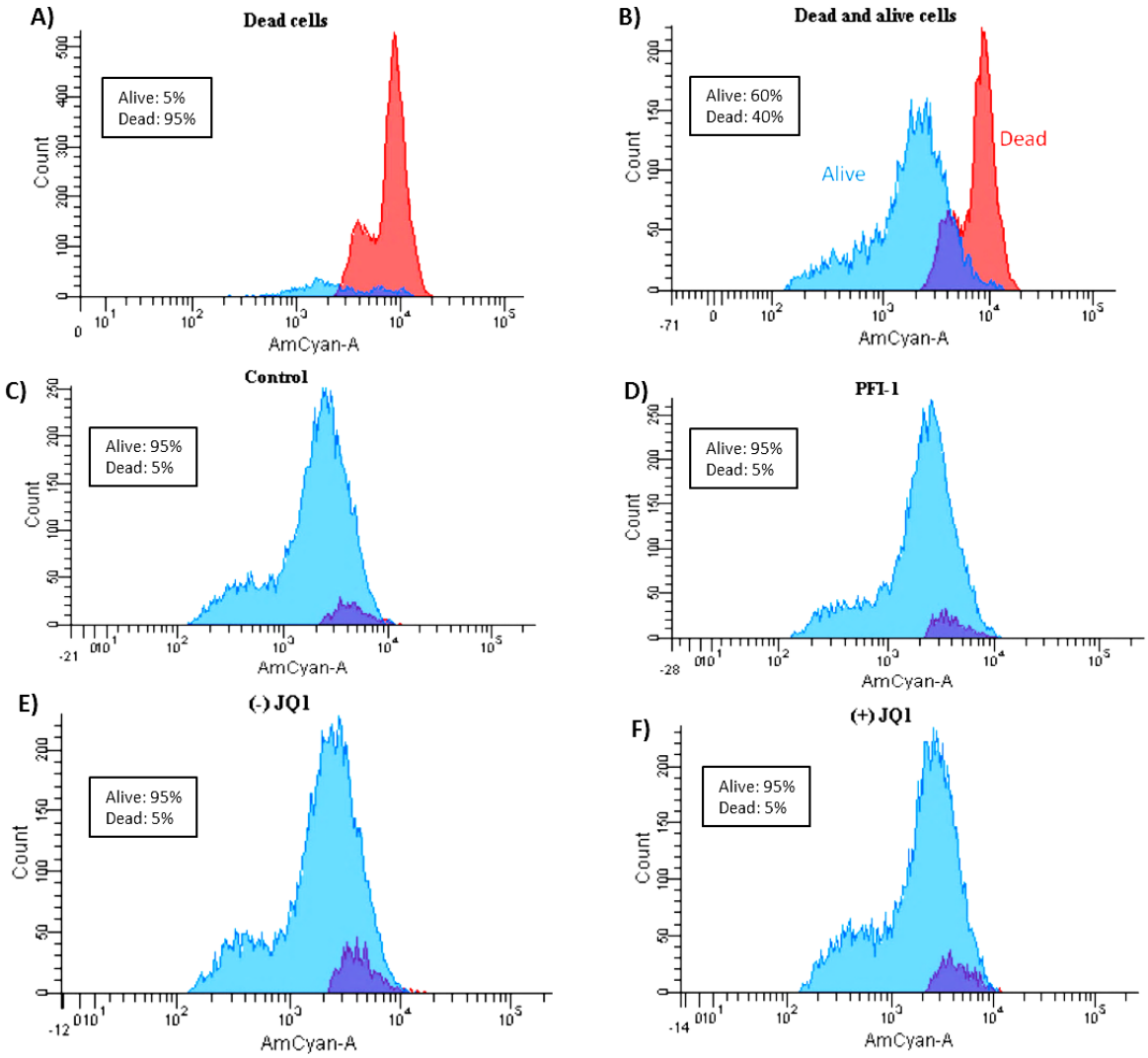


Figure 5.6: Cell viability post JQ1 and PFI-1 treatments. LIVE/DEAD® Fixable Aqua staining of cells treated with heat (80°C), JQ-1 (0.5µM) and PFI-1 (1µM) for 16 hours, followed by flow cytometry analysis. The blue histogram represents percentage viable cells whereas red indicates % dead cells. Heat treated cells alone **(A)** or mixed with live cells **(B)** were used as negative control and to set the parameters for analysis **(C)**. DMSO/Control (<1%) alone **(D)** PFI-1, **(E)** (-)-JQ1 and **(F)** (+)-JQ1 did not affect cell viability. The data is representative of 3 independent experiments.

5.3.4 The effect of JQ-1 on IL-6 and CXCL8 expression

Previously (Chapter 3, section 3.3.8), we demonstrated that H_2O_2 (100μM) alone has minimal effect on IL-6 and CXCL8 expression; however, it enhanced the expression of both cytokines in IL-1β-induced cells. Therefore, we investigated the effect of JQ-1 on IL-6 and CXCL8/IL-8 expression under such conditions. BEAS-2B cells were serum-starved overnight and treated with (+)-JQ1 or (-)-JQ1 enantiomer both at 500nM for 4 hours followed by treatment with H_2O_2 (100μM) and IL-1β (1ng/ml) alone or in combination or left untreated for 16 hours. IL-6 and CXCL8 proteins were assayed in cell supernatant using ELISA and mRNA transcripts were quantified by real-time RT-QPCR. H_2O_2 alone had little or no effect on IL-6 or CXCL8 protein production, however, co-stimulation with H_2O_2 and IL-1-β significantly induced the release of both IL-6 and CXCL8 proteins in comparison with unstimulated cells (Fig. 5.7A and 5.7B). Similarly we observed significant increase in *IL-6* and *IL-8* transcripts (Fig. 5.7C and 5.7D). However, the release of IL-6 and CXCL8 proteins was significantly suppressed in (+)-JQ1 but not (-)-JQ1 treated cells (Fig. 5.7A and 5.7B). Consistent with the ELISA data, cells pre-treated with (+)-JQ1 significantly reduced the expression of both *IL-6* and *IL-8* mRNA induced by IL-1β and H_2O_2 (Fig. 5.7C and 5.7D). The data showed that the expression of *IL-6* and *IL-8* genes can be repressed by JQ-1 but not down to control resting levels.

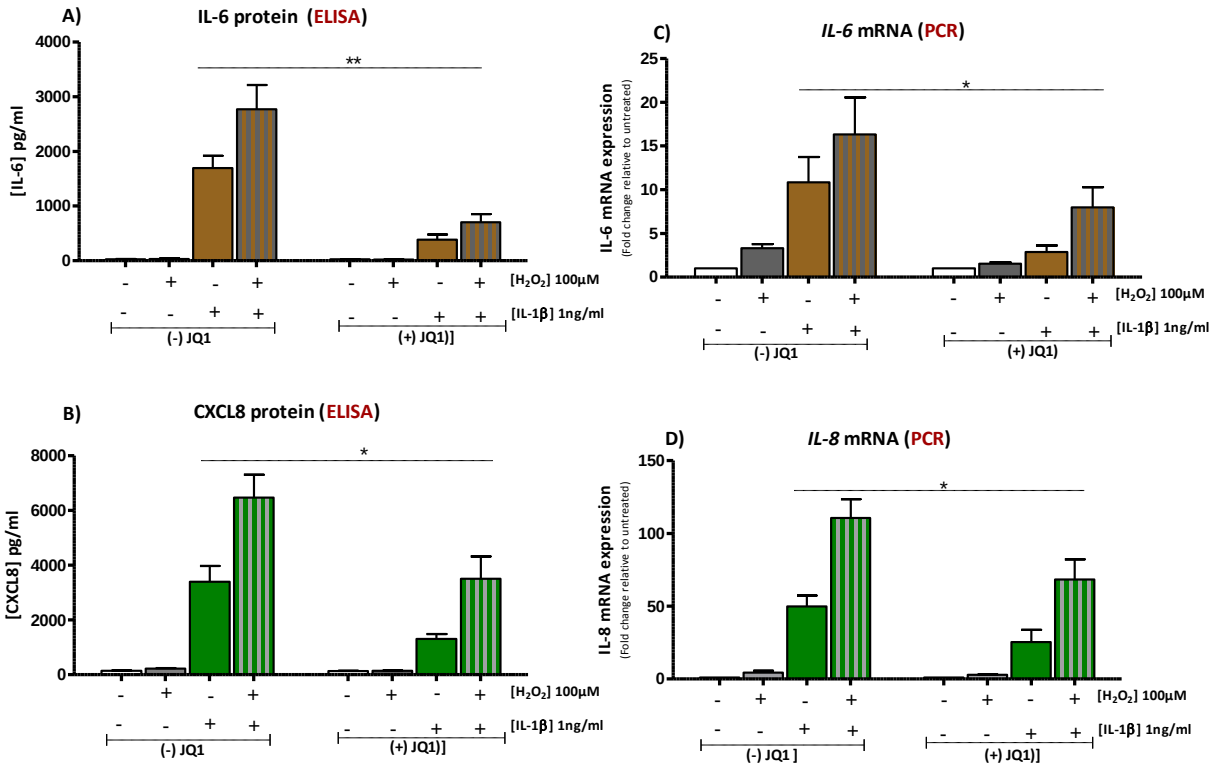


Figure 5.7: The effect of JQ-1 on IL-6 and CXCL8 expression. Cells were pre-treated with either (+)-JQ-1 or (-)-JQ1 both at 500nM for 4 hours followed by stimulating with IL-1β (1ng/ml) H_2O_2 (100µM) or IL-1β (1ng/ml) or both for 16 hours or left unstimulated. IL-6 and CXCL8 proteins were assayed by ELISA **(A-B)**. *IL-6* and *IL-8* transcripts were quantified by RT-PCR **(C-D)**. n=4 independent experiments. Bar graph represent mean ± SEM *p<0.05, **p<0.01, (-)-JQ1 versus (+)-JQ1 treated.

5.3.5 The effect of PFI-1 on IL-6 and CXCL8 expression

Next we examined the inhibitory effect of PFI-1 on IL-6 and CXL8 expression. Cells were treated with PFI-1 (1µM) for 4 hours prior to co-stimulation with IL-1β (1ng/ml) or H_2O_2 (100µM) alone or in combination for 16 hours or left unstimulated. IL-6 and CXCL8 proteins were assayed in cell supernatant using ELISA and mRNA transcripts were quantified by RT-QPCR. We observed similar findings with PFI-1 as with JQ-1. The release of IL-6 and CXCL8 proteins was significantly suppressed with PFI-1 when compared untreated cells (Fig. 5.8A and 5.8B). In accord with ELISA data, pre-treated cells with PFI-1 significantly reduced the expression of *IL-6* and *IL-8* mRNA in IL-1β and H_2O_2 induced cells (Fig. 5.8C and 5.8D). However, the level did not return to baseline.

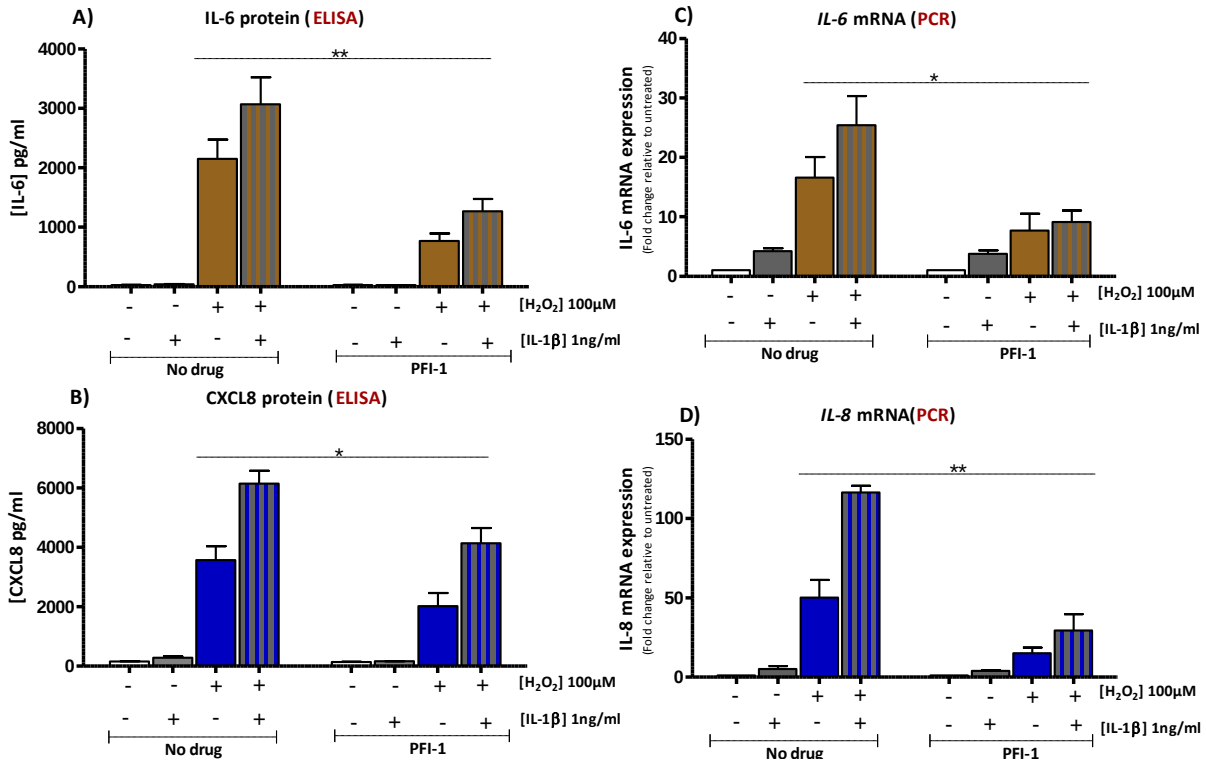


Figure 5.8: The effect of PFI-1 on IL-6 and CXCL8 expression. Cells were pre-treated with PFI-1 (1µM) or without drug for 4 hours before stimulating with IL-1β (1ng/ml) or H_2O_2 (100µM) alone or both for 16 hours or left unstimulated. IL-6 and CXCL8 proteins were assayed by ELISA **(A-B)**. *IL-6* and *IL-8* mRNA was amplified by RT-QPCR **(C-D)**. n=4 independent experiments. Bar graph represent mean ± SEM *p<0.05, **p<0.01, no drug versus PFI-1 treated.

5.3.6 Brd4 knockdown in BEAS-2B cells reduces IL-1β and H_2O_2-induced IL-6 and CXCL8

We demonstrated that (+)-JQ1 significantly reduced H_2O_2 enhanced expression of IL-6 and CXCL8 cytokines in IL-1β-induced cells. (+)JQ1 shows a greater binding affinity to Brd4 than any other BET proteins (Filippakopoulos, Qi et al. 2010). Therefore, we postulated that knocking down Brd4 would reduce IL-6 and CXCL8 release in H_2O_2 and IL-1β stimulated cells. To test this hypothesis, cells were transfected with Brd4 or non-specific small interfering RNAs for 72 hours. Nuclear extracts were examined for Brd4 protein expression levels using Western blotting and *Brd4* mRNA was quantified by RT-QPCR. *Brd4* mRNA expression and protein was significantly decreased by 71.8 ± 3.2% and 63.26 ± 7.5%, respectively (Fig 5.9A and 5.9B)

Cells were serum starved overnight post-transfection and co-treated with H_2O_2 (100μM) and IL-1β (1ng/ml) for 16 hours. Supernatants were analysed for IL-6 and CXCL8 proteins using ELISA. Cells co-stimulated with H_2O_2 and IL-1-β induced the release of IL-6 and CXCL8 proteins in contrast to unstimulated cells. The release of IL-6 and CXCL8 proteins was significantly suppressed in Brd4-depleted cells in comparison with non-specific siRNA transfected cells (Fig. 5.9C and 5.9D). Intriguingly, the suppression of IL-6 (1000pg/ml) and CXCL8 (4000pg/ml) levels were the same as (+)-JQ-1 treated cells (Fig. 5.7A and Fig. 5.7B). These data confirms that Brd4 plays an important role in the expression of *IL-6* and *IL-8* genes.

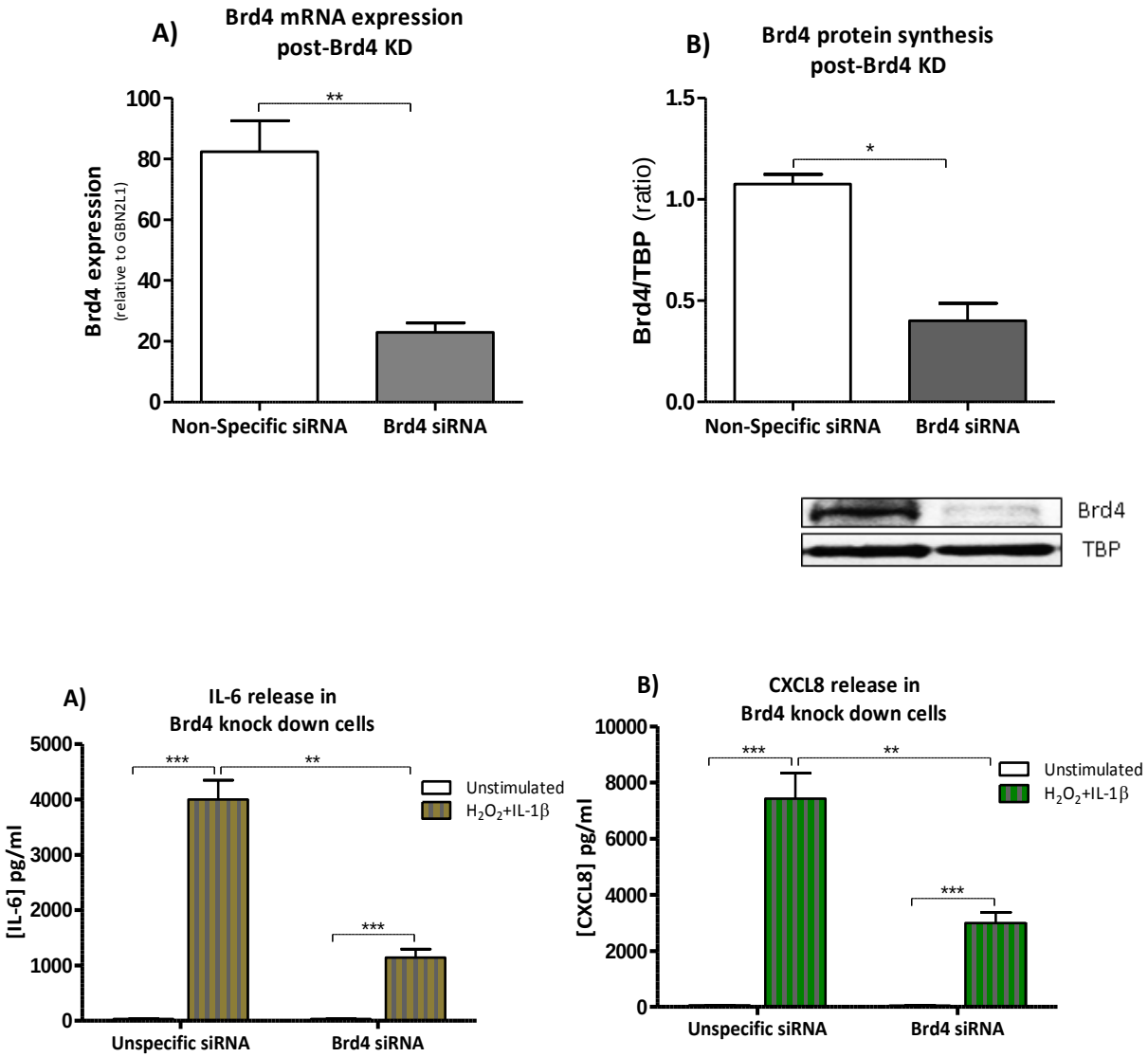


Figure 5.9: The effects of Brd4 knockdown on IL-6 and CXCL8 expression. Cells were transfected with either pooled Brd4 siRNAs (12nM) or a non-specific siRNAs (12nM). **(A-B)** At 72 hours post-trasfection Brd4 mRNA was quantified by RT-QPCR and nuclear extracts were analysed by immunoblotting using anti-Brd4 and -TATA-binding protein (TBP). IL-6 and CXCL8 proteins were measured by ELISA in post-transfected cells following stimulation with H_2O_2 and IL-1β together **(C-D)**. n=4 independent experiments. Results represent mean ± SEM *p<0.05, **p<0.01, ***p<0.001, non-specific siRNA versus Brd4 siRNA.

5.3.7 The effect of Brd2 knockdown in BEAS-2B cells on IL-6 and CXCL8 release

Having shown that Brd4 depletion led to a reduction of IL-6 and CXCL8 release, we wanted to confirm that this reduction is exclusively through Brd4 and not any other BET protein. Since there is 80% structural homology between Brd2 and Brd4 (Belkina and Denis 2012), we wished to assess whether Brd2 knockdown could account for some of the effect seen with (+)-JQ1 and Br4 knock

down on IL-6 and CXCL8 release. Following brd2 knock down, Brd2 mRNA and protein expression was significantly reduced (Fig. 5.10A and 5.10B). Brd2 knockdown had no effect on either IL-6 or CXCL8 release in IL-1β and H_2O_2 co-treated cells (Fig. 5.10C and 5.10D). These data reconfirms that Brd4 is pivotal in regulation of *IL-6* and *IL-8* expression. It also clarifies that JQ1 targets Brd4 and not Brd2 protein.

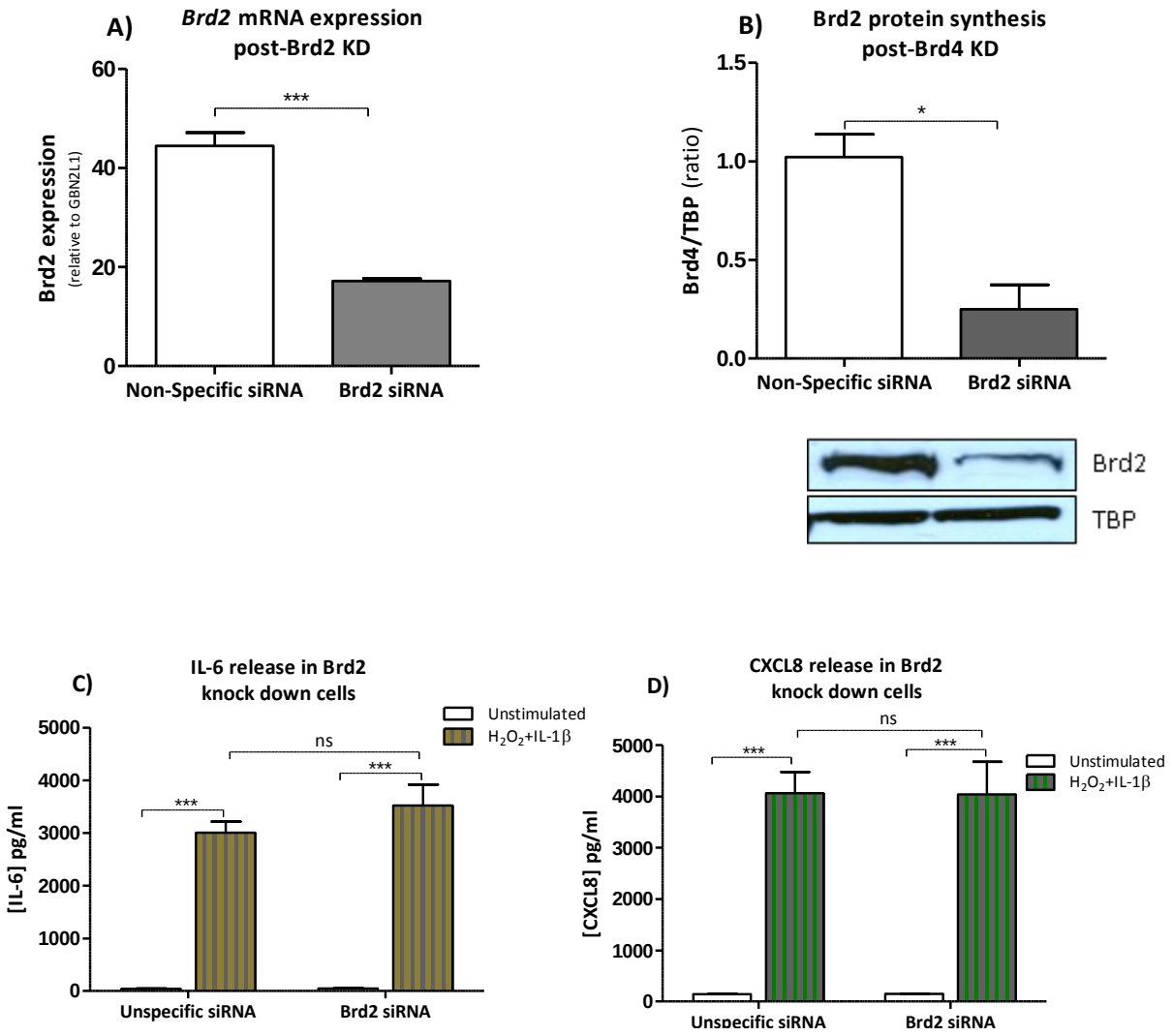


Figure 5.10: The effect of Brd2 knockdown on IL-6 and CXCL8 expression. Cells were transfected with either pooled Brd2 siRNAs (12nM) or a non-specific siRNAs (12nM). **(A-B**) At 72 hours post-trasfection Brd2 mRNA was quantified by RT-QPCR and nuclear extracts were analysed by immunoblotting using anti-Brd2 and -TATA-binding protein (TBP). IL-6 and CXCL8 proteins were measured by ELISA in post-transfected cells following stimulation with H_2O_2 and IL-1β together **(C-D).** n=4 independent experiments. Results represent mean ± SEM *p<0.05, **p<0.01, ***p<0.001, non-specific siRNA versus Brd2 siRNA.

5.3.8 Brd4 and p65 binding to *IL-6* and *IL-8* promoters post-JQ-1

In previous the chapter, we have shown that p65 and Brd4 are recruited to the κB sites in the *IL-6* and *IL-8* promoters following stimulation with H_2O_2 and IL-1β. Nicodeme and colleagues have also shown that Brd4 occupies *IL-6* and *TNF* promoter sites following LPS stimulation. Interestingly, this recruitment is abolished following treatment with I-BET (Nicodeme, Jeffrey et al. 2010). Brd4 enrichment at the *Myc* promoter is also suppressed following JQ-1 treatment (Mertz, Conery et al. 2011). Therefore, we investigated the effect of JQ-1 on the binding of Brd4 and p65 to the *IL-6* and *IL-8* promoters.

BEAS-2B cells were pre-treated with either (-)-JQ1 or (+)-JQ1 both at 500nM for 4 hours followed by co-stimulation with H_2O_2 (100μM) and IL-1β (1ng/ml) or left untreated for 2 hours. ChIP analysis was used to investigate the recruitment of the Brd4 and p65 to the *IL-6* and *IL-8* promoter sites. We observed a significant enrichment of Brd4 and p65 at the *IL-6* promoter following co-stimulation with IL-1β and H_2O_2. The inactive enantiomer (-)-JQ1 had no effect on the binding activity. However, the active form (+)-JQ1 significantly reduced p65 and Brd4 binding (Fig. 5.11A and 5.11B). Similarly, Brd4 and p65 binding increased by 4-fold and 10-fold respectively at *IL-8* promoters in stimulated cells (H_2O_2+IL-1β), when compared with unstimulated cells. This effect was abolished by (+)-JQ1 (Fig. 5.11C and 5.11D). These findings re-affirm the findings of others and signify the importance of Brd4 in the gene transcription of subsets of inflammatory genes. It also shows that although the recruitment of p65 and Brd4 is completely abrogated at both the *IL-6* and *IL-8* promoters the expression of both *IL-6* and *IL-8* did not returned to baseline levels in (+)-JQ1 pre-treated cells. This suggests that either other inflammatory signalling pathways such as p38 MAPK and JAK-STAT may be driving the expression of cytokines in addition to NF-κB pathway in the presence of (+)-JQ1 (Ono and Han 2000; Tron, Samoylenko et al. 2006; Sansone and Bromberg 2012) or that other κB binding sites may recruit Brd4. ChIP-Seq analysis may resolve this latter point.

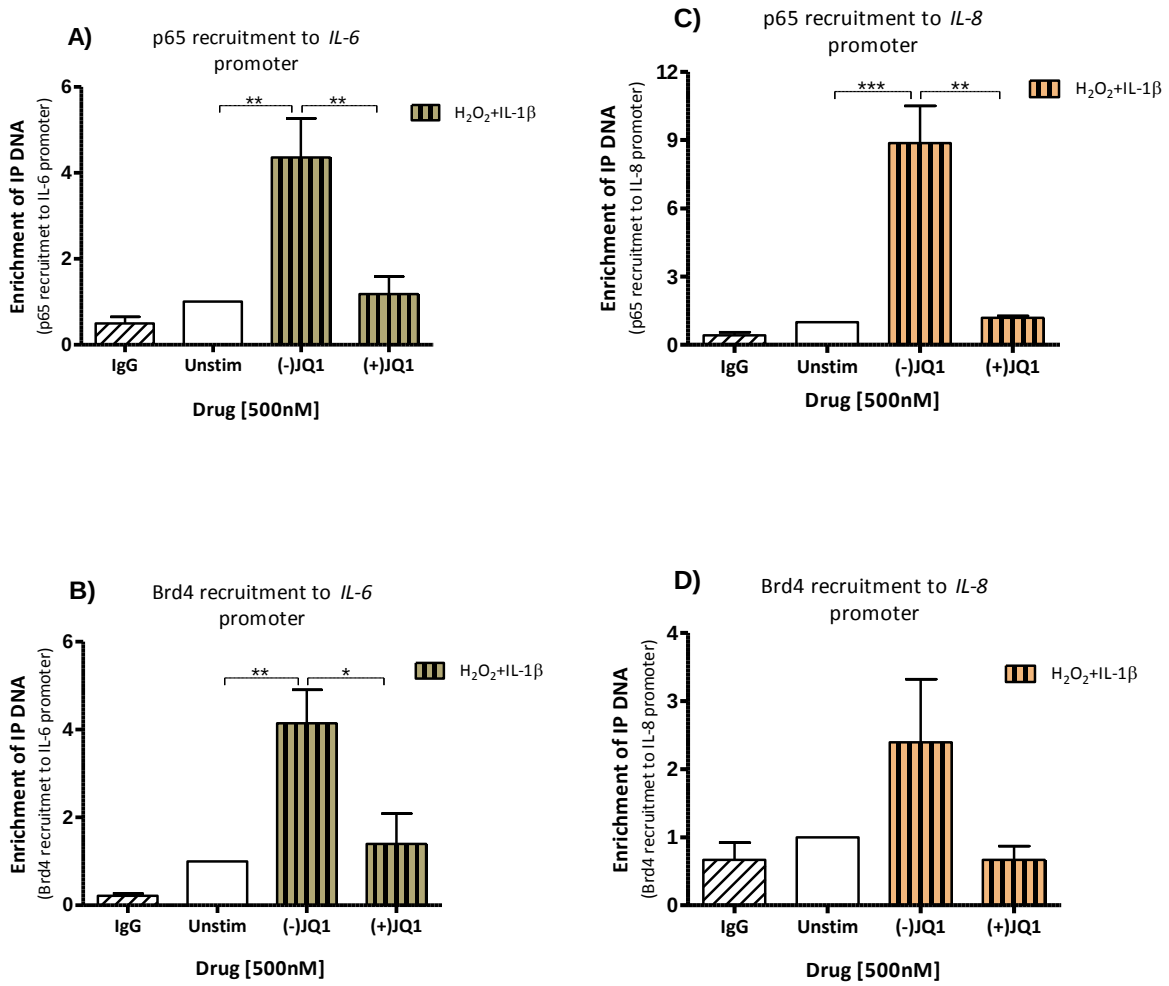


Figure 5.11: The effect of JQ1 (500nM) on Brd4 and p65 binding to *IL-6* and *IL-8* promoters. Chromatin immunoprecipitation assay shows that IL-1β (1ng/ml) and H_2O_2 (100μM) induces p65 and Brd4 DNA binding to the *IL-6* promoter by 5-fold which is abolished by (+)-JQ1 (500nM) **(A-B)**. Similarly, p65 and Brd4 DNA binding at the *IL-8* promoter are increased following H_2O_2 and IL-1β after stimulation in (-)-JQ1 (500nM) pre-treated cells by 10- and 4-fold **(C-D)**. This binding is abolished by the active (+)-JQ1 (500nM). Results are representative n≥ 3 independent experiments.*p<0.05, **p<0.01, ***p<0.001 compared with unstimulated cells.

5.4 Discussion

NF-κB signalling pathway orchestrates the cellular immune response, apoptosis, differentiation and inflammation (Barnes 2006; Watters, Kenny et al. 2007). Selective inhibition of its activity without compromising its protective role through therapeutic intervention is essential in treating inflammation. These results provide evidence that IL-1β-induced NF-κB-mediated expression of IL-6 and CXCL8/IL-8 can be abrogated by the bromodomain inhibitors JQ-1 and PFI-1 in a concentration-dependent manner. We have also demonstrated that these compounds significantly suppressed H_2O_2 enhanced IL-1β-induced expression of these cytokines. This finding confirms that Brd4 plays an important role in the induction of *IL-6* and *IL-8* genes. Knockdown studies revealed that IL-6 and CXCL8 release is significantly reduced in Brd4 depleted cells. Whereas Brd2 has no effect on IL-6 and CXCL8 release. Furthermore, oxidative stress (H_2O_2) enhanced IL-1β-induced recruitment to p65 and Brd4 at *IL-6* and *IL-8* promoters is markedly reduced in (+)-JQ-1 but not (-)-JQ1 pre-treated cells. Therefore, this data strongly suggest that BET bomodomin protein (Brd4) inhibition, also suppresses NF-κB pro-inflammatory activity, providing a potential therapeutic target for treating inflammation in COPD.

DNA and histones are tightly packed together into stable heterochromatin structures in an unstimulated cell (Adcock, Cosio et al. 2005; Adcock, Ford et al. 2006). Cell can employ different strategies to overcome this suppression upon stimulation, one such strategy is a post-translation modification of histones through the addition of an acetyl group to lysine residues on the N-terminal tails of histones (Eberharter and Becker 2002; Ito, Ito et al. 2005; Adcock, Tsaprouni et al. 2007). Acetylation of histones leads to the unwiding of DNA and histones at the transcription site paving the way for transcriptions factors (NF-κB) and co-activator proteins (Brd4) leading to gene expression (Rahman and Adcock 2006; Chiang 2009; Huang, Yang et al. 2009). Hyper-acetylation of H3 and H4 at the promoter regions of inflammatory gene is linked to gene activation (Yang, Valvo et al. 2008; Tsaprouni, Ito et al. 2011; Zhang, Cai et al. 2011). We have also demonstrated in the

previous chapter that H3 is acetylated in cells stimulated with IL-1β alone and in combination with H_2O_2 at both *IL-6* and *IL-8* promoters. Furthermore, BET family proteins such as Brd4 are essential regulatory proteins in the recognition of acetylated histones and p65 (Chiang 2009; Huang, Yang et al. 2009). It has been reported that Brd4 inhibition results in down-regulation of NF-κB targeted genes, suggesting its role in the induction and regulation of gene expression (Zhang, Liu et al. 2012). Indeed, we reported in a previous chapter that both Brd4 and p65 are recruited to both the *IL-6* and *IL-8* promoters. NF-κB activation is central to the induction of pro-inflammatory genes and inhibiting its activity will reduce inflammation. However, complete inhibition of several NF-κB components could potentially cause severe side effects as it is involved in many cellular processes including immune responses and aging (Yamamoto and Gaynor 2001; Hayden, West et al. 2006) Alternatively, manipulating NF-κB function through specific and short-term inhibition of its signalling component or associated proteins during an exacerbation when inflammation is acutely elevated may prove effective (Lounnas, Frelin et al. 2009; Tagoug, Sauty De Chalon et al. 2011). Therefore, bromodomain proteins could represent a viable therapeutic target for limiting the impact of an exacerbation. Consequently, we investigated the effect of bromodomain inhibitors on NF-κB mediated expression on IL-6 and CXCL8 in our oxidative stress model, as oxidative stress is known to be a major component of an exacerbatory attack.

The chemically distinct bromodomain inhibitors, both JQ-1 and PFI-1, significantly suppressed IL-6 and CXCL8 expression in IL-1β alone and in combination with H_2O_2 treated cells in comparison to untreated cells. Importantly, this was associated with the decreased recruitment of both Brd4 and p65 to *IL-6* and *IL-8* promoters in cells pre-treated with (+)-JQ-1. Similar to our findings, Nicodeme and colleagues have shown that I-BET disrupts the interaction of bromodmoain proteins and acetylated histones at the *IL-6* and *TNF* promoters leading to a reduction in the expression of these cytokines in LPS-stimulated macrophages. JQ-1 is extensively studied in cancer where it targets c-Myc-induced proliferation (Filippakopoulos, Qi et al. 2010; Delmore, Issa et al. 2011; Mertz, Conery

et al. 2011; Ott, Kopp et al. 2012) though its anti-inflammatory activity is yet to be reported. A novel finding of our study is that we have elucidated the anti-inflammatory properties of JQ-1 and it's mode of action in BEAS-2B cells. JQ-1 exhibits its anti-inflammatory action through disrupting the interaction between Brd4 and acetylated histones and p65 at the *IL-6* and *IL-8* promoters (Fig. 5.12). This interaction is a vital step for facilitating the recruitment of RNA Pol II, p-TEFb and additional p65 during transcription initiation and elongation (Huang, Yang et al. 2009; Zhang, Liu et al. 2012). Recently, Zhang and colleagues have shown in primary human CD4+ T cells, using genome-wide chromatin immunoprecipitation sequencing analysis that Brd4 co-localizes with RNA pol II at both enhancers and promoters of all active genes (Zhang, Prakash et al. 2012). This interaction is disrupted upon JQ-1 treatment leading to reduced lineage-specific genes expression and acetylated histone-bromodomain association (Zhang, Prakash et al. 2012). The study implies that Brd4 could be used as a tool to identify active promoters and enhancers in a genome-wide manner in human epithelial cells.

These findings also raise questions about the role of other bromodomain proteins such as Brd2, Brd3 and Brtd in inflammatory gene regulation and expression. We have shown that Brd2 depletion in BEAS-2B cells has no effect on *IL-6* and *IL-8* expression following stimulation with IL-1β and H_2O_2. However, in depth analysis and understanding of these proteins are required in order to fully exploit them as potential therapeutic targets in inflammation and cancer. More importantly, inhibition of Brd4 either by (+)-JQ1 or knockdown did not reduced inflammation completely whereas (+)-JQ-1 blocks p65 DNA binding and subsequent Brd4 recruitment, suggesting that other inflammatory signaling pathways such as p38 MAPK and JAK-STAT are equally important in the suppression of inflammation (Ono and Han 2000; Tron, Samoylenko et al. 2006; Sansone and Bromberg 2012). Therefore, the inhibition of several signalling pathways may be necessary as compare to the suppression of a single inflammatory pathway.

In addition, the specificity and side effects of bromodomain inhibitors need to be evaluated extensively as epigenetics play a cardinal role in several cellular function including proliferation, differentiation, self-renewal and development (Dawson, Kouzarides et al. 2012). In this study, we have shown that JQ-1 targets Brd4 in human BEAS-2B cells whereas other investigators have shown that it can inhibit Brdt in mice testes, causing severe defects in germ-line cells (Matzuk, McKeown et al. 2012).

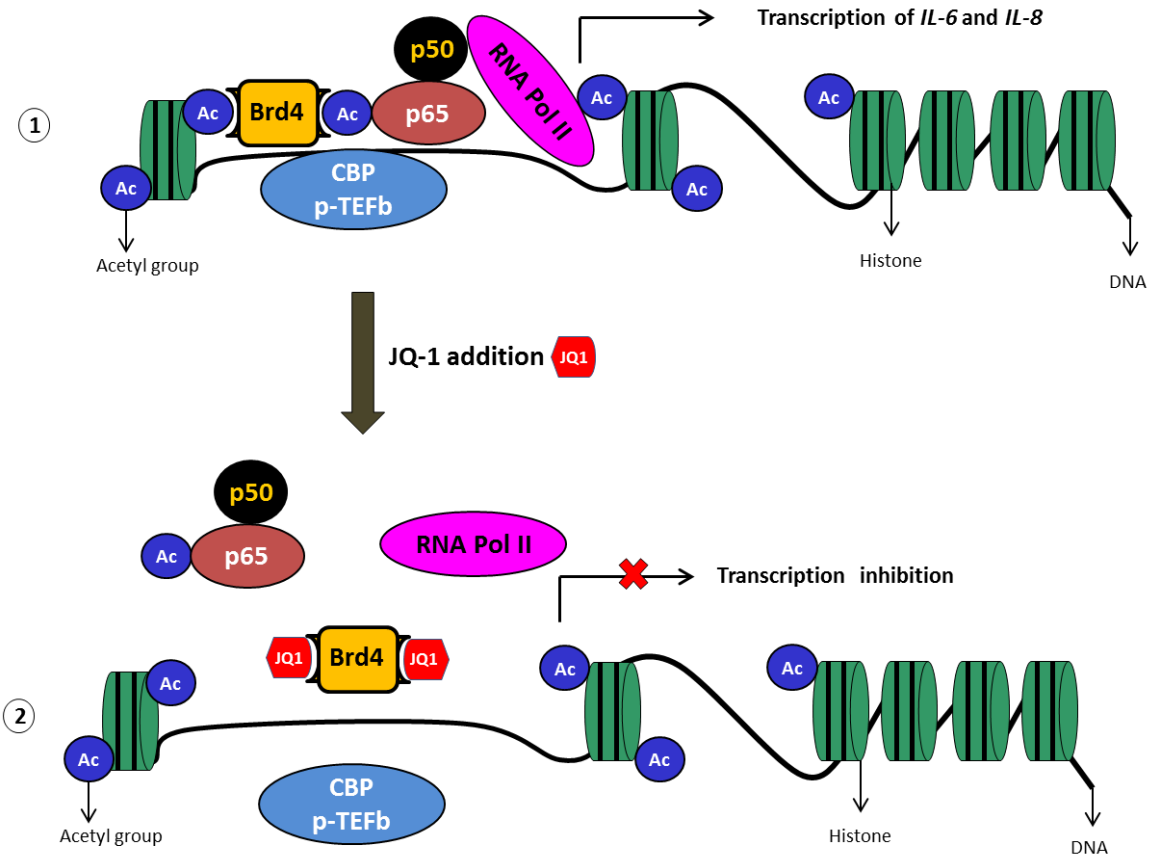


Figure 5.12: The role of JQ-1 in the inhibition of gene expression. (1) Upon stimulation, histones and p65 are acetylated in BEAS-2B cells. These acetylated sites are recognised by Brd4. Subsequently, Brd4 recruits p-TEFb and RNA polymerase to the *IL-6* and *IL-8* promoters forming part of a large transcription complex that results in gene transcription. **(2)** The introduction of JQ-1 disrupts the binding between Brd4 and acetylated proteins (histones and p65), resulting in the dissolution of the transcription complex and reduced gene suppression.

In summary, we have shown that the chemically distinct bromodomain inhibitors, JQ-1 and PFI-1, significantly reduced oxidative stress (H_2O_2) enhanced IL-1β-induced inflammation in BEAS-2B cells. JQ-1 has high binding affinity to Brd4 but not Brd2. This was confirmed by Brd4 knock down studies which lead to a similar significant decrease in IL-6 and CXCL8 release. Furthermore, JQ-1 ablated the occupancy of Brd4 and p65 at both the *IL-6* and *IL-8* promoters. The ability of bromodomain inhibitors to reduce inflammation makes bromodomain proteins an attractive therapeutic target for inflammatory disorders including COPD particularly during an exarcerbation when inflammation is enhanced. However, whilst the development of highly specific pharmacological inhibitors of bromodomain proteins (BET family) will facilitate a better understanding of how these proteins contribute to gene activation and regulation, questions still remain. We still need to understand and address:

1. Why levels of IL-6 and CXCL8 release are still above control levels despite complete ablation of p65 and Brd4 promoter binding?
2. The role of individual bromodomain proteins in cell function, for example, cell proliferation, differentiation etc.
3. Identify bromodomain inhibitors/mimics that can provide the desired anti-inflammatory outcomes upon inhibition with minimum side effects as it has been recently shown that Brd4 is recruited to both promoters and enhancers of all active genes including house-keeping genes (Zhang, Prakash et al. 2012).
4. To developed better and specific inhibitors with high binding specificity towards individual bromodomains.

Chapter 6

General Discussion & Future Directions

6.1 General discussion

Chronic obstructive pulmonary disease (COPD) is caused by chronic exposure to oxidative stress of which prolonged cigarette smoke (CS) exposure is a major aetiological factor. Increased oxidative stress produced by elevated oxidants, derived from cells and CS, or reduced anti-oxidant response is reported in COPD patients (Finkel and Holbrook 2000; GOLD 2011). Oxidative stress is also thought to contribute to the development of many other diseases including those of heart, immune system, brain and cancer (Dalle-Donne, Aldini et al. 2006; Kurien and Scofield 2008). As a result, a number of animals and cell/tissue culture models have been developed to study the impact of oxidative stress (Yatin, Varadarajan et al. 1999; Aksenova, Aksenov et al. 2005; Wijeratne, Cuppett et al. 2005). However, most of these models are based on one off acute ROS exposure which fails to reproduce the disease pathology (Yatin, Varadarajan et al. 1999; Aksenova, Aksenov et al. 2005; Wijeratne, Cuppett et al. 2005) whereas chronic oxidative stress is central in the pathogenesis of these diseases including COPD. Structural cells, such as airway bronchial epithelial cells (ABECs), form a first line of defence against inhaled harmful mixtures such as CS. The inflammatory response, initiated by epithelial cells in the airways, is potentiated by immune cells, resulting in the amplification of the inflammation in airways (Panettieri 2004; Tamimi, Serdarevic et al. 2012). Chronic inflammation is a key characteristic of COPD that drives the pathophysiology of the disease and persists long after smoking cessation (Willemse, Postma et al. 2004). This suggests that oxidative stress could affect the course of gene expression through changes in epigenetic modifications such as DNA methylation and histone acetylation (Adcock, Cosio et al. 2005; Gu, Sun et al. 2012).

The NF-κB inflammatory pathway is one of the main signalling pathways activated in inflammatory cells and a master regulator of the transcription of inflammatory mediators including cytokines, chemokines, growth factors and proteases (Schreck and Baeuerle 1990; Salminen, Huuskonen et al. 2008). In addition, NF-κB is redox-sensitive and regulated by oxidants (Kirkham and Rahman 2006). Hydrogen peroxide (H_2O_2) is one of the many oxidants reported to be elevated in breath condensates of COPD patients and is associated with enhanced inflammation (Montuschi,

Kharitonov et al. 2003; Montuschi 2005). H_2O_2 is a product of the dismutation of superoxide, catalysed by the enzyme superoxide dismutase (SOD) (Imlay 2008). Superoxide is continuously produced as a by-product of mitochondrial respiration, but can also be produced from enzymes such as the NAD oxidases or xanthine oxidase (Elahi, Kong et al. 2009). H_2O_2 exerts its toxic effects through generation of highly reactive hydroxyl radical ($^{\cdot}OH$) that can react and modify macromolecules (DNA, proteins and lipids) (Ahsan, Ali et al. 2003; Negre-Salvayre, Coatrieux et al. 2008) and in doing so enhances an on-going inflammatory response. Inhaled corticosteroids (ICS) are frequently used in the management of inflammation in COPD patients; however, they provide little or no benefits to COPD patients and do not reduce inflammation in these patients due mainly to the continued presence of oxidative stress (Barnes 2011). Understanding the mechanisms underlying this enhanced inflammatory response and the lack of ICS response may provide novel potential therapeutic targets (Barnes 2011; Barnes 2012).

Abnormal histone acetylation profiles have been linked to smoke exposure (Liu, Killian et al. 2010) and to relative steroid unresponsiveness in COPD (Barnes, Adcock et al. 2005; Barnes 2011). The bromodomain and extra terminal domain (BET) protein Brd4 forms a complex with transcription initiation and elongation machinery that includes p-TEFb, RNA pol II and NF-κB transcription factor (Jang, Mochizuki et al. 2005; Yang, Yik et al. 2005). Acetylated histones provide a docking site for Brd4 which subsequently recruits other regulatory proteins to the promoter site of the targeted genes (Belkina and Denis 2012). Brd4 has high binding affinity for acetylated H3 and H4 that direct a diverse set of cellular instructions including the induction of inflammatory genes such as *IL-6* and *IL-8* (Turner 2000; Agalioti, Chen et al. 2002; An, Palhan et al. 2002). Therefore, inhibiting the formation of Brd4 and downstream regulatory complex through the inhibitors JQ-1 and PFI-1 could prevent inflammatory gene transcription.

Airway epithelial cells constitute the inner surface of the airways lumen and protect the airways against irritants such as oxidants (Kirkham, Spooner et al. 2004; Galli, Tsai et al. 2008). We have used

H_2O_2 in our *in vitro* models with the knowledge that it does not reflect the complex mixture of the stimulants seen in CS (Young, Hopkins et al. 2006; Barnes 2007). We hypothesised that the major effect of cigarette smoke is due to ROS thereby allowing use to use a simplifed model. We demonstrated the H_2O_2 (oxidant) significantly induced intracellular oxidative stress in BEAS-2Bs cells in concentration dependent-manner (Fig. 3.6). Therefore, BEAS-2B cells were used to establish both acute and chronic oxidative stress models using H_2O_2.

Acute high concentrations of H_2O_2 significantly exaggerated the response of inflammatory stimuli (TNF-α and IL-1β) on the expression of *IL-6* and *IL-8* mRNA and protein (Fig. 3.7B, 3.8B and 3.12). H_2O_2 alone had a minimal effect on either *IL-6* or *IL-8* expression. Interestingly, we observed that repeated exposure of low concentrations of H_2O_2 together with TNF-α amplified the production of CXCL8 and IL-6 cytokines to a similar extend as that seen with acute high H_2O_2 exposure (Fig. 3.7C and 3.8C). This implies that chronic low oxidative exposure alters the cell sensitivity and subsequent exposure to inflammatory stress resulting in a different pattern of inflammatory/immune gene expression. Oxidants in CS may be modifying the ability of airway epithelial cells to produce antimicrobial peptides such as mucins, defesins and antiproteases (Barnes 2004), making COPD patients and smokers more susceptible to respiratory infection and exacerbation (Dacydchenko and Bova 2007; Hurst, Vestbo et al. 2010). However, this sensitivity was diminished in cells exposed to 10 and 15 days of low concentration of H_2O_2 (Fig.3.7D/E and 3.8D/E).

This may be explained by the resistance developed by the cells over continuous exposure to oxidative stress. It has been reported by several groups that low oxidative stress enhances cell survival through induction of antioxidants systems (Collinson and Dawes 1992; Bose Girigoswami, Bhaumik et al. 2005). It is also possible that oxidative stress sensitive cells went under either necrosis or apoptosis following longer oxidative stress challenges. Others groups have used cell culture media supplemented with sodium pyruvate (110µg/ml) and uridine (100µg/ml), which allow cells to survive through an alternative anaerobic ATP generating source (Wang, Perez et al. 2007; Liu,

Geng et al. 2009) and protect cells against H_2O_2 (Giandomenico, Cerniglia et al. 1997; Jagtap, Chandele et al. 2003). It is also possible that chronic oxidative stress may have affected cell signalling or epigenetics events. Stable isotope labelling by amino acid (SILAC) and genome-wide DNAse 1 hypersensitivity assay might be used to examine cell signalling and epigenetics changes, respectively (Crawford, Holt et al. 2006).

An IKK selective inhibitor (AS602868) was used to confirm that NF-κB mediated the expression of *IL-8* and *IL-6* genes. AS602868 inhibitor completely suppressed the expression of both *IL-6* and *IL-8* cytokines in BEAS-B cells co-treated with IL-1β and H_2O_2 (Fig. 4.2). This was further confirmed by increased nuclear p65 translocation into the nucleus, acetylation and activation following co-stimulation with IL-1β and H_2O_2 (Fig. 4.3 and 4.4A). Post-translation modification (PTM) of histones such as acetylation is important in the regulation of inflammatory genes by the NF-κB pathway (Margueron and Reinberg ; Ito 2007; Kouzarides 2007). However, little is known about either p65 or histone modification by carbonylation/ protein oxidation. Therefore, we postulated that H_2O_2 may modulate gene expression through histone or p65 carbonylation.

There is accumulating evidence associating oxidative stress with the induction of protein carbonylation (England and Cotter 2004; Dalle-Donne, Aldini et al. 2006; Giustarini, Dalle-Donne et al. 2009) either by direct introduction of carbonyl groups (R-C=O-R`) into the side chain of amino acids or by addition of reactive aldehydes (MDA, 4HNE) released from lipid peroxidation process (Wong, Marcocci et al. 2010). The preliminary data showed a trend towards enhancing carbonyl-modified p65 carbonylation which was more prevalent in acute treated cells in comparison to chronic treatment (Fig. 3.9A). Again, we had similar issue as above. Therefore, to clarify the role of acute H_2O_2 in p65 carbonylation, we used higher concentrations of H_2O_2 and noticed a small increase in p65 carbonylation with increasing concentration of H_2O_2 (Fig. 3.10A/B). Intracellular oxidised proteins are constantly subjected to degradation (Grune, Reinheckel et al. 1996; Davies 2001). For example, HDAC2 is flagged for proteosomal degradation once nitrated (Meja, Rajendrasozhan et al.

2008; Osoata, Yamamura et al. 2009). Therefore, cells pre-treated with proteasome inhibitor (MG-132), showed a significant increase in p65-carbonylation (Fig. 3.10D). These findings suggest that carbonylation may be occurring during the "respiratory burst" that leads to excess ROS production during lung inflammation and further contributing to pathophysiology of COPD (Pettersen and Adler 2002; Merry 2004). Indeed, actin and myosin proteins from diaphragm of COPD patients have been reported to be carbonylated (Marin-Corral, Minguella et al. 2009).

In contrast, we did not observe histone carbonylation (3.9C/D). Histones are considered to be highly conserved proteins (Sandman and Reeve 2000) and may not be subjected to ad hoc enzymatic or proteosomal degradation. Alternatively, histone modification may require longer exposure to oxidative stress or any carbonylated histones are rapidly degraded but this could reflect itself an altered acetylation/methylation profile as the histone has been replaced *de nova* and is now reflecting the current environment. Indeed, Liu and colleagues have reported that histone acetylation of H4 at lysine 16 and methylation at lysine 20 is completely abrogated following 5 month exposure to CS in human small airway epithelial cells (SAEC) compared with 72 hours exposure (Gloire, Legrand-Poels et al. 2006; Liu, Killian et al. 2010). Similarly, DNA oxidation increases significantly in mice fibroblast exposed to CS for 6 months compare to acute exposure (3 days and 1 month) (Deslee, Adair-Kirk et al. 2010).

In addition, we also examined the effect of oxidative stress (H_2O_2) alone or in combination with an inflammatory stimulus (TNF-α) on H3 and H4 acetylation. In non-stimulated cells, DNA and histones are tightly packed together into stable heterochromatin structures (Adcock, Cosio et al. 2005; Adcock, Ford et al. 2006). Following stimulation, histones are acetylated, leading to the unwinding of DNA and histones (Eberharter and Becker 2002; Ito, Ito et al. 2005; Adcock, Tsaprouni et al. 2007). This open DNA-histone structure allows transcription factors (NF-κB) and co-regulatory proteins (Brd4) recruitment to the transcription site (Rahman and Adcock 2006; Chiang 2009; Huang, Yang et al. 2009). There was no change in global histone acetylation in either acutely or chronically treated

cells (Fig. 3.11). This wasn't a surprise as acetylation should be only associated with subset of actively transcribed genes (Ito, Hanazawa et al. 2004). Therefore, a gene specific approached (ChIP) was used to examine H3 and H4 acetylation at *IL-6* and *IL-8* promoters.

ChIP analysis revealed that H3 and not H4 is acetylated at the κB sites at both the *IL-6* and *IL-8* promoters following stimulation with H_2O_2 and IL-1β (Fig.4.7). This does not rule out a role for H4 acetylation during *IL-6* and *IL-8* expression. However, we only analyzed H4 acetylation at 2 hours post-stimulation and therefore; further analysis would be required at different time points to show whether H4 acetylation at the κB sites at the *IL-6* and *IL-8* promoters varied temporally. Although, H_2O_2 alone was unable to induce H3 or H4 acetylation directly in BEAS-2B cells, it has been previously shown that H_2O_2 exerts some of its effects by reducing HDAC2 activity in BEAS-2B cells (Ito, Hanazawa et al. 2004). This causes a shift in the balance towards HATs (CBP/p300) and delaying the removal of attached acetyl-groups on histones. The overall result is greater expression of *IL-6* and *IL-8* in cells co-stimulated with H_2O_2 and IL-1β. Hyper-acetylation of H3 and H4 at promoter regions of inflammatory gene is linked to gene activation (Yang, Valvo et al. 2008; Tsaprouni, Ito et al. 2011; Zhang, Cai et al. 2011). ChIP analysis also confirmed that both Brd4 and p65 are recruited to the κB site at the *IL-6* and *IL-8* promoters following combined IL-1β and H_2O_2 stimulation (Fig. 4.5 and 4.6). It has been shown that Brd4 is recruited to the promoter regions of the p65-induced genes TNF-α and E-selectin (Huang, Yang et al. 2009), however, the binding of Brd4 to the *IL-6* and *IL-8* κB-responsive sites in the promoters has not been previously reported to our knowledge. Furthermore, we have shown that acetylated p65 forms a complex with Brd4 (Fig. 4.4B).

BET family proteins such as Brd4 are essential regulatory proteins in the recognition of acetylated histones and p65 (Chiang 2009; Huang, Yang et al. 2009). It has been reported that Brd4 inhibition results in down-regulation of NF-κB targeted genes, suggesting its role in the induction and regulation of gene expression (Zhang, Liu et al. 2012). Bromodomain inhibitors, JQ-1 and PFI-1, significantly suppressed *IL-6* and *IL-8* expression in IL-1β and H_2O_2 treated cells in comparison to

untreated cells (Fig. 5.7 and 5.8). Intriguingly, although the occupancy of both p65 and Brd4 was completely ablated at both the *IL-6* and *IL-8* promoters the expression of neither *IL-6* nor *IL-8* returned to baseline levels in (+)-JQ1 pre-treated cells (Fig. 5.11). This discrepancy may be partly explained by the activation of other inflammatory pathways including p38 MAPK and JAK-STAT signalling pathways (Ono and Han 2000; Tron, Samoylenko et al. 2006; Sansone and Bromberg 2012). For example, the induction of *IL-6* involves the activation of both p38 and NF-κB pathways in BEAS-2B cells (Craig, Larkin et al. 2000; Wang, Wong et al. 2005). A time-course ChIP analysis should be performed to confirm the p65 and Brd4 binding duration in pre- and post- (+)-JQ-1 treatment. Furthermore, a more precise technique such as protein-binding microarray could be used to identify other binding κB sites that might be important (Bulyk 2007). Alternatively, a time course using Brd4-ChIP-Seq could be utilised to see whether all Brd4 DNA association is lost in the presence of (+)-JQ1.

Similar to our results, it has been shown that I-BET (a bromodamain inhibitor) interrupts the association between bromodomain proteins and acetylated histones at the *IL-6* and *TNF-*α promoters in LPS-stimulated macrophages leading to reduced *IL-6* and *TNF-*α expression. A novel finding of this study is that we have reported for the first time the anti-inflammatory properties of JQ-1 in airway epithelial cells. These findings have been confirmed in a number of other cells including smooth muscles cells, A549 and macrophages by other groups in our department. JQ-1 exhibits its anti-inflammatory properties by disrupting the interaction between Brd4 and acetylated histones and p65 on the *IL-6* and *IL-8* promoters. This interaction is vital step for facilitating the recruitment of RNA Pol II, p-TEFb and p65 during transcription initiation and elongation (Huang, Yang et al. 2009; Zhang, Liu et al. 2012). The knockdown studies reported here confirmed that Brd4 is required for maximal induction of *IL-6* and *IL-8* genes. There was significant reduction in both IL-6 and CXCL8 release in Brd4 deficient cells (Fig. 5.9). Furthermore, Brd2 knockdown had no effect on the expression of either cytokine, confirming that Brd4 rather than Brd2 is instrumental in the induction of inflammatory genes in these cells (Fig. 5.10). These findings are reinforced by a recent publication showing the co-localization of Brd4 and RNA pol II at the promoters and enhancer

regions of all active genes could be disrupted by JQ-1 treatment. This study also supported the notion that Brd4 could be used as tool to identify active genes in a genome-wide screen (Zhang, Prakash et al. 2012).

In conclusion (Fig. 6.1), I have shown in this thesis, that H_2O_2 enhances IL-1β- and TNF-α-induced expression of the inflammatory cytokines IL-6 and CXCL8. I have also demonstrated that whilst persistent exposure to low concentrations of H_2O_2 alone has no effect on IL-6 and CXCL8 release, it does however enhance the effect of TNF-α-induced IL-6 and CXCL8 expression.This data may explain the key role of long-term ROS stress on enhancing inflammation in COPD. I have shown that the induction of these cytokines is mediated through activation of the NF-κB pathway. The activation of these genes is associated with p65 and H3 acetylation and subsequent recruitment of both Brd4 and p65 to the appropriate κB sties in the *IL-6* and *IL-8* promoters. I have also shown that this induction can be reversed through either knockdown of Brd4 or by using synthetic Brd4 inhibitors JQ-1 and PFI-1. Brd4 provides a potential therapeutic target for the reduction of inflammation and synthetic compounds such as JQ-1 and PFI-1 may be used in treatment of inflammation where steroids show little benefit. The novel findings of this thesis are:

1. Repeated stimulation with low concentration of H_2O_2 is as potent as acute high concentration of H_2O_2
2. Brd4-p65 association is an integral part of inflammatory gene expression in BEAS-2B cells
3. Brd4 but not Brd2 is involved in the induction of *IL-6* and *IL-8* genes
4. JQ-1 and PFI-1 have anti-inflammatory properties

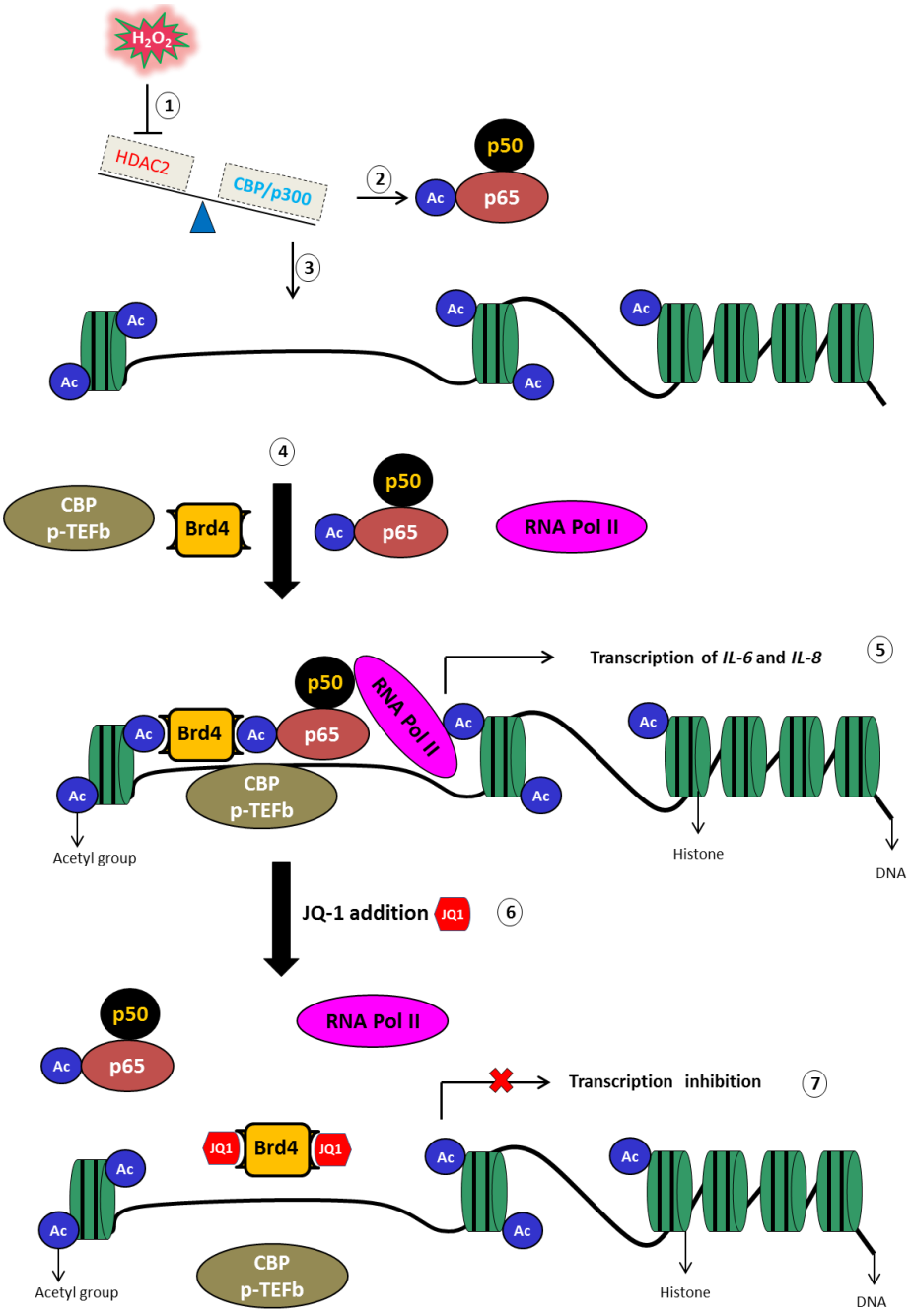


Figure 6.1: A summary of some of the findings in this thesis. (1) H_2O_2 reduces HDAC2 activity in Beas-2B cells (Ito, Hanazawa et al. 2004) allowing CPB/p300 (HAT) to acetylate **(2)** NF-κB p65 and **(3)** histones 3. **(4)** These acetylated proteins are recognised by Brd4 proteins. **(5)** Subsequently, Brd4 recruits p-TEFb and RNA pol II at the *IL-6* and *IL-8* promoters forming a part of transcription apparatus that leads to gene transcription (Jang, Mochizuki et al. 2005; Yang, Yik et al. 2005). **(6)** The introduction of JQ-1 disrupts the binding between Brd4 and acetylated proteins (Histones and p65) and **(7)** resulting in the disintegration of the transcription apparatus and gene suppression.

6.2 Future directions

Due to time constraints, we could not show a statistically significant difference in some experiments despite observing an obvious trend. The "n" numbers would need to be increased in order to perform a valid statistical analysis in these experiments.

We have used BEAS-2B cell lines in throughout this investigation and all findings are based on these cells. However, ideally these findings need to be replicated in primary cells from healthy and COPD patients. This may also establish a differing role/expression of brd4 protein in primary human bronchial epithelial cells (HBECs) in disease patients. In addition; we have used H_2O_2 as the source of oxidant to establish acute and chronic *in vitro* models of oxidative stress. Although, the presence of H_2O_2 in exhaled breath of COPD patients reflects the importance of H_2O_2 as an oxidant (Boots, Haenen et al. 2003), it is but one species observed *in vivo*. A plethora of other oxidants ($HO_2\cdot$, $\cdot OH$, HOCl), nitrites/nitrates (NOx) and carbonyls (4-HNE, MDA) are also generated in the lung of COPD patients (Pryor and Stone 1993; MacNee 2005; Negre-Salvayre, Coatrieux et al. 2008). Together, these harmful mediators may be more likely to drive the pathophysiology of the disease rather than a single oxidant by itself. Furthermore, H_2O_2 has a short-life and degrades quickly in most cell-culture media (Song, Driessens et al. 2007). Therefore, using H_2O_2 to establish a chronic model of oxidative stress may be not an ideal despite being used for chronic treatment previously (Bose, Bhaumik et al. 2003). A number of other H_2O_2 generated agents, for example glucose oxidase, KO_2, or menadione, could be used to maintain the continuous supply of H_2O_2 (Arai, Endo et al. 2001; Loor, Kondapalli et al. 2010; Ho, Asagiri et al. 2011; Choe, Yu et al. 2012). Alternatively, chronic exposure to ozone may provide a good model (Triantaphyllopoulos, Hussain et al. 2011)

It is important to investigate whether JQ-1 and PFI-1 can modulate the expression of other inflammatory genes in addition to *IL-6* and *IL-8*. Microarray analysis or deep sequencing ideally in primary HBECs can be applied to RNA samples isolated from cells pre-treated with bromodomain inhibitors under various experimental conditions. In the experiments described here, we examined

the association between p65 and Brd4 only. However, transcription factors such as AP-1 and Nrf2 may also be examined using Co-IP. Similarly, the role of other BET family proteins as such Brd2, Brd3 and Brtd should be investigated. This will further enhance our understanding of their role in gene regulation. Importantly, the apparent discrepancy between the effects of JQ-1 and PFI-1 and Brd4 knockdown on IL-6 and CXCL8 protein expression and the complete loss of Brd4 and p65 from their respective promoters need to be investigated. It is also important to establish whether another pathway such as p38 MAPK takes over when JQ-1 is present.

The present findings suggest that only H3 is acetylated. However, other studies have shown that individual acetylated-lysine residues (H3K14, H4K5, 12, 18) are crucial for Brd4 binding (Dey, Chitsaz et al. 2003). The diverse acetylated-lysine residues in H3 and H4 are more likely to reflect both gene specific acetylation and regulation at specific time point analysed. Therefore, it would be invaluable to examine in concert a number of individual acetylated lysine residues both on H3 or H4 to give an insight into gene specific activation in relation to acetylation of individual lysine residue. In addition, DNA and histone methylation has been shown to be involved in epigenetic regulation of gene expression (Siegfried and Simon 2010). The use of H3K27me3 mimics, for example GSK05, to block this repressive tag of specific histone methyltrasferases may provide useful data. Increased histone acetylation, modulation of histone methylation and decreased DNA methylation of CpG islands at the promoter regions of inflammatory gene is correlated with elevated gene expression (Kabesch and Adcock 2012).

Parental cigarette smoke exposure has been associated with global and gene-specific DNA methylation changes in children (Breton, Byun et al. 2009). Oxidative stress (H_2O_2) is reported to recruit DNMT1 at the promoters, resulting in changes in DNA methylation patterns and gene expression (O'Hagan, Wang et al. 2011). Similar experiments could be conducted using direct sequencing after bisulphite treatment or by methylation dependent immuneprecipitation (MeDIP) to investigate the effect of H_2O_2 on DNA methylation in the CpG islands and shores across the IL-*6*

and *IL-8* promoters and coding regions. Genome-wide changes in hydroxymethylated cytosines may also provise additional information concern the regulation of inflammatory genes in the presence of oxidative stress.

Steroids are widely used as an anti-inflammatory drug. However, they fail to exert their anti-inflammatory effects in severe asthmatics and COPD patients (Barnes 2012) which has been attributed to the impact of oxidative stress. It would therefore be important to identify the role of oxidative stress in steroid unresponsiveness in our model and to ascertain whether corticosteroids predominantly target the epigenome or rewire/modify intracellular signalling processes. A comparison of ChIP-Seq, transcriptomic and proteomic (SILAC) analysis may help clarify this.

Publications

1. Paul A. Kirkham, Gaetano Caramori, Paolo Casolari, Alberto A. Papi, Matt Edwards, Betty Shamji, Kostas Triantaphyllopoulos, Farhana Hussain, Mariona Pinart, **Younis Khan,** Lucy Heinemann, Laurie Stevens, Mike Yeadon, Peter J. Barnes, Kian F. Chung and Ian M. Adcock. Oxidative Stress-induced antibodies to Carbonyl-modified Protein correlate with Severity of Chronic Obstructive Pulmonary Disease. *Am. J. Respir.Crit. Care Med. 2011; 184: 796-802.*

2. **Younis Khan**, Ian M. Adcock and Paul Kirkham. Acute and Chronic Hydrogen Peroxide (H_2O_2) Treatement induced proinflammatory mediators in Epithelial Cells (BEAS-2B) *Am. J. Respir. Crit. Care Med. 2011; 183: A2796.*

3. Brd4 but not Brd2, is essential for oxidative stress driven inflammation in human epithelial cells (in *preparation*).

4. Bromodomain inhibitors and the control of inflammation in COPD and Severe Asthma. (in preparation)

References:

Aaron, S. D., J. B. Angel, et al. (2001). "Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **163**(2): 349-355.

Abboud, R. T., A. F. Ofulue, et al. (1998). "Relationship of alveolar macrophage plasminogen activator and elastase activities to lung function and CT evidence of emphysema." Chest **113**(5): 1257-1263.

Abboud, R. T. and S. Vimalanathan (2008). "Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema." Int J Tuberc Lung Dis **12**(4): 361-367.

Adcock, I. M. (1997). "Transcription factors as activators of gene transcription: AP-1 and NF-kappa B." Monaldi Arch Chest Dis **52**(2): 178-186.

Adcock, I. M., P. C. Chou, et al. (2009). "Overcoming steroid unresponsiveness in airways disease." Biochem Soc Trans **37**(Pt 4): 824-829.

Adcock, I. M., B. Cosio, et al. (2005). "Redox regulation of histone deacetylases and glucocorticoid-mediated inhibition of the inflammatory response." Antioxid Redox Signal **7**(1-2): 144-152.

Adcock, I. M., P. Ford, et al. (2006). "Epigenetics and airways disease." Respir Res **7**: 21.

Adcock, I. M. and K. Ito (2005). "Glucocorticoid pathways in chronic obstructive pulmonary disease therapy." Proc Am Thorac Soc **2**(4): 313-319; discussion 340-311.

Adcock, I. M., K. Ito, et al. (2004). "Glucocorticoids: effects on gene transcription." Proc Am Thorac Soc **1**(3): 247-254.

Adcock, I. M., L. Tsaprouni, et al. (2007). "Epigenetic regulation of airway inflammation." Curr Opin Immunol **19**(6): 694-700.

Agalioti, T., G. Y. Chen, et al. (2002). "Deciphering the transcriptional histone acetylation code for a human gene." Cell **111**(3): 381-392.

Agusti, A., W. MacNee, et al. (2003). "Hypothesis: does COPD have an autoimmune component?" Thorax **58**(10): 832-834.

Ahsan, H., A. Ali, et al. (2003). "Oxygen free radicals and systemic autoimmunity." Clin Exp Immunol **131**(3): 398-404.

Aksenova, M. V., M. Y. Aksenov, et al. (2005). "Cell culture models of oxidative stress and injury in the central nervous system." Curr Neurovasc Res **2**(1): 73-89.

Almqvist, N. and I. L. Martensson (2012). "The pre-B cell receptor; selecting for or against autoreactivity." Scand J Immunol **76**(3): 256-262.

Amir-Zilberstein, L., E. Ainbinder, et al. (2007). "Differential regulation of NF-kappaB by elongation factors is determined by core promoter type." Mol Cell Biol **27**(14): 5246-5259.

An, W. J., V. B. Palhan, et al. (2002). "Selective requirements for histone H3 and H4N termini in p300-dependent transcriptional activation from chromatin." Molecular Cell **9**(4): 811-821.

Antunes, F. and E. Cadenas (2001). "Cellular titration of apoptosis with steady state concentrations of H(2)O(2): submicromolar levels of H(2)O(2) induce apoptosis through Fenton chemistry independent of the cellular thiol state." Free Radic Biol Med **30**(9): 1008-1018.

Arai, T., N. Endo, et al. (2001). "6-formylpterin, a xanthine oxidase inhibitor, intracellularly generates reactive oxygen species involved in apoptosis and cell proliferation." Free Radic Biol Med **30**(3): 248-259.

Arcavi, L. and N. L. Benowitz (2004). "Cigarette smoking and infection." Arch Intern Med **164**(20): 2206-2216.

Awane, M., P. G. Andres, et al. (1999). "NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells." J Immunol **162**(9): 5337-5344.

Bannister, A. J. and E. A. Miska (2000). "Regulation of gene expression by transcription factor acetylation." Cell Mol Life Sci **57**(8-9): 1184-1192.

Barboric, M., R. M. Nissen, et al. (2001). "NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II." Mol Cell **8**(2): 327-337.

Barnes, P. J. (2003). "New concepts in chronic obstructive pulmonary disease." Annu Rev Med **54**: 113-129.

Barnes, P. J. (2004). "Alveolar macrophages as orchestrators of COPD." COPD **1**(1): 59-70.

Barnes, P. J. (2004). "COPD: is there light at the end of the tunnel?" Curr Opin Pharmacol **4**(3): 263-272.

Barnes, P. J. (2004). "Mediators of chronic obstructive pulmonary disease." Pharmacol Rev **56**(4): 515-548.

Barnes, P. J. (2006). "How corticosteroids control inflammation: Quintiles Prize Lecture 2005." Br J Pharmacol **148**(3): 245-254.

Barnes, P. J. (2007). "Chronic obstructive pulmonary disease: a growing but neglected global epidemic." PLoS Med **4**(5): e112.

Barnes, P. J. (2008). "The cytokine network in asthma and chronic obstructive pulmonary disease." J Clin Invest **118**(11): 3546-3556.

Barnes, P. J. (2008). Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol. **8:** 183-192.

Barnes, P. J. (2009). "The cytokine network in chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **41**(6): 631-638.

Barnes, P. J. (2009). "Targeting the epigenome in the treatment of asthma and chronic obstructive pulmonary disease." Proc Am Thorac Soc **6**(8): 693-696.

Barnes, P. J. (2010). "New therapies for chronic obstructive pulmonary disease." Med Princ Pract **19**(5): 330-338.

Barnes, P. J. (2011). "Glucocorticosteroids: current and future directions." Br J Pharmacol **163**(1): 29-43.

Barnes, P. J. (2012). "Development of New Drugs for COPD." Curr Med Chem. Sep 3. [Epub ahead of print] PMID: 22963554

Barnes, P. J. and I. M. Adcock (2003). "How do corticosteroids work in asthma?" Ann Intern Med **139**(5 Pt 1): 359-370.

Barnes, P. J. and I. M. Adcock (2009). "Glucocorticoid resistance in inflammatory diseases." Lancet **373**(9678): 1905-1917.

Barnes, P. J., I. M. Adcock, et al. (2005). "Histone acetylation and deacetylation: importance in inflammatory lung diseases." Eur Respir J **25**(3): 552-563.

Barnes, P. J., S. D. Shapiro, et al. (2003). "Chronic obstructive pulmonary disease: molecular and cellular mechanisms." Eur Respir J **22**(4): 672-688.

Barnes, P. J. and R. A. Stockley (2005). "COPD: current therapeutic interventions and future approaches." Eur Respir J **25**(6): 1084-1106.

Bednarek, M., D. Gorecka, et al. (2006). "Smokers with airway obstruction are more likely to quit smoking." Thorax **61**(10): 869-873.

Belkina, A. C. and G. V. Denis (2012). "BET domain co-regulators in obesity, inflammation and cancer." Nat Rev Cancer **12**(7): 465-477.

Berndt, A., A. S. Leme, et al. (2012). "Emerging genetics of COPD." EMBO Mol Med **4**(11): 1144-1155.

Betsuyaku, T., S. Fuke, et al. (2012). "Bronchiolar epithelial catalase is diminished in smokers with mild COPD." Eur Respir J. Oct 25. [Epub ahead of print] PMID: 23100509.

Bhome, A. B. (2012). "COPD in India: Iceberg or volcano?" J Thorac Dis **4**(3): 298-309.

Biddie, S. C., S. John, et al. (2011). "Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding." Mol Cell **43**(1): 145-155.

Biswas, S., J. W. Hwang, et al. (2012). "Pharmacologial and Dietary Antioxidant Therapies for Chronic Obstructive Pulmonary Disease." Curr Med Chem. Sep 3. [Epub ahead of print] PMID: 22963552.

Bonarius, H. P., C. A. Brandsma, et al. (2011). "Antinuclear autoantibodies are more prevalent in COPD in association with low body mass index but not with smoking history." Thorax **66**(2): 101-107.

Boone, D. L., E. E. Turer, et al. (2004). "The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses." Nat Immunol **5**(10): 1052-1060.

Boots, A. W., G. R. Haenen, et al. (2003). "Oxidant metabolism in chronic obstructive pulmonary disease." Eur Respir J Suppl **46**: 14s-27s.

Borger, P., B. Oliver, et al. (2012). "Beyond the Immune System: The Role of Resident Cells in Asthma and COPD." J Allergy (Cairo) **2012**: 968039.

Borregaard, N. (2010). "Neutrophils, from marrow to microbes." Immunity **33**(5): 657-670.

Bose Girigoswami, K., G. Bhaumik, et al. (2005). "Induced resistance in cells exposed to repeated low doses of H2O2 involves enhanced activity of antioxidant enzymes." Cell Biol Int **29**(9): 761-767.

Bose, K., G. Bhaumik, et al. (2003). "Chronic low dose exposure to hydrogen peroxide changes sensitivity of V79 cells to different damaging agents." Indian J Exp Biol **41**(8): 832-836.

Bosse, Y. (2012). "Updates on the COPD gene list." Int J Chron Obstruct Pulmon Dis **7**: 607-631.

Breton, C. V., H. M. Byun, et al. (2009). "Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation." Am J Respir Crit Care Med **180**(5): 462-467.

Brightling, C. E., S. Gupta, et al. (2011). "Immunopathogenesis of severe asthma." Curr Pharm Des **17**(7): 667-673.

Broaddus, V. C., C. A. Hebert, et al. (1992). "Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema." Am Rev Respir Dis **146**(4): 825-830.

Brode, S. K., S. C. Ling, et al. (2012). "Alpha-1 antitrypsin deficiency: a commonly overlooked cause of lung disease." CMAJ **184**(12): 1365-1371.

Brown, K. D., E. Claudio, et al. (2008). "The roles of the classical and alternative nuclear factor-kappaB pathways: potential implications for autoimmunity and rheumatoid arthritis." Arthritis Res Ther **10**(4): 212.

Brusselle, G. G., G. F. Joos, et al. (2011). "New insights into the immunology of chronic obstructive pulmonary disease." Lancet **378**(9795): 1015-1026.

Bugl, S., S. Wirths, et al. (2012). "Current insights into neutrophil homeostasis." Ann N Y Acad Sci **1266**: 171-178.

Buist, A. S., M. A. McBurnie, et al. (2007). "International variation in the prevalence of COPD (the BOLD Study): a population-based prevalence study." Lancet **370**(9589): 741-750.

Bulyk, M. L. (2007). "Protein binding microarrays for the characterization of DNA-protein interactions." Adv Biochem Eng Biotechnol **104**: 65-85.

Cai, C., F. N. Jiang, et al. (2011). "Classical and alternative nuclear factor-kappaB pathways: a comparison among normal prostate, benign prostate hyperplasia and prostate cancer." Pathol Oncol Res **17**(4): 873-878.

Calder, P. C. (2009). "Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale." Biochimie **91**(6): 791-795.

Camoretti-Mercado, B. (2009). "Targeting the airway smooth muscle for asthma treatment." Transl Res **154**(4): 165-174.

Capelli, A., A. Di Stefano, et al. (1999). "Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics." Eur Respir J **14**(1): 160-165.

Carroll, T. P., C. M. Greene, et al. (2005). "Viral inhibition of IL-1- and neutrophil elastase-induced inflammatory responses in bronchial epithelial cells." J Immunol **175**(11): 7594-7601.

Cazzola, M. and M. G. Matera (2012). "IL-17 in chronic obstructive pulmonary disease." Expert Rev Respir Med **6**(2): 135-138.

Chan, H. L., H. C. Chou, et al. (2010). "Major role of epidermal growth factor receptor and Src kinases in promoting oxidative stress-dependent loss of adhesion and apoptosis in epithelial cells." J Biol Chem **285**(7): 4307-4318.

Chang, P. J., P. K. Bhavsar, et al. (2012). "Corticosteroid insensitivity of chemokine expression in airway smooth muscle of patients with severe asthma." J Allergy Clin Immunol **130**(4): 877-885 e875.

Chaouat, A., L. Savale, et al. (2009). "Role for interleukin-6 in COPD-related pulmonary hypertension." Chest **136**(3): 678-687.

Chen, L. F., Y. Mu, et al. (2002). "Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB." EMBO J **21**(23): 6539-6548.

Chen, L. F., S. A. Williams, et al. (2005). "NF-kappaB RelA phosphorylation regulates RelA acetylation." Mol Cell Biol **25**(18): 7966-7975.

Chen, Y., P. Chen, et al. (2008). "Enhanced levels of prostaglandin E2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD." Respirology **13**(7): 1014-1021.

Chiang, C. M. (2009). "Brd4 engagement from chromatin targeting to transcriptional regulation: selective contact with acetylated histone H3 and H4." F1000 Biol Rep **1**: 98.

Choe, Y., J. Y. Yu, et al. (2012). "Continuously generated H2O2 stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts." J Cell Biochem **113**(4): 1426-1436.

Chung, K. F. (2001). "Cytokines in chronic obstructive pulmonary disease." Eur Respir J Suppl **34**: 50s-59s.

Chung, K. F. (2005). "The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease." Proc Am Thorac Soc **2**(4): 347-354; discussion 371-342.

Chung, K. F. and I. M. Adcock (2008). "Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction." Eur Respir J **31**(6): 1334-1356.

Chung, K. F. and J. A. Marwick "Molecular mechanisms of oxidative stress in airways and lungs with reference to asthma and chronic obstructive pulmonary disease." Ann N Y Acad Sci **1203**: 85-91.

Chung, K. F. and P. J. Sterk (2000). "The airway smooth muscle cell: a major contributor to asthma?" Eur Respir J **15**(3): 438-439.

Church, D. F. and W. A. Pryor (1985). "Free-radical chemistry of cigarette smoke and its toxicological implications." Environ Health Perspect **64**: 111-126.

Churg, A., M. Cosio, et al. (2008). "Mechanisms of cigarette smoke-induced COPD: insights from animal models." Am J Physiol Lung Cell Mol Physiol **294**(4): L612-631.

Clancy, J. and M. Nobes (2012). "Chronic obstructive pulmonary disease: nature-nurture interactions." Br J Nurs **21**(13): 772-781.

Collinson, L. P. and I. W. Dawes (1992). "Inducibility of the response of yeast cells to peroxide stress." J Gen Microbiol **138**(2): 329-335.

Conti, P. and M. DiGioacchino (2001). "MCP-1 and RANTES are mediators of acute and chronic inflammation." Allergy Asthma Proc **22**(3): 133-137.

Craig, R., A. Larkin, et al. (2000). "p38 MAPK and NF-kappa B collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system." J Biol Chem **275**(31): 23814-23824.

Crawford, G. E., I. E. Holt, et al. (2006). "Genome-wide mapping of DNase hypersensitive sites using massively parallel signature sequencing (MPSS)." Genome Res **16**(1): 123-131.

Crooks, S. W., D. L. Bayley, et al. (2000). "Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4." Eur Respir J **15**(2): 274-280.

Culpitt, S. V., D. F. Rogers, et al. (2003). "Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **167**(1): 24-31.

Dacydchenko, S. V. and A. A. Bova (2007). "[The role of respiratory infection in exacerbation of chronic obstructive pulmonary disease]." Klin Med (Mosk) **85**(5): 10-15.

Daldegan, M. B., M. M. Teixeira, et al. (2005). "Concentration of CCL11, CXCL8 and TNF-alpha in sputum and plasma of patients undergoing asthma or chronic obstructive pulmonary disease exacerbation." Braz J Med Biol Res **38**(9): 1359-1365.

Dalle-Donne, I., G. Aldini, et al. (2006). "Protein carbonylation, cellular dysfunction, and disease progression." J Cell Mol Med **10**(2): 389-406.

Dalle-Donne, I., R. Rossi, et al. (2003). "Protein carbonyl groups as biomarkers of oxidative stress." Clin Chim Acta **329**(1-2): 23-38.

Daly, M. M., A. E. Mirsky, et al. (1951). "The amino acid composition and some properties of histones." J Gen Physiol **34**(4): 439-450.

Davies, K. J. (2001). "Degradation of oxidized proteins by the 20S proteasome." Biochimie **83**(3-4): 301-310.

Dawson, M. A., T. Kouzarides, et al. (2012). "Targeting epigenetic readers in cancer." N Engl J Med **367**(7): 647-657.

Decramer, M., W. Janssens, et al. (2012). "Chronic obstructive pulmonary disease." Lancet **379**(9823): 1341-1351.

Dejardin, E. (2006). "The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development." Biochem Pharmacol **72**(9): 1161-1179.

Dekhuijzen, P. N., K. K. Aben, et al. (1996). "Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease." Am J Respir Crit Care Med **154**(3 Pt 1): 813-816.

Delmore, J. E., G. C. Issa, et al. (2011). "BET bromodomain inhibition as a therapeutic strategy to target c-Myc." Cell **146**(6): 904-917.

Department of Health (2010)."Facts about COPD(2010)." from www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_113006.

Department of Health (2011). "An outcomes strategy for people with chronic obstructive pulmonary disease (COPD) and asthma in England." from http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_127974.

Deslee, G., T. L. Adair-Kirk, et al. (2010). "Cigarette smoke induces nucleic-Acid oxidation in lung fibroblasts." Am J Respir Cell Mol Biol **43**(5): 576-584.

Deveci, Y., F. Deveci, et al. (2010). "[Serum ghrelin, IL-6 and TNF-alpha levels in patients with chronic obstructive pulmonary disease.]." Tuberk Toraks **58**(2): 162-172.

Dewar, M. and R. W. Curry, Jr. (2006). "Chronic obstructive pulmonary disease: diagnostic considerations." Am Fam Physician **73**(4): 669-676.

Dey, A., F. Chitsaz, et al. (2003). "The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis." Proc Natl Acad Sci U S A **100**(15): 8758-8763.

Di Stefano, A., G. Caramori, et al. (2009). "Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD." Thorax **64**(11): 968-975.

Di Stefano, A., G. Caramori, et al. (2009). "T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients." Clin Exp Immunol **157**(2): 316-324.

Di Stefano, A., G. Caramori, et al. (2002). "Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD." Eur Respir J **20**(3): 556-563.

Di Stefano, A., G. Caramori, et al. (2004). "Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview." Clin Exp Allergy **34**(8): 1156-1167.

Dinarello, C. A. (2000). "Proinflammatory cytokines." Chest **118**(2): 503-508.

Donnelly, L. E. and P. J. Barnes (2006). "Chemokine receptors as therapeutic targets in chronic obstructive pulmonary disease." Trends Pharmacol Sci **27**(10): 546-553.

Donnelly, L. E. and P. J. Barnes (2012). "Defective phagocytosis in airways disease." Chest **141**(4): 1055-1062.

Donohue, J. F. (2006). "Ageing, smoking and oxidative stress." Thorax **61**(6): 461-462.
Durham, A., I. M. Adcock, et al. (2011). "Steroid resistance in severe asthma: current mechanisms and future treatment." Curr Pharm Des **17**(7): 674-684.
Eberharter, A. and P. B. Becker (2002). "Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics." EMBO Rep **3**(3): 224-229.
Edwards, M. R., N. W. Bartlett, et al. (2009). "Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease." Pharmacol Ther **121**(1): 1-13.
Elahi, M. M., Y. X. Kong, et al. (2009). "Oxidative stress as a mediator of cardiovascular disease." Oxid Med Cell Longev **2**(5): 259-269.
Enesa, K., K. Ito, et al. (2008). "Hydrogen peroxide prolongs nuclear localization of NF-kappaB in activated cells by suppressing negative regulatory mechanisms." J Biol Chem **283**(27): 18582-18590.
England, K. and T. Cotter (2004). "Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER." Biochem Biophys Res Commun **320**(1): 123-130.
Eustace, A., L. J. Smyth, et al. (2011). "Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD." Chest **139**(5): 1089-1100.
Feghali-Bostwick, C. A., A. S. Gadgil, et al. (2008). "Autoantibodies in patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **177**(2): 156-163.
Feghali, C. A. and T. M. Wright (1997). "Cytokines in acute and chronic inflammation." Front Biosci **2**: d12-26.
Fernandez, E. J. and E. Lolis (2002). "Structure, function, and inhibition of chemokines." Annu Rev Pharmacol Toxicol **42**: 469-499.
Filippakopoulos, P. and S. Knapp (2012). "The bromodomain interaction module." FEBS Lett **586**(17): 2692-2704.
Filippakopoulos, P., J. Qi, et al. (2010). "Selective inhibition of BET bromodomains." Nature **468**(7327): 1067-1073.
Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature **408**(6809): 239-247.
Fischer, B. M., E. Pavlisko, et al. (2011). "Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation." Int J Chron Obstruct Pulmon Dis **6**: 413-421.
Flattery-O'Brien, J. A. and I. W. Dawes (1998). "Hydrogen peroxide causes RAD9-dependent cell cycle arrest in G2 in Saccharomyces cerevisiae whereas menadione causes G1 arrest independent of RAD9 function." J Biol Chem **273**(15): 8564-8571.
Fletcher, C. and R. Peto (1977). "The natural history of chronic airflow obstruction." Br Med J **1**(6077): 1645-1648.
Flohe, L. (2012). "The fairytale of the GSSG/GSH redox potential." Biochim Biophys Acta. Nov 2. doi:pii: S0304-4165(12)00306-6. 10.1016/j.bbagen.2012.10.020. [Epub ahead of print]
Foran, E., M. M. Garrity-Park, et al. "Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6." Mol Cancer Res **8**(4): 471-481.
Foreman, M. G., M. Campos, et al. (2012). "Genes and chronic obstructive pulmonary disease." Med Clin North Am **96**(4): 699-711.
Forteza, R. M., S. M. Casalino-Matsuda, et al. (2012). "Hyaluronan and Layilin Mediate Loss of Airway Epithelial Barrier Function Induced by Cigarette Smoke by Decreasing E-cadherin." J Biol Chem **287**(50): 42288-42298.
Fox, L., S. Hegde, et al. (2010). "Natural killer T cells: innate lymphocytes positioned as a bridge between acute and chronic inflammation?" Microbes Infect **12**(14-15): 1125-1133.
Franco, R., O. Schoneveld, et al. (2008). "Oxidative stress, DNA methylation and carcinogenesis." Cancer Lett **266**(1): 6-11.

Frankenberger, M., M. Menzel, et al. (2004). "Characterization of a population of small macrophages in induced sputum of patients with chronic obstructive pulmonary disease and healthy volunteers." Clin Exp Immunol **138**(3): 507-516.

French, C. A. (2010). "NUT midline carcinoma." Cancer Genet Cytogenet **203**(1): 16-20.

Fujimoto, K., M. Yasuo, et al. (2005). "Airway inflammation during stable and acutely exacerbated chronic obstructive pulmonary disease." Eur Respir J **25**(4): 640-646.

Fukuda, H., N. Sano, et al. (2006). "Simple histone acetylation plays a complex role in the regulation of gene expression." Brief Funct Genomic Proteomic **5**(3): 190-208.

Gadek, J. E., G. A. Fells, et al. (1981). "Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema." J Clin Invest **68**(4): 889-898.

Galli, S. J., M. Tsai, et al. (2008). "The development of allergic inflammation." Nature **454**(7203): 445-454.

Garcia-Gimenez, J. L., A. M. Ledesma, et al. (2012). "Histone carbonylation occurs in proliferating cells." Free Radic Biol Med **52**(8): 1453-1464.

Geissmann, F., M. G. Manz, et al. (2010). "Development of monocytes, macrophages, and dendritic cells." Science **327**(5966): 656-661.

Gerondakis, S. and U. Siebenlist (2010). "Roles of the NF-kappaB pathway in lymphocyte development and function." Cold Spring Harb Perspect Biol **2**(5): a000182.

Gershon, A. S., C. Wang, et al. (2010). "Trends in chronic obstructive pulmonary disease prevalence, incidence, and mortality in ontario, Canada, 1996 to 2007: a population-based study." Arch Intern Med **170**(6): 560-565.

Gershon, A. S., L. Warner, et al. (2011). "Lifetime risk of developing chronic obstructive pulmonary disease: a longitudinal population study." Lancet **378**(9795): 991-996.

Ghavami, A., K. L. Stark, et al. (1999). "Differential addressing of 5-HT1A and 5-HT1B receptors in epithelial cells and neurons." J Cell Sci **112 (Pt 6)**: 967-976.

Ghio, A. J., E. D. Hilborn, et al. (2008). "Particulate matter in cigarette smoke alters iron homeostasis to produce a biological effect." Am J Respir Crit Care Med **178**(11): 1130-1138.

Ghio, A. J., J. G. Stonehuerner, et al. (2008). "Iron homeostasis and oxidative stress in idiopathic pulmonary alveolar proteinosis: a case-control study." Respir Res **9**: 10.

Giandomenico, A. R., G. E. Cerniglia, et al. (1997). "The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide." Free Radic Biol Med **23**(3): 426-434.

Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-6684.

Giustarini, D., I. Dalle-Donne, et al. (2009). "Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers." Crit Rev Clin Lab Sci **46**(5-6): 241-281.

Gloire, G., E. Charlier, et al. (2006). "Restoration of SHIP-1 activity in human leukemic cells modifies NF-kappaB activation pathway and cellular survival upon oxidative stress." Oncogene **25**(40): 5485-5494.

Gloire, G., S. Legrand-Poels, et al. (2006). "NF-kappaB activation by reactive oxygen species: fifteen years later." Biochem Pharmacol **72**(11): 1493-1505.

GOLD. (2011). "Global Initiative for Chronic Obstructive Lung Disease (2011) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease."from:http://www.goldcopd.org/uploads/users/files/GOLD_Report_2011_Feb21.pdf.

Goransson, M., E. Elias, et al. (2005). "Myxoid liposarcoma FUS-DDIT3 fusion oncogene induces C/EBP beta-mediated interleukin 6 expression." Int J Cancer **115**(4): 556-560.

Gosman, M. M., B. W. Willemse, et al. (2006). "Increased number of B-cells in bronchial biopsies in COPD." Eur Respir J **27**(1): 60-64.

Greene, C. M. and N. G. McElvaney (2009). "Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery." Br J Pharmacol **158**(4): 1048-1058.

Grubek-Jaworska, H., M. Paplinska, et al. (2012). "IL-6 and IL-13 in induced sputum of COPD and asthma patients: correlation with respiratory tests." Respiration **84**(2): 101-107.

Gruffydd-Jones, K. (2008). "A national strategy for the management of chronic obstructive pulmonary disease (COPD) in England: aiming to improve the quality of care for patients." Prim Care Respir J **17 Suppl 1**: S1-8.

Grune, T., T. Reinheckel, et al. (1996). "Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome." J Biol Chem **271**(26): 15504-15509.

Gu, X., J. Sun, et al. (2012). "Oxidative stress induces DNA demethylation and histone acetylation in SH-SY5Y cells: potential epigenetic mechanisms in gene transcription in Abeta production." Neurobiol Aging. Nov 8. pii: S0197-4580(12)00524-6. doi: 10.1016/j.neurobiolaging.2012.10.013. [Epub ahead of print].

Guilleret, I. and J. Benhattar (2003). "Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres." Exp Cell Res **289**(2): 326-334.

Hakim, A., I. M. Adcock, et al. (2012). "Corticosteroid resistance and novel anti-inflammatory therapies in chronic obstructive pulmonary disease: current evidence and future direction." Drugs **72**(10): 1299-1312.

Hall, E. H., J. L. Balsbaugh, et al. (2010). "Comprehensive analysis of phosphorylation sites in Tensin1 reveals regulation by p38MAPK." Mol Cell Proteomics **9**(12): 2853-2863.

Hallgren, O., K. Nihlberg, et al. (2010). "Altered fibroblast proteoglycan production in COPD." Respir Res **11**: 55.

Hanania, N. A., N. Ambrosino, et al. (2005). "Treatments for COPD." Respir Med **99**: S28-S40.

Hayashi, R., H. Wada, et al. (2004). "Effects of glucocorticoids on gene transcription." Eur J Pharmacol **500**(1-3): 51-62.

Hayden, M. S. and S. Ghosh (2004). "Signaling to NF-kappaB." Genes Dev **18**(18): 2195-2224.

Hayden, M. S., A. P. West, et al. (2006). "NF-kappaB and the immune response." Oncogene **25**(51): 6758-6780.

Hayes, J. D., J. U. Flanagan, et al. (2005). "Glutathione transferases." Annu Rev Pharmacol Toxicol **45**: 51-88.

He, J. Q., M. G. Foreman, et al. (2009). "Associations of IL6 polymorphisms with lung function decline and COPD." Thorax **64**(8): 698-704.

Hewings, D. S., T. P. Rooney, et al. (2012). "Progress in the Development and Application of Small Molecule Inhibitors of Bromodomain-Acetyl-lysine Interactions." J Med Chem. Nov 26;55(22):9393-413.

Ho, J. Q., M. Asagiri, et al. (2011). "NF-kappaB potentiates caspase independent hydrogen peroxide induced cell death." PLoS One **6**(2): e16815.

Ho, S. C., K. Y. Lee, et al. (2009). "Neutrophil elastase represses IL-8/CXCL8 synthesis in human airway smooth muscle cells through induction of NF-kappa B repressing factor." J Immunol **183**(1): 411-420.

Hoberg, J. E., A. E. Popko, et al. (2006). "IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300." Mol Cell Biol **26**(2): 457-471.

Hodge, D. R., W. Xiao, et al. (2001). "Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells." J Biol Chem **276**(43): 39508-39511.

Hogg, J. C. (2004). "Pathophysiology of airflow limitation in chronic obstructive pulmonary disease." Lancet **364**(9435): 709-721.

Hogg, J. C. (2006). "Why does airway inflammation persist after the smoking stops?" Thorax **61**(2): 96-97.

Holgate, S. T. (2007). "Epithelium dysfunction in asthma." J Allergy Clin Immunol **120**(6): 1233-1244; quiz 1245-1236.

Hollingshead, B. D., T. V. Beischlag, et al. (2008). "Inflammatory signaling and aryl hydrocarbon receptor mediate synergistic induction of interleukin 6 in MCF-7 cells." Cancer Res **68**(10): 3609-3617.

Huang, B., X. D. Yang, et al. (2010). "Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway." Cell Signal **22**(9): 1282-1290.

Huang, B., X. D. Yang, et al. (2009). "Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA." Mol Cell Biol **29**(5): 1375-1387.

Huang, M., S. Sharma, et al. (2002). "IL-7 inhibits fibroblast TGF-beta production and signaling in pulmonary fibrosis." J Clin Invest **109**(7): 931-937.

Hurst, J. R., J. Vestbo, et al. (2010). "Susceptibility to exacerbation in chronic obstructive pulmonary disease." N Engl J Med **363**(12): 1128-1138.

Imlay, J. A. (2008). "Cellular defenses against superoxide and hydrogen peroxide." Annu Rev Biochem **77**: 755-776.

Ingram, J. L., M. J. Huggins, et al. (2011). "Airway fibroblasts in asthma manifest an invasive phenotype." Am J Respir Crit Care Med **183**(12): 1625-1632.

Inoue, K., H. Takano, et al. (2005). "Protease-antiprotease imbalance in inflammatory diseases in the lung." Chest **128**(2): 1069; author reply 1069.

Invitrogen. (2012). "LIVE/DEAD® Fixable Dead Cell Stains." from http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/LIVE-DEAD-Viability-Brand-Page/Live-Dead-Fixable-Dead-Cell-Stains.html.

Ito, K. (2007). "Impact of post-translational modifications of proteins on the inflammatory process." Biochem Soc Trans **35**(Pt 2): 281-283.

Ito, K., P. J. Barnes, et al. (2000). "Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12." Mol Cell Biol **20**(18): 6891-6903.

Ito, K., C. E. Charron, et al. (2007). "Impact of protein acetylation in inflammatory lung diseases." Pharmacol Ther **116**(2): 249-265.

Ito, K., T. Hanazawa, et al. (2004). "Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration." Biochem Biophys Res Commun **315**(1): 240-245.

Ito, K., M. Ito, et al. (2005). "Decreased histone deacetylase activity in chronic obstructive pulmonary disease." N Engl J Med **352**(19): 1967-1976.

Ito, K., S. Yamamura, et al. (2006). "Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression." J Exp Med **203**(1): 7-13.

Jagtap, J. C., A. Chandele, et al. (2003). "Sodium pyruvate protects against H(2)O(2) mediated apoptosis in human neuroblastoma cell line-SK-N-MC." J Chem Neuroanat **26**(2): 109-118.

James, A. and N. Carroll (2000). "Airway smooth muscle in health and disease; methods of measurement and relation to function." Eur Respir J **15**(4): 782-789.

Jang, M. K., K. Mochizuki, et al. (2005). "The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription." Mol Cell **19**(4): 523-534.

Jee, Y. K., J. Gilmour, et al. (2005). "Repression of interleukin-5 transcription by the glucocorticoid receptor targets GATA3 signaling and involves histone deacetylase recruitment." J Biol Chem **280**(24): 23243-23250.

Ji, H., L. I. Ehrlich, et al. "Comprehensive methylome map of lineage commitment from haematopoietic progenitors." Nature **467**(7313): 338-342.

John, S., P. J. Sabo, et al. (2011). "Chromatin accessibility pre-determines glucocorticoid receptor binding patterns." Nat Genet **43**(3): 264-268.

Jomova, K. and M. Valko (2011). "Importance of iron chelation in free radical-induced oxidative stress and human disease." Curr Pharm Des **17**(31): 3460-3473.

Jones, P. A. and S. B. Baylin (2002). "The fundamental role of epigenetic events in cancer." Nat Rev Genet **3**(6): 415-428.

Kabesch, M. and I. M. Adcock (2012). "Epigenetics in asthma and COPD." Biochimie **94**(11): 2231-2241.

Kalsheker, N. and S. Chappell (2008). "The new genetics and chronic obstructive pulmonary disease." COPD **5**(4): 257-264.

Kawanishi, S. and S. Oikawa (2004). "Mechanism of telomere shortening by oxidative stress." Ann N Y Acad Sci **1019**: 278-284.

Keatings, V. M., P. D. Collins, et al. (1996). "Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma." Am J Respir Crit Care Med **153**(2): 530-534.

Kersul, A. L., A. Iglesias, et al. (2011). "Molecular mechanisms of inflammation during exacerbations of chronic obstructive pulmonary disease." Arch Bronconeumol **47**(4): 176-183.

Kirkham, P. and I. Rahman (2006). "Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy." Pharmacol Ther **111**(2): 476-494.

Kirkham, P. A., G. Caramori, et al. (2011). "Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **184**(7): 796-802.

Kirkham, P. A., G. Spooner, et al. (2003). "Cigarette smoke triggers macrophage adhesion and activation: role of lipid peroxidation products and scavenger receptor." Free Radic Biol Med **35**(7): 697-710.

Kirkham, P. A., G. Spooner, et al. (2004). "Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products." Biochem Biophys Res Commun **318**(1): 32-37.

Knight, D. (2001). "Epithelium-fibroblast interactions in response to airway inflammation." Immunol Cell Biol **79**(2): 160-164.

Knox, A. J. (1994). "Airway re-modelling in asthma: role of airway smooth muscle." Clin Sci (Lond) **86**(6): 647-652.

Koblizek, V., M. Tomsova, et al. (2011). "Impairment of nasal mucociliary clearance in former smokers with stable chronic obstructive pulmonary disease relates to the presence of a chronic bronchitis phenotype." Rhinology **49**(4): 397-406.

Kohli, P. and B. D. Levy (2009). "Resolvins and protectins: mediating solutions to inflammation." Br J Pharmacol **158**(4): 960-971.

Kong, X., M. H. Cho, et al. (2011). "Genome-wide association study identifies BICD1 as a susceptibility gene for emphysema." Am J Respir Crit Care Med **183**(1): 43-49.

Konigshoff, M., N. Kneidinger, et al. (2009). "TGF-beta signaling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimen." Swiss Med Wkly **139**(39-40): 554-563.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Kraaij, M. D., S. W. van der Kooij, et al. (2011). "Dexamethasone increases ROS production and T cell suppressive capacity by anti-inflammatory macrophages." Mol Immunol **49**(3): 549-557.

Kukkonen, M. K., E. Tiili, et al. (2011). "SERPINE2 haplotype as a risk factor for panlobular type of emphysema." BMC Med Genet **12**: 157.

Kurien, B. T. and R. H. Scofield (2008). "Autoimmunity and oxidatively modified autoantigens." Autoimmun Rev **7**(7): 567-573.

Lasch, P., T. Petras, et al. (2001). "Hydrogen peroxide-induced structural alterations of RNAse A." J Biol Chem **276**(12): 9492-9502.

Lawrence, T., D. A. Willoughby, et al. (2002). "Anti-inflammatory lipid mediators and insights into the resolution of inflammation." Nat Rev Immunol **2**(10): 787-795.

Lazaar, A. L., L. E. Sweeney, et al. (2011). "SB-656933, a novel CXCR2 selective antagonist, inhibits ex vivo neutrophil activation and ozone-induced airway inflammation in humans." Br J Clin Pharmacol **72**(2): 282-293.

Lebre, M. C., C. E. Vergunst, et al. (2011). "Why CCR2 and CCR5 blockade failed and why CCR1 blockade might still be effective in the treatment of rheumatoid arthritis." PLoS One **6**(7): e21772.

Lee, S. H., S. Goswami, et al. (2007). "Antielastin autoimmunity in tobacco smoking-induced emphysema." Nat Med **13**(5): 567-569.
Levine, R. L., D. Garland, et al. (1990). "Determination of carbonyl content in oxidatively modified proteins." Methods Enzymol **186**: 464-478.
Li, L. C. (2008). "The multifaceted small RNAs." RNA Biol **5**(2): 61-64.
Li, L. L., S. T. Hu, et al. (2010). "Positive transcription elongation factor b (P-TEFb) contributes to dengue virus-stimulated induction of interleukin-8 (IL-8)." Cell Microbiol **12**(11): 1589-1603.
Li, Q. and J. F. Engelhardt (2006). "Interleukin-1beta induction of NFkappaB is partially regulated by H2O2-mediated activation of NFkappaB-inducing kinase." J Biol Chem **281**(3): 1495-1505.
Li, Q. and I. M. Verma (2002). "NF-kappaB regulation in the immune system." Nat Rev Immunol **2**(10): 725-734.
Li, Y., Y. Wang, et al. (2012). "The role of airway epithelial cells in response to mycobacteria infection." Clin Dev Immunol **2012**: 791392.
Liu, B., R. Yang, et al. (2005). "Negative regulation of NF-kappaB signaling by PIAS1." Mol Cell Biol **25**(3): 1113-1123.
Liu, F., J. K. Killian, et al. (2010). "Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate." Oncogene **29**(25): 3650-3664.
Liu, Y. and Y. P. Di (2012). "Effects of second hand smoke on airway secretion and mucociliary clearance." Front Physiol **3**: 342.
Liu, Y., L. Geng, et al. (2009). "Differentiation effect of pyruvate and uridine on cultured U937-rho degrees cells." Ultrastruct Pathol **33**(4): 160-164.
Loor, G., J. Kondapalli, et al. (2010). "Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis." Free Radic Biol Med **49**(12): 1925-1936.
Lou, X., S. Sun, et al. (2011). "Negative feedback regulation of NF-kappaB action by CITED2 in the nucleus." J Immunol **186**(1): 539-548.
Louhelainen, N., P. Rytila, et al. (2009). "Persistence of oxidant and protease burden in the airways after smoking cessation." BMC Pulm Med **9**: 25.
Lounnas, N., C. Frelin, et al. (2009). "NF-kappaB inhibition triggers death of imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells including T315I Bcr-Abl mutants." Int J Cancer **125**(2): 308-317.
Lovinsky-Desir, S. and R. L. Miller (2012). "Epigenetics, asthma, and allergic diseases: a review of the latest advancements." Curr Allergy Asthma Rep **12**(3): 211-220.
Mackay, A. J. and J. R. Hurst (2012). "COPD exacerbations: causes, prevention, and treatment." Med Clin North Am **96**(4): 789-809.
MacNee, W. (2005). "Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease." Proc Am Thorac Soc **2**(1): 50-60.
Maher, S. A., M. A. Birrell, et al. (2009). "Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy." Am J Respir Crit Care Med **180**(10): 923-928.
Malerba, M., F. Ricciardolo, et al. (2006). "Neutrophilic inflammation and IL-8 levels in induced sputum of alpha-1-antitrypsin PiMZ subjects." Thorax **61**(2): 129-133.
Mannino, D. M. and A. S. Buist (2007). "Global burden of COPD: risk factors, prevalence, and future trends." Lancet **370**(9589): 765-773.
Margueron, R. and D. Reinberg "Chromatin structure and the inheritance of epigenetic information." Nat Rev Genet **11**(4): 285-296.
Marin-Corral, J., J. Minguella, et al. (2009). "Oxidised proteins and superoxide anion production in the diaphragm of severe COPD patients." Eur Respir J **33**(6): 1309-1319.
Marwick, J. A., K. Ito, et al. (2007). "Oxidative stress and steroid resistance in asthma and COPD: pharmacological manipulation of HDAC-2 as a therapeutic strategy." Expert Opin Ther Targets **11**(6): 745-755.

Marwick, J. A., P. A. Kirkham, et al. (2004). "Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs." Am J Respir Cell Mol Biol **31**(6): 633-642.

Mathers, C. D. and D. M. Fat. (2008). "The global burden of disease: 2004 update." Retrieved 08/11/2012, 2012, from http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf.

Mathers, C. D. and D. Loncar (2006). "Projections of global mortality and burden of disease from 2002 to 2030." PLoS Med **3**(11): e442.

Matthay, M. A. and J. A. Clements (2004). "Coagulation-dependent mechanisms and asthma." J Clin Invest **114**(1): 20-23.

Matzuk, M. M., M. R. McKeown, et al. (2012). "Small-molecule inhibition of BRDT for male contraception." Cell **150**(4): 673-684.

Meja, K. K., S. Rajendrasozhan, et al. (2008). "Curcumin restores corticosteroid function in monocytes exposed to oxidants by maintaining HDAC2." Am J Respir Cell Mol Biol **39**(3): 312-323.

Menendez, A. and B. Brett Finlay (2007). "Defensins in the immunology of bacterial infections." Curr Opin Immunol **19**(4): 385-391.

Menezes, A. M., R. Perez-Padilla, et al. (2008). "Worldwide burden of COPD in high- and low-income countries. Part II. Burden of chronic obstructive lung disease in Latin America: the PLATINO study." Int J Tuberc Lung Dis **12**(7): 709-712.

Merry, B. J. (2004). "Oxidative stress and mitochondrial function with aging--the effects of calorie restriction." Aging Cell **3**(1): 7-12.

Mertz, J. A., A. R. Conery, et al. (2011). "Targeting MYC dependence in cancer by inhibiting BET bromodomains." Proc Natl Acad Sci U S A **108**(40): 16669-16674.

Meshi, B., T. Z. Vitalis, et al. (2002). "Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response." Am J Respir Cell Mol Biol **26**(1): 52-57.

Mets, O. M., P. A. de Jong, et al. (2012). "Quantitative computed tomography in COPD: possibilities and limitations." Lung **190**(2): 133-145.

Milara, J., G. Juan, et al. (2012). "Neutrophil activation in severe, early-onset COPD patients versus healthy non-smoker subjects in vitro: effects of antioxidant therapy." Respiration **83**(2): 147-158.

Milot, E. and J. G. Filep (2011). "Regulation of neutrophil survival/apoptosis by Mcl-1." ScientificWorldJournal **11**: 1948-1962.

Min, T., M. Bodas, et al. (2011). "Critical role of proteostasis-imbalance in pathogenesis of COPD and severe emphysema." J Mol Med (Berl) **89**(6): 577-593.

Montuschi, P. (2005). "Exhaled breath condensate analysis in patients with COPD." Clin Chim Acta **356**(1-2): 22-34.

Montuschi, P., S. A. Kharitonov, et al. (2003). "Exhaled leukotrienes and prostaglandins in COPD." Thorax **58**(7): 585-588.

Morgan, M. J. and Z. G. Liu (2011). "Crosstalk of reactive oxygen species and NF-kappaB signaling." Cell Res **21**(1): 103-115.

Mortaz, E., M. V. Rad, et al. (2008). "Salmeterol with fluticasone enhances the suppression of IL-8 release and increases the translocation of glucocorticoid receptor by human neutrophils stimulated with cigarette smoke." J Mol Med **86**(9): 1045-1056.

Mukaida, N. (2003). "Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases." Am J Physiol Lung Cell Mol Physiol **284**(4): L566-577.

Negre-Salvayre, A., C. Coatrieux, et al. (2008). "Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors." Br J Pharmacol **153**(1): 6-20.

Nettles, K. W., G. Gil, et al. (2008). "CBP Is a dosage-dependent regulator of nuclear factor-kappaB suppression by the estrogen receptor." Mol Endocrinol **22**(2): 263-272.

Neurath, M. F. and S. Finotto (2011). "IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer." Cytokine Growth Factor Rev **22**(2): 83-89.

Nicodeme, E., K. L. Jeffrey, et al. (2010). "Suppression of inflammation by a synthetic histone mimic." Nature **468**(7327): 1119-1123.

Nikota, J. K. and M. R. Stampfli (2012). "Cigarette smoke-induced inflammation and respiratory host defense: Insights from animal models." Pulm Pharmacol Ther **25**(4): 257-262.

Noguera, A., S. Batle, et al. (2001). "Enhanced neutrophil response in chronic obstructive pulmonary disease." Thorax **56**(6): 432-437.

Nowak, D., K. Berger, et al. (2005). "Epidemiology and health economics of COPD across Europe: a critical analysis." Treat Respir Med **4**(6): 381-395.

Nowak, D., M. Kasielski, et al. (1998). "Cigarette smoking does not increase hydrogen peroxide levels in expired breath condensate of patients with stable COPD." Monaldi Arch Chest Dis **53**(3): 268-273.

Nowak, D. E., B. Tian, et al. (2008). "RelA Ser276 phosphorylation is required for activation of a subset of NF-kappaB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes." Mol Cell Biol **28**(11): 3623-3638.

O'Donnell, R. A., C. Peebles, et al. (2004). "Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD." Thorax **59**(10): 837-842.

O'Hagan, H. M., W. Wang, et al. (2011). "Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG Islands." Cancer Cell **20**(5): 606-619.

Ober, C., A. J. Butte, et al. (2010). "Getting from genes to function in lung disease: a National Heart, Lung, and Blood Institute workshop report." Am J Respir Crit Care Med **182**(6): 732-737.

Oeckinghaus, A. and S. Ghosh (2009). "The NF-kappaB family of transcription factors and its regulation." Cold Spring Harb Perspect Biol **1**(4): a000034.

Ofulue, A. F. and M. Ko (1999). "Effects of depletion of neutrophils or macrophages on development of cigarette smoke-induced emphysema." Am J Physiol **277**(1 Pt 1): L97-105.

Oliveira-Marques, V., H. S. Marinho, et al. (2009). "Role of hydrogen peroxide in NF-kappaB activation: from inducer to modulator." Antioxid Redox Signal **11**(9): 2223-2243.

Olloquequi, J., J. F. Montes, et al. (2011). "Significant increase of CD57+ cells in pulmonary lymphoid follicles of COPD patients." Eur Respir J **37**(2): 289-298.

Ono, K. and J. Han (2000). "The p38 signal transduction pathway: activation and function." Cell Signal **12**(1): 1-13.

Osoata, G. O., S. Yamamura, et al. (2009). "Nitration of distinct tyrosine residues causes inactivation of histone deacetylase 2." Biochem Biophys Res Commun **384**(3): 366-371.

Ott, C. J., N. Kopp, et al. (2012). "BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia." Blood **120**(14): 2843-2852.

Paats, M. S., I. M. Bergen, et al. (2012). "Systemic CD4+ and CD8+ T-cell cytokine profiles correlate with GOLD stage in stable COPD." Eur Respir J **40**(2): 330-337.

Panettieri, R. A., Jr. (2004). "Effects of corticosteroids on structural cells in asthma and chronic obstructive pulmonary disease." Proc Am Thorac Soc **1**(3): 231-234.

Pettersen, C. A. and K. B. Adler (2002). "Airways inflammation and COPD: epithelial-neutrophil interactions." Chest **121**(5 Suppl): 142S-150S.

Pickering, A. M., A. L. Koop, et al. (2010). "The immunoproteasome, the 20S proteasome and the PA28alphabeta proteasome regulator are oxidative-stress-adaptive proteolytic complexes." Biochem J **432**(3): 585-594.

Pitchford, S. C. (2007). "Novel uses for anti-platelet agents as anti-inflammatory drugs." Br J Pharmacol **152**(7): 987-1002.

Plumb, J., L. J. Smyth, et al. (2009). "Increased T-regulatory cells within lymphocyte follicles in moderate COPD." Eur Respir J **34**(1): 89-94.

Polverino, F., S. Baraldo, et al. (2010). "A novel insight into adaptive immunity in chronic obstructive pulmonary disease: B cell activating factor belonging to the tumor necrosis factor family." Am J Respir Crit Care Med **182**(8): 1011-1019.

Pryor, W. A. and K. Stone (1993). "Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite." Ann N Y Acad Sci **686**: 12-27; discussion 27-18.

Puchelle, E., J. M. Zahm, et al. (2006). "Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease." Proc Am Thorac Soc **3**(8): 726-733.

Putova, I., C. Dostal, et al. (2007). "Prevalence of antinucleosome antibodies by enzyme-linked immunosorbent assays in patients with systemic lupus erythematosus and other autoimmune systemic diseases." Ann N Y Acad Sci **1109**: 275-286.

Qaseem, A., T. J. Wilt, et al. (2011). "Diagnosis and management of stable chronic obstructive pulmonary disease: a clinical practice guideline update from the American College of Physicians, American College of Chest Physicians, American Thoracic Society, and European Respiratory Society." Ann Intern Med **155**(3): 179-191.

Rahman, I. and I. M. Adcock (2006). "Oxidative stress and redox regulation of lung inflammation in COPD." Eur Respir J **28**(1): 219-242.

Rahman, I., A. A. van Schadewijk, et al. (2002). "4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **166**(4): 490-495.

Rahman, S., M. E. Sowa, et al. (2011). "The Brd4 extraterminal domain confers transcription activation independent of pTEFb by recruiting multiple proteins, including NSD3." Mol Cell Biol **31**(13): 2641-2652.

Reese, J. C. (2003). "Basal transcription factors." Curr Opin Genet Dev **13**(2): 114-118.

Rennard, S. I. and K. Wachenfeldt (2011). "Rationale and emerging approaches for targeting lung repair and regeneration in the treatment of chronic obstructive pulmonary disease." Proc Am Thorac Soc **8**(4): 368-375.

Retamales, I., W. M. Elliott, et al. (2001). "Amplification of inflammation in emphysema and its association with latent adenoviral infection." Am J Respir Crit Care Med **164**(3): 469-473.

Rinaldi, M., A. Lehouck, et al. (2012). "Antielastin B-cell and T-cell immunity in patients with chronic obstructive pulmonary disease." Thorax **67**(8): 694-700.

Rodriguez, R. M., C. Huidobro, et al. (2012). "Aberrant epigenetic regulation of bromodomain BRD4 in human colon cancer." J Mol Med (Berl) **90**(5): 587-595.

Rogers, D. F. (2005). "Mucociliary dysfunction in COPD: effect of current pharmacotherapeutic options." Pulm Pharmacol Ther **18**(1): 1-8.

Rosseau, S., J. Selhorst, et al. (2000). "Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines." J Immunol **164**(1): 427-435.

Rothgiesser, K. M., M. Fey, et al. (2010). "Acetylation of p65 at lysine 314 is important for late NF-kappaB-dependent gene expression." BMC Genomics **11**: 22.

Ruland, J. (2011). "Return to homeostasis: downregulation of NF-kappaB responses." Nat Immunol **12**(8): 709-714.

Russell, R. E., S. V. Culpitt, et al. (2002). "Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **26**(5): 602-609.

Rusznak, C., P. R. Mills, et al. (2000). "Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **23**(4): 530-536.

Sacco, O., M. Silvestri, et al. (2004). "Epithelial cells and fibroblasts: structural repair and remodelling in the airways." Paediatr Respir Rev **5 Suppl A**: S35-40.

Saetta, M., A. Di Stefano, et al. (1998). "CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **157**(3 Pt 1): 822-826.

Salminen, A., J. Huuskonen, et al. (2008). "Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging." Ageing Res Rev **7**(2): 83-105.

Salvi, S. S. and P. J. Barnes (2009). "Chronic obstructive pulmonary disease in non-smokers." Lancet **374**(9691): 733-743.

Sanchez, R. and M. M. Zhou (2009). "The role of human bromodomains in chromatin biology and gene transcription." Curr Opin Drug Discov Devel **12**(5): 659-665.

Sandman, K. and J. N. Reeve (2000). "Structure and functional relationships of archaeal and eukaryal histones and nucleosomes." Arch Microbiol **173**(3): 165-169.

Sansone, P. and J. Bromberg (2012). "Targeting the interleukin-6/Jak/stat pathway in human malignancies." J Clin Oncol **30**(9): 1005-1014.

Sapey, E., A. Ahmad, et al. (2009). "Imbalances between interleukin-1 and tumor necrosis factor agonists and antagonists in stable COPD." J Clin Immunol **29**(4): 508-516.

Sapey, E. and R. A. Stockley (2006). "COPD exacerbations . 2: aetiology." Thorax **61**(3): 250-258.

Sasaki, C. Y., T. J. Barberi, et al. (2005). "Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway." J Biol Chem **280**(41): 34538-34547.

Savale, L., A. Chaouat, et al. (2009). "Shortened telomeres in circulating leukocytes of patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **179**(7): 566-571.

Schreck, R. and P. A. Baeuerle (1990). "NF-kappa B as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene." Mol Cell Biol **10**(3): 1281-1286.

Schwartz, B. E. and K. Ahmad (2005). "Transcriptional activation triggers deposition and removal of the histone variant H3.3." Genes Dev **19**(7): 804-814.

Sen, P., S. Mukherjee, et al. (2003). "Enhancement of catalase activity by repetitive low-grade H2O2 exposures protects fibroblasts from subsequent stress-induced apoptosis." Mutat Res **529**(1-2): 87-94.

Shapiro, S. D. (2002). "Neutrophil elastase: path clearer, pathogen killer, or just pathologic?" Am J Respir Cell Mol Biol **26**(3): 266-268.

Shapiro, S. D. (2002). "Proteinases in chronic obstructive pulmonary disease." Biochem Soc Trans **30**(2): 98-102.

Shiratsuchi, N., K. Asai, et al. (2011). "Measurement of soluble perforin, a marker of CD8+ T lymphocyte activation in epithelial lining fluid." Respir Med **105**(12): 1885-1890.

Siegfried, Z. and I. Simon (2010). "DNA methylation and gene expression." Wiley Interdiscip Rev Syst Biol Med **2**(3): 362-371.

Siena, L., M. Gjomarkaj, et al. (2011). "Reduced apoptosis of CD8+ T-lymphocytes in the airways of smokers with mild/moderate COPD." Respir Med **105**(10): 1491-1500.

Sin, D. D. and S. F. Man (2008). "Interleukin-6: a red herring or a real catch in COPD?" Chest **133**(1): 4-6.

Sin, D. D. and J. Vestbo (2009). "Biomarkers in chronic obstructive pulmonary disease." Proc Am Thorac Soc **6**(6): 543-545.

Sinden, N. J. and R. A. Stockley "Systemic inflammation and comorbidity in COPD: a result of 'overspill' of inflammatory mediators from the lungs? Review of the evidence." Thorax.

Singh, B., S. Arora, et al. (2010). "Association of severity of COPD with IgE and interleukin-1 beta." Monaldi Arch Chest Dis **73**(2): 86-87.

Singh, D., L. Edwards, et al. (2010). "Sputum neutrophils as a biomarker in COPD: findings from the ECLIPSE study." Respir Res **11**: 77.

So, A. Y., S. B. Cooper, et al. (2008). "Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at glucocorticoid-induced genes." Proc Natl Acad Sci U S A **105**(15): 5745-5749.

Soler Artigas, M., L. V. Wain, et al. (2011). "Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function." Am J Respir Crit Care Med **184**(7): 786-795.

Song, Y., N. Driessens, et al. (2007). "Roles of hydrogen peroxide in thyroid physiology and disease." J Clin Endocrinol Metab **92**(10): 3764-3773.

Soriano, J. B. and R. Rodriguez-Roisin (2011). "Chronic obstructive pulmonary disease overview: epidemiology, risk factors, and clinical presentation." Proc Am Thorac Soc **8**(4): 363-367.

Stimson, L., M. G. Rowlands, et al. (2005). "Isothiazolones as inhibitors of PCAF and p300 histone acetyltransferase activity." Mol Cancer Ther **4**(10): 1521-1532.

Storz, P., H. Doppler, et al. (2004). "Protein kinase Cdelta selectively regulates protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling." Mol Cell Biol **24**(7): 2614-2626.

Storz, P. and A. Toker (2003). "Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway." EMBO J **22**(1): 109-120.

Structural Genomics Consortium (2012). "PFI-1 - Selective chemical probe for BET Bromodomains." Retrieved 29 September 2012, 2012, from http://www.thesgc.org/scientists/chemical_probes/PFI-1/.

Struthers, M. and A. Pasternak (2010). "CCR2 antagonists." Curr Top Med Chem **10**(13): 1278-1298.

Sun, L. and R. D. Ye (2012). "Role of G protein-coupled receptors in inflammation." Acta Pharmacol Sin **33**(3): 342-350.

Szulakowski, P., A. J. Crowther, et al. (2006). "The effect of smoking on the transcriptional regulation of lung inflammation in patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **174**(1): 41-50.

Tagoug, I., A. Sauty De Chalon, et al. (2011). "Inhibition of IGF-1 signalling enhances the apoptotic effect of AS602868, an IKK2 inhibitor, in multiple myeloma cell lines." PLoS One **6**(7): e22641.

Takada, Y., A. Mukhopadhyay, et al. (2003). "Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase." J Biol Chem **278**(26): 24233-24241.

Tamimi, A., D. Serdarevic, et al. (2012). "The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications." Respir Med **106**(3): 319-328.

Taylor, J. D. (2010). "COPD and the response of the lung to tobacco smoke exposure." Pulm Pharmacol Ther **23**(5): 376-383.

Terlecky, S. R., L. J. Terlecky, et al. (2012). "Peroxisomes, oxidative stress, and inflammation." World J Biol Chem **3**(5): 93-97.

Tetley, T. D. (2002). "Macrophages and the pathogenesis of COPD." Chest **121**(5 Suppl): 156S-159S.

Thompson-Wicking, K., R. W. Francis, et al. (2012). "Novel BRD4-NUT fusion isoforms increase the pathogenic complexity in NUT midline carcinoma." Oncogene.

Thorley, A. J. and T. D. Tetley (2007). "Pulmonary epithelium, cigarette smoke, and chronic obstructive pulmonary disease." Int J Chron Obstruct Pulmon Dis **2**(4): 409-428.

Togo, S., O. Holz, et al. (2008). "Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms." Am J Respir Crit Care Med **178**(3): 248-260.

Tokiwa, H., N. Sera, et al. (1999). "8-Hydroxyguanosine formed in human lung tissues and the association with diesel exhaust particles." Free Radic Biol Med **27**(11-12): 1251-1258.

Traves, S. L., S. V. Culpitt, et al. (2002). "Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD." Thorax **57**(7): 590-595.

Triantaphyllopoulos, K., F. Hussain, et al. (2011). "A model of chronic inflammation and pulmonary emphysema after multiple ozone exposures in mice." Am J Physiol Lung Cell Mol Physiol **300**(5): L691-700.

Tron, K., A. Samoylenko, et al. (2006). "Regulation of rat heme oxygenase-1 expression by interleukin-6 via the Jak/STAT pathway in hepatocytes." J Hepatol **45**(1): 72-80.

Tsaprouni, L. G., K. Ito, et al. (2011). "Differential patterns of histone acetylation in inflammatory bowel diseases." J Inflamm (Lond) **8**(1): 1.

Tsoumakidou, M., N. Tzanakis, et al. (2003). "Induced sputum in the investigation of airway inflammation of COPD." Respir Med **97**(8): 863-871.

Tsuchiya, A., K. Imai, et al. (2010). "Inhibition of inflammatory cytokine production from rheumatoid synovial fibroblasts by a novel IkappaB kinase inhibitor." J Pharmacol Exp Ther **333**(1): 236-243.

Turner, B. M. (2000). "Histone acetylation and an epigenetic code." Bioessays **22**(9): 836-845.

Tzouvelekis, A., K. Kostikas, et al. (2012). "Autoimmunity and chronic obstructive pulmonary disease: thinking beyond cigarette smoke." Am J Respir Crit Care Med **185**(11): 1248-1249.

Urbanowicz, R. A., J. R. Lamb, et al. (2010). "Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients." Respir Res **11**: 76.

van der Vaart, H., D. S. Postma, et al. (2004). "Acute effects of cigarette smoke on inflammation and oxidative stress: a review." Thorax **59**(8): 713-721.

Vanaudenaerde, B. M., S. E. Verleden, et al. (2011). "Innate and adaptive interleukin-17-producing lymphocytes in chronic inflammatory lung disorders." Am J Respir Crit Care Med **183**(8): 977-986.

Vargas-Rojas, M. I., A. Ramirez-Venegas, et al. (2011). "Increase of Th17 cells in peripheral blood of patients with chronic obstructive pulmonary disease." Respir Med **105**(11): 1648-1654.

Vereecke, L., R. Beyaert, et al. (2009). "The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology." Trends Immunol **30**(8): 383-391.

Vestbo, J., S. S. Hurd, et al. (2012). "Global Strategy for the Diagnosis, Management and Prevention of Chronic Obstructive Pulmonary Disease, GOLD Executive Summary." Am J Respir Crit Care Med.

Vlahos, R., P. A. Wark, et al. (2012). "Glucocorticosteroids differentially regulate MMP-9 and neutrophil elastase in COPD." PLoS One **7**(3): e33277.

Voelkel, N. F., J. Gomez-Arroyo, et al. (2011). "COPD/emphysema: The vascular story." Pulm Circ **1**(3): 320-326.

Vogelmeier, C. and R. Bals (2007). "Chronic obstructive pulmonary disease and premature aging." Am J Respir Crit Care Med **175**(12): 1217-1218.

Voss, T. C., R. L. Schiltz, et al. (2011). "Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism." Cell **146**(4): 544-554.

Waddinton, C. (1942). "The epigenotype." Endeavor (1): 18-24.

Wang, C. B., C. K. Wong, et al. (2005). "Induction of IL-6 in co-culture of bronchial epithelial cells and eosinophils is regulated by p38 MAPK and NF-kappaB." Allergy **60**(11): 1378-1385.

Wang, P. and S. R. Powell. (2010). "Decreased sensitivity associated with an altered formulation of a commercially available kit for detection of protein carbonyls." Free Radic Biol Med **49**(2): 119-121.

Wang, X., E. Perez, et al. (2007). "Pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells." Brain Res **1132**(1): 1-9.

Wang, Y. L., C. X. Bai, et al. (2008). "Role of airway epithelial cells in development of asthma and allergic rhinitis." Respir Med **102**(7): 949-955.

Watters, T. M., E. F. Kenny, et al. (2007). "Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins." Immunol Cell Biol **85**(6): 411-419.

Wells, R. G., E. Kruglov, et al. (2004). "Autocrine release of TGF-beta by portal fibroblasts regulates cell growth." FEBS Lett **559**(1-3): 107-110.

Wen, Y., D. W. Reid, et al. (2010). "Assessment of airway inflammation using sputum, BAL, and endobronchial biopsies in current and ex-smokers with established COPD." Int J Chron Obstruct Pulmon Dis **5**: 327-334.

WHO. (2008). "The Global burden of disease: 2004 update " Retrieved 02 Nov 2012, 2012, from http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html

Wijeratne, S. S., S. L. Cuppett, et al. (2005). "Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells." J Agric Food Chem **53**(22): 8768-8774.

Wilk, J. B., N. R. Shrine, et al. (2012). "Genome-wide association studies identify CHRNA5/3 and HTR4 in the development of airflow obstruction." Am J Respir Crit Care Med **186**(7): 622-632.

Willemse, B. W., D. S. Postma, et al. (2004). "The impact of smoking cessation on respiratory symptoms, lung function, airway hyperresponsiveness and inflammation." Eur Respir J **23**(3): 464-476.

Williams, T. J. and P. J. Jose (2001). "Neutrophils in chronic obstructive pulmonary disease." Novartis Found Symp **234**: 136-141; discussion 141-138.

Winkelman, C. (2008). "Inflammation and genomics in the critical care unit." Crit Care Nurs Clin North Am **20**(2): 213-221, vi.

Wong, C. M., L. Marcocci, et al. (2010). "Cell signaling by protein carbonylation and decarbonylation." Antioxid Redox Signal **12**(3): 393-404.

Wood, A. M. and R. A. Stockley (2006). "The genetics of chronic obstructive pulmonary disease." Respir Res **7**: 130.

Workman, J. L. (2006). "Nucleosome displacement in transcription." Genes Dev **20**(15): 2009-2017.

Wright, J. L., M. Cosio, et al. (2008). "Animal models of chronic obstructive pulmonary disease." Am J Physiol Lung Cell Mol Physiol **295**(1): L1-15.

Yaghi, A., A. Zaman, et al. (2012). "Ciliary beating is depressed in nasal cilia from chronic obstructive pulmonary disease subjects." Respir Med **106**(8): 1139-1147.

Yamamoto, Y. and R. B. Gaynor (2001). "Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer." J Clin Invest **107**(2): 135-142.

Yan, J., J. Diaz, et al. (2011). "Perturbation of BRD4 protein function by BRD4-NUT protein abrogates cellular differentiation in NUT midline carcinoma." J Biol Chem **286**(31): 27663-27675.

Yang, S. R., S. Valvo, et al. (2008). "IKK alpha causes chromatin modification on pro-inflammatory genes by cigarette smoke in mouse lung." Am J Respir Cell Mol Biol **38**(6): 689-698.

Yang, Z., J. H. Yik, et al. (2005). "Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4." Mol Cell **19**(4): 535-545.

Yao, H. and I. Rahman (2009). "Current concepts on the role of inflammation in COPD and lung cancer." Curr Opin Pharmacol **9**(4): 375-383.

Yatin, S. M., S. Varadarajan, et al. (1999). "In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta-peptide (1-42)." Neurobiol Aging **20**(3): 325-330; discussion 339-342.

Young, R. P., R. Hopkins, et al. (2006). "Functional variants of antioxidant genes in smokers with COPD and in those with normal lung function." Thorax **61**(5): 394-399.

Zanini, A., A. Chetta, et al. (2010). "The role of the bronchial microvasculature in the airway remodelling in asthma and COPD." Respir Res **11**: 132.

Zhang, G., R. Liu, et al. (2012). "Down-regulation of NF-kappaB transcriptional activity in HIV-associated kidney disease by BRD4 inhibition." J Biol Chem **287**(34): 28840-28851.

Zhang, J., J. He, et al. (2012). "Delayed apoptosis by neutrophils from COPD patients is associated with altered bak, bcl-xl, and mcl-1 mRNA expression." Diagn Pathol **7**: 65.

Zhang, J., G. Johnston, et al. (2001). "Hydrogen peroxide activates NFkappaB and the interleukin-6 promoter through NFkappaB-inducing kinase." Antioxid Redox Signal **3**(3): 493-504.

Zhang, J., L. Wu, et al. (2011). "Pulmonary fibroblasts from COPD patients show an impaired response of elastin synthesis to TGF-beta1." Respir Physiol Neurobiol **177**(3): 236-240.

Zhang, J. Q., Q. F. Lao, et al. (2010). "[Interleukin-17 expression and significance in normal lung function smokers and chronic obstructive pulmonary disease patients]." Zhonghua Yi Xue Za Zhi **90**(20): 1431-1435.

Zhang, W. S., C. Prakash, et al. (2012). "Bromodomain-Containing-Protein 4 (BRD4) Regulates RNA Polymerase II Serine 2 Phosphorylation in Human CD4+ T Cells." J Biol Chem.

Zhang, Z., Y. Q. Cai, et al. (2011). "Epigenetic suppression of GAD65 expression mediates persistent pain." Nat Med **17**(11): 1448-1455.

Zhou, J. and Y. Du (2012). "Acquisition of Resistance of Pancreatic Cancer Cells to 2-Methoxyestradiol Is Associated with the Upregulation of Manganese Superoxide Dismutase." Mol Cancer Res.

Zhou, X., Q. Dai, et al. (2012). "Neutrophils in acute lung injury." Front Biosci **17**: 2278-2283.

Zuber, J., J. Shi, et al. (2011). "RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia." Nature **478**(7370): 524-528.