Molecular mechanisms of enhanced expression of the chemokine Interleukin 8 (CXCL8) in cystic fibrosis (CF) airway epithelial cells

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ABSTRACT

Cystic fibrosis (CF) is a fatal disease caused by a mutation of the CFTR gene and severe inflammation of the lungs. The inflammatory process is characterised by increased production of the potent neutrophil-attracting chemokine interleukin 8 (CXCL8), but the mechanism responsible is poorly understood. We tested the hypothesis that altered epigenetic regulation is responsible for the basal and cytokine-induced CXCL8 upregulation in CF airway epithelial cells. We found that CXCL8 protein levels and mRNA expression were higher in CF as compared to normal cells both basally and following cytokine stimulation. The difference in the expression was independent of increased mRNA stability or increased transcription factor activation and/or expression in CF cells. We found increased basal, but not cytokine-induced transcription factor binding to the CXCL8 promoter in a chromatin environment in CF cells in comparison with normal cells, increased histone H3 lysine 4 trimethylation, hypomethylation of CpG sites and increased binding of BRD3 and BRD4 to the CXCL8 promoter. Disruption of BRD4 association with chromatin using the selective BET bromodomain inhibitor JQ1 decreased CXCL8 protein release from CF cells to the levels observed in normal cells. Our observations suggest that epigenetic alterations are responsible for the upregulation of CXCL8 in CF and could become potential targets in the development of new therapeutic strategies.

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LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

aGM1	AsialoGM1 receptor	LPS	Lipopolysaccharide
ARE	AU-rich element	LZ	Leucine zipper dimerization domain
ASL	Airway surface liquid	MAPK	Mitogen-activated protein kinase
ATP	Adenosine triphosphate	MBD	Methyl-CpG-binding domain
β_2-M	Beta $_2$-microglobulin	MBP	Methyl CpG binding protein
BAL	Bronchoalveolar	MBT	Malignant brain tumour
BCA	Bicinchoninic acid protein assay	MDB	Membrane desalting buffer
BCC	*Burkholderia cepacia complex*	MEM	Minimum essential medium Eagle
BET	Bromodomains and extra-terminal	mg	Milligram
BR	Basic region	µl	Microliter
BRD	Bromodomain	mL	Millilitre
BSA	Bovine serum albumin	mM	mmol
Ca^{2+}	Calcium	mM/L	Mmol/Litre
cAMP	Cyclic adenosine monophosphate	M-MLV RT	Moloney murine leukaemia virus reverse transcriptase
CARM1	Co-activator-associated arginine methyltransferase 1	MMP	Matrix metalloproteinase
CBP	cAMP response element binding protein (CREB) binding protein	mRNA	Messenger ribonucleic acid
cDNA	Complimentary deoxyribonucleic acid	MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
C/EBPβ	CCAAT/enhancer binding protein beta	Na^+	Sodium
CF	Cystic fibrosis	$NaCl^-$	Sodium chloride
CFF	Cystic Fibrosis Foundation	NE	Neutrophil elastase
CFTR	Cystic fibrosis transmembrane conductance regulator	NF-κB	Nuclear factor kappa B
CG	Cytosine-guanine	ng	Nanogram
ChIP	Chromatin immunoprecipitation	NLS	Nuclear localisation signal
Cl^-	Chloride	nm	Nanometre
cm^2	Square centimetre	NO	Nitric oxide
Co-IP	Co-Immunoprecipitation	NPD	Nasal potential difference
COPD	Chronic obstructive pulmonary disease	NRE	Negative regulatory element
CT	Computed tomography	NRF	NF-κB-repressing factor
CXCL8	Interleukin 8	Oct-1	Octamer 1
ddH_2O	Double distilled water	OligoDT	Oligodeoxythymidylic

			acid
DEPC	Diethyl pyrocarbonate	P_o	Open probability
DMSO	Dimethyl sulfoxide	P.aeruginosa	*Pseudomonas aeruginosa*
DNMT	DNA methyltransferase	PAMP	Pathogen-associated molecular pattern
DNA	Deoxyribonucleic acid	PBS	Phosphate buffered saline
dNTP	Deoxynucleoside triphosphate	PCAF	p300-CBP associated factor
DTT	Dithiothreitol	PCR	Polymerase chain reaction
ECL™	Western Lightning™ Chemiluminescence Reagent	pg	Picogram
EDTA	Ethylenediaminetetra-acetic acid	PGE2	Prostaglandin E2
ELISA	Enzyme-linked Immunosorbent assay	PIC	Proteinase inhibitor cocktail
ENaC	Amiloride-sensitive epithelial sodium channel	PMN	Polymorphonuclear neutrophil
FAD	Flavin adenine dinucleotide	PMSF	Phenylmethylsulfonyl fluoride
FCS	Foetal calf serum	Pol II	Polymerase II
g	Relative centrifugal force	PRMT	Arginine methyltransferase
GNAT	Gcn5-related N-acetyltransferase	PRR	Pattern recognition receptor
H	Histone	PVDF	Polyvinylidene difluoride
H_2O_2	Hydrogen peroxide	qPCR	Quantitative real-time polymerase chain reaction
H2A	Histone 2A	R	Arginine
H2B	Histone 2B	RAW1,2	Wash buffer 1 and 2
H3K4	Histone 3 lysine 4	RCF	Relative centrifugal force
H3K4me	Histone 3 lysine 4 trimethylation	RD	Regulatory domain
H3K9me	Histone 3 lysine 9 trimethylation	rDNase	Recombinant deoxyribonuclease
H3K27	Histone 3 lysine 27	Re-ChIP	Re-Chromatin immunoprecipitation precipitation
H3K27me3	Histone 3 lysine 27 trimethylation	RHD	Rel homology domain
H3K36	Histone 3 lysine 36	RNA	Ribonucleic acid
H4K20	Histone 4 lysine 20	RNAase	Ribonuclease
H4R3	Histone 4 arginine 3	RNA-se A	Rinobuclease A
HAT	Histone acetyltransferase	ROS	Reactive oxygen species
HCO_{3-}	Bicarbonate	RPM	Rotations per minute

HDAC	Histone deacetylase	RT	Reverse transcription
HDM	Histone demethylase	RT-QPCR	Real-time quantitative polymerase chain reaction
H.influenza	*Haemophilus influenzae*	S	Serine
HMT	Histone methyltransferase	S.aureus	*Staphylococcus aureus*
IFN-γ	Interferon gamma	SDS	Sodium dodecyl sulphate
IgG	Immunoglobulin G	SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
IKK	IκB kinase	SEM	Standard error of the mean
IL	Interleukin	SET	Suppressor of variegation-Enhancer of zeste-Trithorax
IL1-β	Interleukin 1 beta	SRC-1	Steroid receptor coactivator-1
IL-6	Interleukin 6	Streptavidin-HRP	Streptavidin-horseradish-peroxidase
IL-10	Interleukin 10	SUMO	Small Ubiquitin-related MOdifier protein
IP	Immunoprecipitation	TBS-T	Tris buffered saline plus Tween-20
JmjC	Jumonji	TD/TAD	Transactivation domain
JNK	Jun N-terminal kinase	TF	Transcription factor
K	Lysine	TGF-ß	Transforming growth factor-beta
K+	Potassium	TIF-2	Transcriptional intermediary factor-2
Kac	Epsilon-N-acetyl lysine	TNF-α	Tumour necrosis factor-alpha
kb	Kilobase	TNFR	Tumour necrosis factor receptor
KC	Keratinocyte chemoattractant	USF	Upstream stimulatory factors
kDa	KiloDalton	UV	Ultraviolet
LAR II	Luciferase assay reagent II	V	Volt
LF	Lipofectamine	WT	Wild type

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1 INTRODUCTION

1.1 Overview of cystic fibrosis

1.1.1 Aetiology

Cystic fibrosis (CF) is a life-shortening inherited disease occurring in people of all ethnic and racial backgrounds, but mostly widespread among the Caucasians. CF affects 1 in 3500 newborn babies in the USA (CFF, 2012) and 1 in 2000-3000 live newborns in the European Union (WHO, 2010). The median predicted age of survival of patients with CF has risen progressively with improvements in treatment.

CF is an autosomal recessive disorder caused by mutations in a gene encoding the 1480-residue cystic fibrosis transmembrane conductance regulator (CFTR) protein. The CFTR gene is located on the long arm of chromosome 7 at q31.2. It contains approximately 170 000 base pairs and comprises 27 coding exons. The molecular weight of the CFTR protein is 170 kDa and the transcript is 6.5 kb (Kerem et al., 1989, NIH, Zielenski J, 1995, Bartling, 2009, Home and Reference, 2013). To date, 1939 mutations of the CFTR gene have been identified (Database, 2001). CFTR is expressed in epithelial cells of several organs including the skin, lungs, liver, pancreas, sweat glands, salivary glands, kidney, digestive and reproductive tracts (Guo et al. 2009, Cozens et al., 1994, Chmiel and Davis, 2003).

The CFTR protein is a phosphorylation-dependent cyclic adenosine monophosphate (cAMP)-controlled adenosine triphosphate (ATP)-gated chloride (Cl-) channel located at the apical membrane of secretory epithelial cells and exocrine glands (Terheggen-Lagro et al., 2005, Sheppard and Welsh, 1999, Xu et al., 2003). CFTR functions both as an ion channel and a regulator of ion transport by suppressing

sodium (Na^+) permeability across epithelial apical surfaces and activating non-CFTR Cl^- channels. CFTR also possesses the ability to regulate other membrane proteins (Kunzelmann, 2003): the best studied and documented is the negative regulation of the amiloride-sensitive epithelial Na^+ channel (ENaC). ENaCs are primarily expressed in the airways and alveolar epithelium: they are the major regulators of electrolyte and water exchange in the airways (Catalán et al., 2010, Rubenstein et al., 2011). Several studies have shown that lack of CFTR in CF airways results in an increased open probability (P_o) and amplified conductance of Na^+ due to dysfunction or absence of negatively regulated ENaCs and, consequently, increased levels of basal Na^+ absorption (Berdiev et al., 2009, Nagel et al., 2001).

CFTR dysfunction disrupts transepithelial ion transport and results in increased water reabsorption, reduced airway surface liquid (ASL) volume and impaired ciliary clearance followed by development of chronic lung disease (Kunzelmann and Mall, 2003, Welsh et al., 1995, Boucher, 2007). There is growing evidence that CFTR is involved in the regulation of other membrane proteins responsible for several ion transporters such as non-CFTR Cl^-, Cl^-, Na^+, potassium (K^+), ATP and glutathione channels, and the Cl^-/HCO_3^- exchanger (Bear et al., 1992, Carroll et al., 2005).

1.1.2 Symptoms and diagnosis

CF is equally diagnosed in males and females (Nick et al., 2010, CFF, 2011). Average survival depends on the nature and progression of the lung disease, and correlates with CFTR genotype and mutation type. Patients with mutations causing a milder lung disease have significantly better survival.

CFTR deficiency leads to the development of broad-spectrum hallmark CF symptoms: they include elevated sweat chloride levels, thick and dehydrated airway mucus production resulting in bronchial obstruction and persistent lung infection, chronic sinusitis and nasal polyp formation, pancreatic insufficiency, bile duct and intestinal obstruction, and urogenital abnormalities causing infertility in men (Carroll et al., 2005). Although, CF has multiple clinical manifestations, lung disease is the most serious complication resulting in 90% of the morbidity and mortality in CF patients (CFF, 2011, Bartling, 2009). Despite enormous progress in the field of CF pathophysiology and treatment since the discovery of CFTR gene in 1989 (Kerem et al., 1989), the median life expectancy remains short estimated at 41.1 in 2012 (CFF, 2012) compared to 31.3 at the beginning of the 21st century (FitzSimmons, 1998).

Lung disease in CF is best characterised as a perpetuating circle of bronchial obstruction, permanent bacterial colonisation leading to excessive inflammation and resulting in airway remodelling followed by respiratory failure and death (Katkin, 2014, Ratjen and Döring, 2003). Several studies have reported that the lungs of newborns developing CF are sterile in utero with only minor enlargement of tracheal submucosal glands within first few months of their life (Meyerholz et al., 2012, De Rose, 2002). Shortly after birth, the airways and bronchoalveolar (BAL) fluid of CF newborns display signs of bacterial colonisation characterised by increased levels of Interleukin 8 (CXCL8), neutrophil elastase (NE) and profound neutrophil infiltration compared to control subjects (Peterson-Carmichael et al., 2009, Armstrong et al., 2005). Post-mortem examination of these infants reveals

abnormal mucus secretion, existing inflammation and increased levels of pro-inflammatory cytokines (Nixon et al., 2002, Armstrong et al., 1997). These findings suggest that CFTR-deficient airways are prone to plugging with thick mucus in its turn leading to bronchial obstruction and chronic bacterial infection characterised by excessive inflammation eventually resulting in airway remodelling, scarring, and fibrosis crowned with respiratory failure and ultimate death.

In 1996, the US Cystic Fibrosis Foundation developed criteria for the diagnosis of CF (Farrell et al., 2008) that have been revised later on (Dequeker et al., 2008, Ooi et al., 2012). Currently, a diagnosis of CF is suggested based on a presence of at least two major clinical symptoms such as:

- an abnormal sweat test with Cl^- concentration over 60mM/L with borderline levels of 30-59 mM/L (CFTR.INFO, 2014),
- family history accompanied by genetic confirmation of the existence of one or more characteristic mutations,
- at least, two distinctive clinical symptoms such as chronic sinopulmonary disease, gastrointestinal and nutritional abnormalities, salt loss syndromes and/or genital abnormalities,
- basic and ancillary testing including exocrine pancreatic function tests and imaging, respiratory tract culture for CF-associated pathogens (especially *P. aeruginosa*), genital evaluation in males, pulmonary function testing, bronchoalveolar lavage, high-resolution chest CT, nasal potential difference (NPD) testing and exclusionary testing for ciliary dyskinesia and immune deficiency confirming CF diagnosis.

Despite the existence of several diagnostic tests, a precise and reliable CF diagnostic method still does not exist. 2-10% of all CF diagnoses are atypical cases not detectable by the current gold standard CF diagnostic techniques such as quantitative pilocarpine iontophoresis and NPD test (Mishra et al., 2005, Wang and Freedman, 2002).

1.1.3 Therapy

The Cystic Fibrosis Foundation (CFF) guidelines suggest aggressive treatment of pulmonary exacerbations (defined as a progressive decline of lung function with episodes of acute deterioration of respiratory symptoms) with intravenous antibiotics aiming to control severe inflammation in the lungs, to improve pulmonary outcomes and extend life expectancy (Flume et al., 2009, Conese et al., 2009).

Once a pulmonary exacerbation is diagnosed, current treatment includes antibacterial drugs against *P. aeruginosa* activity, other anti-inflammatory medicines such as corticosteroids and non-steroid anti-inflammatory drugs (Narasimhan and Cohen, 2011, Hoiby, 2011), airway clearance techniques, improved nutrition (Milla, 2007) and relief of various symptoms (Donaldson et al., 2006). Although aggressive approaches using continuous courses of high-dose antibiotics every three months are designed to avoid permanent pulmonary damage, this regimen can lead to the development of drug resistance. Furthermore, while antibiotics can improve lung function and delay tissue

remodelling, developing resistance restricts the long-term use of these medicines (Konstan and Davis, 2002, CFF, 2009).

Double lung or heart-lung transplantation is considered as a treatment option for patients with progressive and/or end-stage lung disease. Despite the fact that modern techniques have lowered post-transplant mortality levels to 5%, infection and graft rejection still remain major problems in patients undergoing lung transplantation (Chan et al., 2006, Hirche, 2014). An ultimate cure for CF would be a restoration of CFTR function via transfecting cells with the wild type CFTR gene. Although some progress has been achieved in the field of gene therapy, it is still in its developmental stage and is not widely used in CF patients (Conese et al., 2009, Mallory, 1996).

Recently, development of potentiator molecules restoring CFTR protein function has been acknowledged to successfully improve the outcomes of lung exacerbation in patients with CF. Several clinical studies in patients 6 years and older with CF have reported, that Ivacaftor (VX-770), a newly developed compound, possesses the ability to improve CFTR's channel function and consequently improve Cl^- transport (Van Goor et al., 2009) as well as to potentiate the open-channel probability of the CFTR protein (Ramsey et al., 2011).

To date, it is the only known effective medication, and yet the safety and long-term effects of the drug are to be evaluated in larger scale clinical trials, phase III completed studies have reported successful use of this molecule associated with significantly improved pulmonary lung function (FEV1), decline in the frequency of exacerbations, decrease in sweat chloride levels (Flume et al., 2012) as well as

improved weight and walking distance in patients with CF who have G551D-CFTR mutation (Harrison et al., 2013, Condren and Bradshaw, 2013, Bobadilla et al., 2002). It would seem likely that other drugs targeting specific CF genotypes will be developed in the future.

1.2 Inflammation in cystic fibrosis

1.2.1 CFTR deficiency and lung pathology

Several hypotheses have been developed to associate the loss of CFTR with changes in CF airways' structure, physiology and increased susceptibility to bacterial infection.

One, proposed by Smith in 1996 and confirmed by Zabner in 1998, is a salt-defensins (high salt) hypothesis. The core statement of this theory is that CFTR protein is considered to function mostly as an anion channel: lack or absence of functional CFTR results in disproportionate accumulation of Na^+ (≥100 mM NaCl) and Cl^- ions in airway surface liquid (ASL) as a result of altered Cl^- conductance (Smith et al., 1996, Zabner et al., 1998). These changes consequently alter functioning of innate defensive mechanisms inactivating salt-sensitive antibacterial peptides and β-defensins 1 and 2. The activity of these salt-sensitive proteins is significantly reduced in the ASL of CF patients due to ion disbalance (Smith et al., 1996) allowing increased bacterial colonisation on the airway surfaces of CF patients (De Rose, 2002)(Figure 1-1).

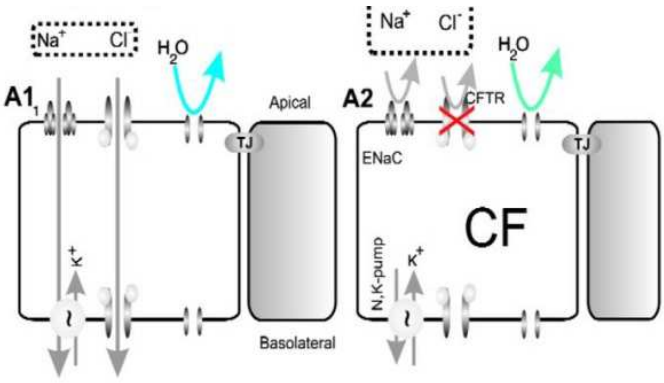


Figure 1-1. The high salt/defensins hypothesis. In healthy lungs, ASL has low salt levels maintained by a combination of surface tension and impermeant anions. In CF lungs, salt is poorly absorbed resulting in extremely salty ASL. The most important features of this model are impaired CFTR Cl^- conductance and development of hypertonic salt absorption due to a thin surface layer and residual water trapping. No any specific role for the inhibition of ENaCs by CFTR is observed (Wine, 1999).

This hypothesis was challenged by Matsui who suggested that changes in the CF lungs are due to CF airway epithelium absorbing isotonic fluid at accelerated rates compared to control cell lines rather than a result of differences in Na^+ and Cl^- levels and/or altered osmolality (Matsui et al., 1998). These findings were confirmed by another research group that highlighted the role of CFTR as a regulator of other channels, namely ENaCs. Loss of CFTR, negatively regulating ENaCs, results in an increased Na^+ absorption, excessive Cl^- flow via shunt pathways and transcellular water absorption leading to a reduction in ASL volume. These changes result in further impairment of mucociliary clearance and development of thick and dry mucus promoting airway infection by CF-associated pathogens (De Rose, 2002, O'Sullivan and Freedman, 2009, Matsui et al., 1998) (Figure 1-2).

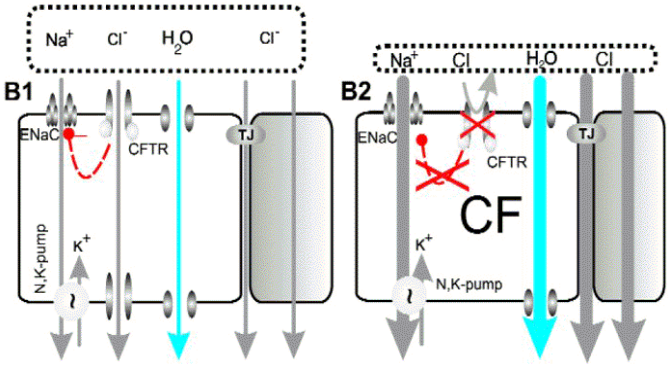


Figure 1-2. The low volume hypothesis. ASL of healthy subjects contains salt levels almost equal to plasma. In CF lungs, lack of ENaCs inhibition due to impaired CFTR function leads to abnormally elevated isotonic fluid absorption depleting ASL and resulting in reduced mucociliary clearance. The main characteristics of this model are Cl^- channel shunt pathway(s) and inhibition of ENaC via CFTR (Wine, 1999).

Another hypothesis, linking CFTR deficiency and amplified bacterial susceptibility in CF, is a cell-receptor theory suggesting that acidification (Poschet et al., 2001) or alkalisation (Imundo et al., 1995) of organelles is responsible for increased susceptibility to P. aeruginosa via amplification of asialoglycolipid (aGM1) molecules on the cell surface serving as receptors for bacteria (Poschet et al., 2001, Imundo et al., 1995). An alternative hypothesis considers the CFTR as a receptor for *P. aeruginosa* indicating that whilst accurately functioning CFTR assimilates and destroys the bacteria, the mutated gene is not able to bind the pathogen allowing growth and multiplication of the latter in the lumen of CF airways (Pier et al., 1996).

However, although all the above mentioned hypotheses are debatable, it is inarguable, that lung disease in CF is characterised by progressive and uncontrollable inflammatory response to bacterial and other stimuli accompanied by neutrophil influx and pro-inflammatory cytokines release (Ratjen and Döring, 2003).

1.2.2 Bacterial presence in the lungs

Several studies have shown that soon after birth, CF patients become infected with bacteria and develop severe lung inflammation. A variety of microorganisms such as *Staphylococcus aureus* (*S.aureus*), *Haemophilus influenzae* (*H.influenza*), *Pseudomonas aeruginosa* (*P.aeruginosa*) and *Burkholderia cepacia complex* (*BCC*) can colonise the endobronchial lumen of patients with CF (Harrison, 2007, Lyczak et al., 2002, Coutinho et al., 2011). CF patients are characterised by *S. aureus* and *H. influenzae* early in life followed by replacement with *P.aeruginosa* in adolescence or adulthood. After initial colonisation with non-mucoid strains, untreated patients become chronically infected with alginate-coated mucoid strains of *P.aeruginosa* (Callaghan and Mcclean, 2011, Delhaes et al., 2012, Bragonzi et al., 2005). Transformation into this type as well as impaired mucociliary bacterial clearance alongside with secreted toxins makes the eradication of *P.aeruginosa* difficult. This pathogen causes long-term impairment of lung function via a release of numerous tissue-damaging mediators such as proteases, neutrophil elastase (NE), and other agents resulting in a decline in lung function and a worse prognosis (Nichols et al., 2008). Though the exact mechanisms of increased susceptibility to *P.aeruginosa* in CF are unclear, there is increasing evidence that altered CFTR function, increased number of asialylated pseudomonal receptors on the cell surface and compromised mucociliary clearance may be involved (Lyczak et al., 2002, Starner and McCray, 2005).

The epidemiology of pulmonary infection has changed during the past few years and now encompasses newly emerging pathogens such as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Aspergillus spp*, *Klebsiella spp* and non-tuberculous mycobacteria. The identification of new pathogens and increased complexity of the bacterial environment changing the manifestation and course of CF can be in part explained by advances in medical care, continuous research, and improved management (Lambiase et al., 2006).

1.3 Inflammatory response

1.3.1 Overview of an inflammatory process

Inflammation is a non-specific immune response developing in reply to injury (Ferrero-Miliani et al., 2007). This protective process is normally initiated by cells such as macrophages, dendritic cells, histiocytes, Kupffer cells and mastocytes. These cells present receptors named pattern recognition receptors (PRRs) on their surfaces that recognise particles shared by pathogens but different from the host molecules called pathogen-associated molecular patterns (PAMPs). Upon activation, cells release inflammatory mediators altering blood vessel permeability and allowing leukocytes (mostly neutrophils) migration along a chemotactic gradient. The inflammation is potentiated by cell-derived mediators and activated biochemical cascade systems (Cotran et al., 1998, Abdel-Azim, 2011, Ricciotti and FitzGerald, 2011). Pro-inflammatory mediators such as lysosomal enzymes, histamine, interferon (IFN)-γ, interleukin 8 (CXCL8), leukotriene B4, nitric oxide, prostaglandins, tumor necrosis factor (TNF)-α and CXCL1 are responsible for clinical

symptoms and pathophysiological changes. Chemokines such as CXCL8 are responsible for activation, recruitment and chemotaxis of neutrophils, their migration across the epithelium and further production of cytokines (Eming et al., 2007, Rottner et al., 2009).

In healthy subjects, the inflammatory process is self-limiting due to the short half-life of released mediators quickly degrading in the inflammatory focus. Once the stimulus has been removed, the inflammation resolves (Cotran et al., 1998, Soehnlein and Lindbom, 2010) through several mechanisms including production and release of anti-inflammatory cytokines such as transforming growth factor (TGF) ß (Ashcroft, 1999, Soehnlein and Lindbom, 2010), CXCL10 (Sato et al., 1999, Asadullah et al., 2003, Ouyang et al., 2011) and anti-inflammatory lipoxins (Serhan, 2008, Soehnlein and Lindbom, 2010). Downregulation of pro-inflammatory mediators such as leukotrienes and upregulation of anti-inflammatory molecules including CXCL1 receptor agonist or soluble tumour necrosis factor receptor (TNFR) (Eming et al., 2007) along with apoptosis of pro-inflammatory cells (Greenhalgh, 1998, Aggarwal et al., 2014) also contribute to resolution. Desensitisation and downregulation of receptors and cleavage of chemokines via matrix metalloproteinases (MMPs) 8 and 9 (McQuibban et al., 2000) are other mechanisms contributing to the resolution of inflammation (Figure 1-3).

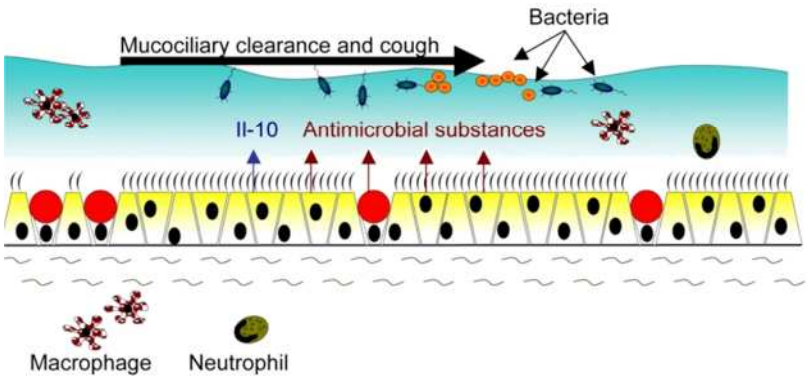


Figure 1-3. Inflammation in non-CF airways. In healthy subjects bacterial invasion results in activation of protective immunological mechanisms in the airways: macrophages, neutrophils and other competent cells migrate to the inflammatory focus and release pro-inflammatory mediators. This is followed by active gene transcription and increased expression of anti-inflammatory and decreased production of pro-inflammatory cytokines with further resolution of the process through cough and mucociliary clearance (adapted from http://www.cfgenetherapy.org.uk/cysticfibrosis/causes.html).

1.3.2 Inflammatory chemokines

Chemokines (chemotactic cytokines) are a family of small (8-15 kDa) proteins sharing common structural and functional motifs which traffic leukocytes to areas of injury. Chemokines are divided into four subfamilies: CXC, CC, CX3C and XC based on the number and position of four conserved cysteine residues in the N-terminal end of the protein (Zlotnik and Yoshie, 2000, Russo et al., 2014). To date, around 50 chemokines and 18 chemokine receptors have been identified (Steinke and Borish, 2006, Colobran et al., 2007, Balkwill, 2004).

The majority of chemokines perform their functions via binding of their N-terminal region (Deshmane et al., 2009) to G-protein coupled receptors present on different cells including leukocytes and endothelial cells (Murphy, 1994, Mélik-Parsadaniantz and Rostène, 2008). This reaction initiates various intracellular processes activating different signalling pathways and corresponding physiological effects. An additional

complexity is achieved as cells express receptors for several chemokines and are a target for several mediators with overlapping effects (Murdoch and Finn, 2000, Viola and Luster, 2008).

Chemokines play a pivotal role in the immune response due to their ability to sample antigen and recruit/direct leukocytes to the site of injury or infection by trans-endothelial migration (Van Coillie et al., 1999, Zlotnik and Yoshie, 2000, Speyer and Ward, 2011). Chemokines also play a role in host immune responses, homeostasis, T cell development, angiogenesis, wound healing, and immune surveillance (Zlotnik and Yoshie, 2000, Steinke and Borish, 2006, Speyer and Ward, 2011).

Chemokines are classified as inducible (inflammatory) or constitutive (homeostatic). Inducible chemokines are induced by bacterial products, growth factors such as TGF-ß, pro-inflammatory mediators such as IL-1 and several pathophysiologic conditions both independently and in cooperation with other stimuli (Brat et al., 2005). In contrast, constitutive chemokines are expressed in the absence of infection or damage (Colobran et al., 2007).

1.4 CXCL8 and its role in CF inflammation

1.4.1 Excessive inflammation in CF

Inflammation is the major driver of airway pathology in CF and is characterised by excessive influx of polymorphonuclear neutrophils (PMNs), macrophages and monocytes. Lung secretions as well as sputum obtained from patients with CF have large concentrations of TNF-α, IL-1, IL-6, CXCL8 and other pro-inflammatory

mediators (Cohen-Cymberknoh et al., 2013, Elizur et al., 2008). BAL fluid and sputum of CF patients already have higher levels of CXCL8 compared to non-CF subjects by the age of 6-7 months (Flume and Van Devanter, 2012). Furthermore, infants with CF have disproportionate expression of pro-inflammatory cytokines including CXCL8 in response to bacterial overload (Noah et al., 1997, Starner and McCray, 2005, Heijerman, 2005, Chmiel and Davis, 2003), but also in the absence of lung infection (Khan et al., 1995a, Verhaeghe et al., 2007a, Cohen and Prince, 2012).

Endogenous activation of CF airways together with excessive bacterial overload and increased number of aGM1 receptors are thought to be responsible for the distinctive inflammatory response in CF (DiMango et al., 1998, McClean and Callaghan, 2009). CF airways are infiltrated with neutrophils that excessively produce pro-inflammatory mediators and reactive oxygen species (ROS) causing damage. Decomposition of neutrophils is the major source of the deoxyribonucleic acid (DNA) that makes the sputum of CF patients viscous and difficult to expectorate (De Rose, 2002, Livraghi and Randell, 2007). Altered inflammation is worsened by electrolyte misbalance and dehydration maintaining and amplifying bronchoconstriction and impairing airway clearance. Collectively these studies suggest that disproportionate and persistent inflammation is a key component of the CF lung pathology. Furthermore, there is evidence that it is initiated and governed by constitutive alterations in the regulation of cytokine production by airway epithelial cells (De Rose, 2002, Cohen-Cymberknoh et al., 2013).

Accumulation of mediators and imbalance of pro- and anti-inflammatory cytokines contribute to further damage (Corvol et al., 2003). Neutrophils are a source of prostaglandin E2 (PGE2) that has an anti-inflammatory effect through lowering levels of endothelial adhesion and chemotaxis (Nakanishi and Rosenberg, 2013). CF lungs are also deficient in IL-10, a major anti-inflammatory cytokine, and nitric oxide (NO) leading to an activation of pro-inflammatory signalling pathways (Saadane et al., 2005, Nakanishi and Rosenberg, 2013) resulting in lung injury (Cohen-Cymberknoh et al., 2013, Serhan, 2008, Sagel et al., 2007, Corvol et al., 2003) (Figure 1-4).

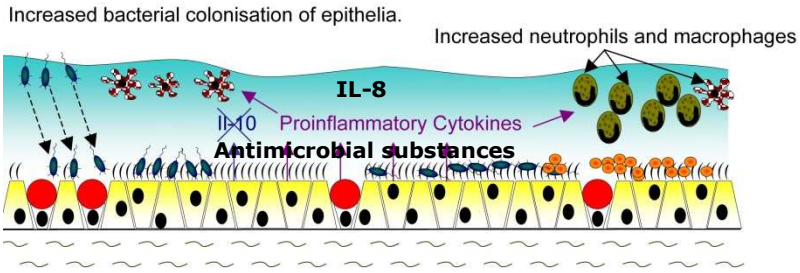


Figure 1-4. Inflammation in CF airways. In CF lungs, impairment of local defensive mechanisms in conjunction with ASL pathology results in bacterial overload leading to continuous neutrophil recruitment with excessive release of pro-inflammatory cytokines and potent chemoattractant CXCL8. Failure of protective mechanisms including lack of IL-10, local defensins, electrolyte misbalance and compromised mucociliary clearance results in a development of a vicious circle of inflammation and lung damage (adapted from http://www.cfgenetherapy.org.uk/cysticfibrosis/causes.html).

1.4.2 CXCL8 and other pathologies

1.4.2.1 CXCL8 and asthma

Several studies have reported increased CXCL8 levels in BAL fluid and sputum obtained from asthmatic patients implicating the importance of this chemokine in the pathogenesis of bronchial asthma (Norzila et al., 2000, Yalcin et al., 2012).

Biopsies of bronchial mucosa obtained from patients with mild and asymptomatic asthma have demonstrated increased secretion of MCP-1, RANTES, IL-5 resulting in eosinophils' recruitment to the airways and submucosal infiltration with activated lymphocytes and eosinophils resulting in development of fibrosis and oedema. These changes lead to an enhanced activation and release of leukotrienes and eosinophilic proteins further damaging airway epithelium and promoting bronchial hyper responsiveness. Several studies have demonstrated increased levels of eosinophils, macrophages and overexpression of pro-inflammatory cytokines such as CXCL8, TNF-α, IL-6, IFN-γ promoting local inflammation (Adcock and Caramori, 2001). Studies using CXCR2-deficient mice have showed increased levels of IgE and CXCL8 in serum suggesting selective inhibition of IL-4-induced IgE production by CXCL8 with an establishment of a negative feedback for IgE expression (Mukaida, 2003). Although further research has demonstrated an association between excessive inflammation in the airways of asthmatic patients and severity of the disease (Pukelsheim et al., 2010), the exact mechanisms and role of CXCL8 in the pathogenesis of bronchial asthma are not clear yet.

1.4.2.2 CXCL8 and COPD

COPD is another inflammatory lung disease that has been characterised by an increased expression of CXCL8: BAL fluid, sputum and plasma of COPD patients have been reported to have higher levels of CXCL8 and CXCL6 compared to normal controls (Hacievliyagil et al., 2013, Chan et al., 2010, Sin and Man, 2008, Culpitt et al., 2003). Greater CXCL8 protein secretion has been linked to an increased mRNA

expression (de Boer et al., 2000) and associated with higher basal CXCL8 production from airway epithelial cells of COPD patients (Profita et al., 2003, Schulz et al., 2003). Oxidative stress existing in the airways results in an activation of NF-κB-mediated signalling and synthesis of pro-inflammatory cytokines promoting neutrophil influx and stimulating transcription of CXCL8 and other chemokines including IL-6, TNF-α, and MMP-9 protease (Van Eeden and Sin, 2013). Excessive inflammation characterised by increased levels of macrophages, T-lymphocytes, and neutrophils in the bronchial lumen (Barnes and Cosio, 2006, Barnes, 2013) promote thickening of a bronchial wall and increased smooth muscle tone, remodelling of small airways, and destruction of lung parenchyma as result of loss of elastic structures due to protease/antiprotease imbalance (Roche et al., 2011). These changes result in a development and establishment of vicious circle: the level of lung inflammation directly correlates with disease severity (Chan et al., 2010).

1.4.2.3 CXCL8 and IPF

Several in vivo and in vitro studies have reported increased CXCL8 production by alveolar macrophages and higher levels of this chemokine in serum and BAL fluid of patients with IPF demonstrating a direct correlation between level of inflammation and disease severity. The inflammatory stage in IPF is characterised by an increased influx of monocytes, neutrophils, T-lymphocytes and eosinophils to the lungs secreting high levels of CXCL8, CCL2 and CCL5 (Razzaque and Taguchi, 2003). BAL fluid of patients with IPF has been reported to contain enhanced levels of CXCL8 and lower levels of CXCL10 as compared to normal controls. It has been suggested

that these cytokines regulate angiogenesis in IPF as CXCL8 possesses potent angiogenic properties as opposed to angiostatic activity of CXCL10 (Schwiebert, 2005). Administration of anti-mouse CXCR2 antibodies to bleomycin-induced IPF mice has resulted in reduced angiogenesis, but not neutrophil infiltration, whereas an inhibition of CXCL10 transcription has repressed angiogenesis process, but not neutrophil migration (Mukaida, 2003). The impaired balance between CXCL8 and CXCL10 results in an excessive accumulation of matrix proteins, destruction of the alveolar wall, loss of airway elasticity and development of severe angiogenesis and decreased pulmonary function that are hallmarks of IPF (Mukaida, 2003, Schwiebert, 2005).

1.4.2.4 CXCL8 and cancer

Enhanced CXCL8 production by tumour cells has been reported in several animal models of various cancer types (Li et al., 2005). BAL fluid of patients with bronchial carcinoma has demonstrated increased levels of neutrophils and CXCL8 and IL-6 correlating with poor outcome (Mukaida, 2003). Existing knowledge suggests an increased expression of CXCR1 and CXCR2 receptors on cancer cells, endothelial cells, neutrophils/tumour-associated macrophages (Waugh and Wilson, 2008) and in in vivo models of breast cancer (Singh et al., 2013, Russo et al., 2014). CXCL8 is thought to be involved in tumour progression via recruitment and activation of macrophages producing growth factors, cytokines CXCL1, CXCL2, CXCL5, CXCL6, CXCL8 and CXCL7 promoting migration of tumour-associated leukocytes and endothelial cells. Some in vivo studies using animal models of non-small lung and

gastric cancer have suggested a direct correlation between CXCL8 transcription and level of neovascularisation in tumour tissues via increased expression of metastasis-related genes, such as MMPs (Mukaida, 2003). Blocking CXCL8 activity with a monoclonal antibody in murine cancer models has led to a reduction in tumour growth (Mian et al., 2003, Qazi et al., 2011). Further research in androgen-independent prostate cancer and melanoma cells has proposed a direct correlation between CXCL8 levels and tumorogenicity and metastatic potential in in vivo models. The ability of CXCL8 to upregulate MMP2 results in increased collagenase activity and increased tumour cell invasiveness in in vitro models (Schwiebert, 2005). Although some progress has been made, the exact role of CXCL8 in cell differentiation, neovascularisation, fibrosis and metastasis in cancer still remain elusive.

1.4.3 CXCL8 structure

CXCL8 is secreted from leukocytes and other granulocytes, T cells, fibroblasts, airway smooth muscle cells, endothelial and epithelial cells (Russo et al., 2014, Brat et al., 2005). It is induced by TNF-α, IFN-γ, other chemokines including IL-1, bacterial flagella and the lipopolysaccharide (LPS) component of the bacterial wall, and viruses (Hoffmann et al., 2002, Shi et al., 2004, Venza et al., 2009).

CXCL8 acts on two heterotrimeric G protein-coupled surface receptors, CXCR1 and CXCR2 (Nasser et al., 2009) expressed on the surfaces of leukocytes (mostly granulocytes) and endothelial cells. CXCL8 receptors share 78% homology, but differences in the N-terminal domains result in different binding peculiarities (Russo

et al., 2014). Whereas CXCR1 binds CXCL6 and CXCL8, CXCL1, 2, 3, 5, 6, 7 and 8 have higher affinity towards CXCR2 (Balkwill, 2004). The classical chemotactic CXCL8 response implicates involvement and activation of pertussis toxin-sensitive $G_{\alpha i}$-proteins (Thelen, 2001, Campbell et al., 2013), while non-classical CXCL8 response involves stimulation of pertussis-insensitive G_{α}-proteins (Schraufstatter et al., 2001, Campbell et al., 2013).

1.4.4 CXCL8 functions

CXCL8 has a range of biological functions including promotion of directed chemotaxis in target cells and their migration to the site of inflammation (Qazi et al., 2011). The sequence of physiological reactions prerequisite for migration and phagocytosis includes an increase in intracellular calcium (Ca^{2+}) levels, exocytosis, release of a variety of lysosomal enzymes from activated neutrophils, and the respiratory burst (Brat et al., 2005). The latter is vital in allowing phagocytes to degrade bacteria through the swift release of ROS from immune cells including neutrophils and monocytes coming into contact with bacterial particles. CXCL8 can also promote neutrophil adhesion to endothelial cells and their trans-endothelial migration (Mukaida, 2003, Qazi et al., 2011) as well as neutrophil activation (Qazi et al., 2011) and histamine liberation from human basophils (Brat et al., 2005).

CXCL8 is also involved in the regulation of ion transport, activation and proliferation of cells including epithelial cells, phagocytosis, angiogenesis and tumorigenesis (Rossi and Zlotnik, 2000, Brat et al., 2005). Collectively, all these properties and

functions indicate that CXCL8 is a key component of the inflammatory response

CXCL8 transcription and regulation.

1.4.5 CXCL8 transcription and regulation

Gene expression is tightly regulated by well-established mechanisms resulting in the transcription of target genes in response to stimulation by specific signal transduction pathways that can either activate or silence gene expression (Venters and Pugh, 2009). The majority of genes are regulated at the transcriptional level by synchronised binding of transcription factors (TFs) to cis-acting DNA elements in the promoter region of the relevant gene. Gene expression is mediated via a coordinated binding of different TFs rather than by sole presence or absence of a single TF (Hoffmann et al., 2002). CXCL8 expression is also regulated post-transcriptionally by stabilisation of mRNA transcripts (via the p38 mitogen-activated protein kinase (MAPK) pathway); stationary mRNA levels are usually comparative to CXCL8 secretion (Hoffmann et al., 2002, Li et al., 2002, Shi et al., 2004).

Sequencing analysis of the CXCL8 promoter region has shown that the 5'-flanking region encompassing an area from -425 to -70 (Hoffmann et al., 2002, Mukaida, 2003) relative to the transcription start site comprises binding sites for various TFs including CCAAT/enhancer binding protein (C/EBP)β, nuclear factor (NF)-κB, activator protein (AP)-1, and octamer (Oct)-1 binding proteins (Campbell et al., 2013, Brat et al., 2005, John et al., 2010)(Figure 1-5).

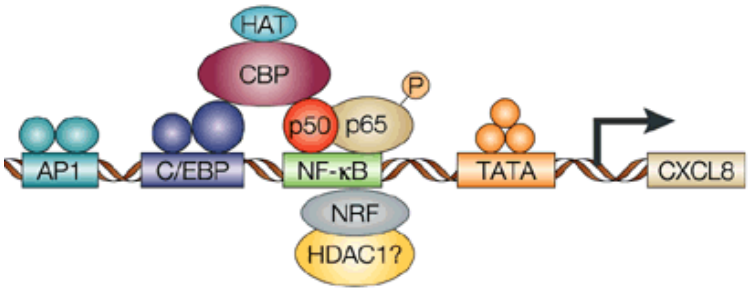


Figure 1-5. Schematic representation of the human CXCL8 promoter region. The CXCL8 gene promoter contains binding sites for C/EBPβ, NF-κB, and AP-1 located in close proximity to each other (Richmond, 2002).

Synchronised binding of NF-κB, AP-1, and C/EBPß is required for the integrated effect and ultimate activation of CXCL8 transcription upon induction by inflammatory stimuli (John et al., 2009, Verhaeghe et al., 2007b, Hoffmann et al., 2002). Though all three factors are involved in the regulation and transcription of CXCL8, studies using transient transfections in cancer cell lines have demonstrated that CXCL8 expression is NF-κB-driven. Although different members of the κB/RelA family have different DNA-binding affinity, it is RelA that influences CXCL8 gene transcription (John et al., 2009, Chen et al., 2002). NF-κB then causes recruitment of a large co-activator complex incorporating histone acetyltransferase (HAT) proteins such as cAMP response element binding protein (CREB) binding protein (CBP) and p300/CBP (PCAF), transcriptional intermediary factor-2 (TIF-2), p160 family members and steroid receptor coactivator-1 (SRC-1) (Jenkins et al., 2001, Adcock et al., 2006). Histone acetylation and/or DNA methylation can also influence CXCL8 transcription (Muselet-Charlier et al., 2007, Bartling and Drumm, 2009).

1.5 NF-κB

1.5.1.1 NF-κB role and functions

Studies in immortalised cell lines, patient samples and animal models have shown that lung inflammation in CF is associated with increased NF-κB signalling which contributes to the excessive CXCL8 expression (Saadane et al., 2005, Knorre et al., 2002, Joseph et al., 2005).

NF-κB is an inducible potent transcriptional activator of a vast number of genes involved in the regulation of stress-induced, inflammation and immunological responses (Gallagher et al., 2014). NF-κB is activated by LPS, inflammatory cytokines including TNF-α and IL-1ß, growth factors, lymphokines, oxidant-free radicals, B or T-cell activation, viral infections, inhaled particles and UV radiation (Pomerantz and Baltimore, 2002). NF-κB is constitutively expressed in several cells playing a key role in gene control and regulation (Barnes, 2006, DiMango et al., 1998).

1.5.1.2 NF-κB structure and regulation

NF-κB is a heterodimeric protein belonging to the NF-κB/Rel protein family and is composed of various combinations of members of the Rel family. The NF-κB/Rel family is characterised by the presence of the Rel homology domain (RHD) responsible for the DNA binding, nuclear localisation, and protein dimerisation and a 300-amino acid N-terminal region (Hoesel and Schmid, 2013, Huxford and Ghosh, 2009). The N-terminal region encompasses the DNA-binding domain, whilst the C-terminal contains the dimerisation domain of the RHD and nuclear localization signal (NLS) responsible for the translocation of active NF-κB complexes to the

nucleus. These proteins are responsible for the regulation of cytokines and other modulators of the host immune response (Hoesel and Schmid, 2013, Hayden et al., 2006).

The mammalian NF-κB family consists of p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) proteins and can be divided into two groups. P65, RelB and c-Rel contain powerful transactivation domains (TDs) and are rich in serine, acidic and hydrophobic amino acids necessary for transactivation activity. The second group comprising p50 and p52 proteins do not possess TDs, and, thus, cannot function as independent transcriptional catalysts (Heissmeyer et al., 2001, Hoesel and Schmid, 2013). NF-κB is composed of homo- and heterodimers of the five members which possess different DNA binding characteristics (Gilmore, 2006).

The active form of human NF-κB represents a dimer composed of two DNA binding subunits: a 50-kDa subunit (initially known as p50 and then renamed NF-κB1), and a 65-kDa subunit (previously called p65 and now titled RelA) (Hayden et al., 2006, Huxford and Ghosh, 2009). Activity of the NF-κB heterodimer depends on the coordinated functioning of the components and any minor changes alter NF-κB activity. The p65 subunit is responsible for the expressed transcriptional activation of genes, while p50 serves as a regulator of trans-activated p65 subunit increasing its' DNA-binding affinity (Baldwin, 2001, Oeckinghaus and Ghosh, 2009). The NF-κB dimer binds to DNA sequences of the consensus S'-GGGPuNNPyPyCC-3'(S) via p65 interacting directly with the basal transcription apparatus (Schmitz and Baeuerle, 1991, Ruben et al., 1992). Whilst overexpression of p65 leads to a constitutive activation, excessive expression of p50 results in the production of a constitutive

DNA-binding protein with no or low trans-activating potential (Nakamura et al., 2002, Guan et al., 2005).

NF-dB exists in 2 forms: one in the cytoplasm of non-stimulated cells which requires dissociation from mediators for its activation, and another in the nucleus, which does not need any factors for its DNA binding activity. In intact cells, NF-κB is retained in the cytoplasm in a complex with inhibitory ankyrin repeat-containing IκB proteins (Grimm and Baeuerle, 1993, Gilmore, 2006). Although this family has several members (IκBα, IκBß, IκBγ, IκBε, IκBζ, Bcl-3, and the Drosophila Cactus protein), most mammalian cells contain IκBα and IκBß proteins (Arenzana-Seisdedos et al., 1997, Bergqvist et al., 2008). These proteins vary in their affinity for specific Rel/NF-κB complexes and regulatory mechanisms and have tissue-specific distribution (Baldwin, 1996).

The main role of the IκB family members is to prevent the nuclear translocation of NF-κB via multiple contacts with the NF-κB heterodimer, and, thereby, downregulating κB-dependent gene expression in the nucleus (Gilmore, 2006). The best studied NF-κB/IκB interaction is association of NF-κB p50/p65 dimer with IκBα inhibitory protein. In unstimulated cells, the NF-κB dimer is stored in the cytosol via non-covalent interactions with IκBα (Blaecke et al., 2002). Upon stimulation, IκB proteins, associated with NF-κB, undergo targeted phosphorylation and ubiquitination. The NF-κB/IκB complex disassociates releasing NF-κB as a homodimer exposing its nuclear localisation sequence and allowing nuclear translocation of NF-κB (Ferreiro and Komives, 2010). The components of the NF-κB/IκBα complex easily dissociate and re-associate, signifying that inhibition by IκB

inhibitory protein is reversible (Hoffmann and Baltimore, 2006, Trask, 2012). Thus, the NF-κB/IκBα complex continuously shuttles between the nucleus and the cytoplasm, but due to prevalence of the nuclear export over the import rate, the complex mainly remains in the cytoplasm (Hoffmann and Baltimore, 2006).

1.5.1.3 NF-κB activation and target gene regulation

Once in the nucleus, the p65 subunit binds to the κB sites in the target gene promoter either independently or in a complex with other (Moreno et al., 2010, Ferreiro and Komives, 2010). NF-κB initiates target gene transcription through the activation of a high molecular weight complex containing a serine-specific IκB kinase (IKK). There are three known subunits of IKK - IKKα, IKKß and IKKγ; IKKα and IKKß are associated catalytic kinase subunits and IKKγ (aka NEMO) is a principal subunit identifying and governing upstream signals. Classical or canonical NF-κB pathway includes activation of the IKK complex via phosphorylation of two specific serines near the N-terminus of IκBα by IKKß that results in the ubiquitination and degradation of IkBa by the 26S proteasome. During the non-canonical (alternative) pathway, activation of the p100/RelB complex takes place via phosphorylation of the C-terminal region of p100 by an IKKα homodimer that consequently leads to the ubiquitination and degradation of p100 IκB-like C-terminal sequences to create p52/RelB. In both cases, the unrevealed NF-κB complex translocates to the nucleus and activates target gene transcription (Figure 1-6). The classical pathway includes activation and regulation of NF-κB-induced target genes through penetration of newly-synthesised IκBα into a nucleus, removal of NF-κB from DNA and relocation

of NF-κB/IκBα complex to the cytoplasm to recover the initial dormant status (Pomerantz and Baltimore, 2002, O'Dea and Hoffmann, 2010, Gilmore, 2006, Ferreiro and Komives, 2010).

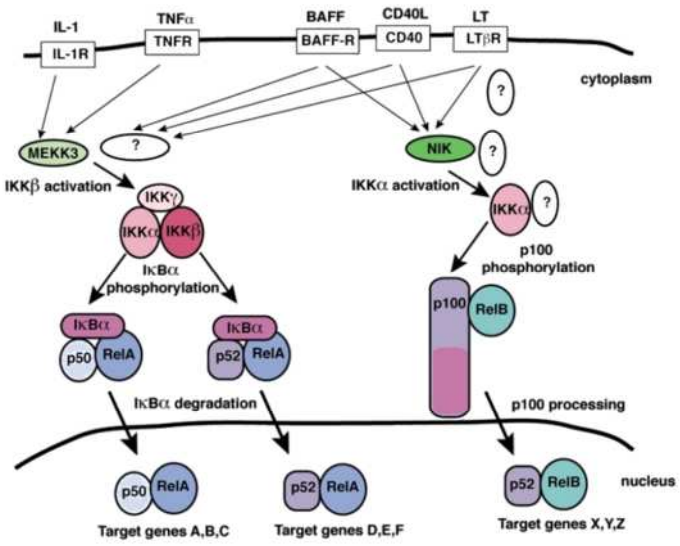


Figure 1-6. Canonical (left) and non-canonical (right) pathways for the activation of NF-κB (Pomerantz and Baltimore, 2002).

However, regulation of the NF-κB pathway is complex and can be modified by other mechanisms such as DNA methylation and/or histone acetylation, methylation, phosphorylation, oxidation/reduction, and prolyl isomerisation. Some studies have shown that ligand binding, its phosphorylation, and integration with regulatory subunits can considerably affect DNA binding capacity and/or transcriptional activity of NF-κB-mediated genes (Moreno et al., 2010). Finally, proteins involved in the NF-κB signalling pathway and protein-protein interactions with non-NF-κB proteins such as p300 (Yu et al., 2004), HDACs (Chen et al., 2001) and TFIIB (Xia et al., 2004), make the regulation process even more complex.

1.5.2 Other transcription factors involved in CXCL8 transcription

Although NF-κB is a vital regulator of inflammatory cytokines and CXCL8 in particular in all cell types (Hoffmann et al., 2002), it is unlikely, that NF-κB alone is able to cause excessive CXCL8 transcription in CF. Several studies have suggested, that CXCL8 is under multifactorial control and synchronised coordination by C/EBPß, and AP-1 along with NF-κB is required (Blau et al., 2007, Hoffmann et al., 2002, Li et al., 2002).

1.5.2.1 C/EBPß and its role in CXCL8 regulation

C/EBPß or CCAAT/enhancer-binding protein ß is a protein belonging to the C/EBP-related family of nuclear TFs also called bZIP proteins (Ramji and Foka, 2002, Gene Entrez, 2012). The most prominent characteristic of this class of proteins is their ability to bind the CCAAT nucleotide consensus sequence and prompt either transcriptional activation or repression of target genes (Cloutier et al., 2009). The C/EBP family comprises six proteins C/EBPα, C/EBPß, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ. All contain a functionally related leucine zipper dimerization domain (LZ) at the C-terminus in addition to a shared highly conserved basic region (BR) facilitating the sequence-specific DNA binding. The N-terminus of C/EBPα, C/EBPβ, C/EBPδ and C/EBPε also encompasses transactivation domains (TADs) and a regulatory domain (RD). Members of the C/EBP family can form homo- and heterodimers with each other and/or with other TFs. The dimerisation is indispensable for C/EBP proteins to bind to specific DNA palindromic sequence (Johnson, 2005, Cloutier et al., 2009).

C/EBP proteins have wide tissue distribution and can be found in hepatocytes, adipocytes, hematopoietic cells, spleen, kidney, brain, and some other organs. These multifunctional proteins are vital components of numerous cellular processes including regulation of cellular proliferation, growth and differentiation, liver regeneration, metabolism and immune reactions. Experiments in mice have shown that C/EBPß is crucial for the regular functioning of macrophages and their ability to differentiate and execute their biological functions (Ruffell et al., 2009). C/EBPβ has been reported to be involved in the anti-inflammatory response, glial and neuronal cell functioning, and neurotrophic factor expression (Ramji and Foka, 2002).

The majority of the C/EBP family proteins, except C/EBPγ that lacks a functional TAD, can initiate target gene transcription via activation of TADs through cooperation with elements of the basal transcriptional machinery. C/EBP proteins are regulated at multiple levels governed by hormones, mitogens, and cytokines. Histone modifications and/or DNA methylation can influence C/EBPβ signalling including DNA binding, transcriptional activity, protein-protein interactions and intracellular localisation. In an unstimulated cell, C/EBPβ is retained in the cytoplasm in a repressed condition via dimensional inhibition of TADs by regulatory domains. Upon activation, phosphorylation of the inhibitory domains results in termination of the repressive effect and leads to an increase in transcriptional activation of C/EBPβ and enhanced DNA binding followed by either activation or inhibition of gene activity (Ramji and Foka, 2002).

C/EBPß is involved in the regulation of immune and inflammatory genes. Its' binding sequences are found in the regulatory regions of a number of pro-inflammatory cytokines such as IL-1ß, CXCL8, TNF-α and IL-6 (Gene Entrez, 2012). There is a direct physical association of the bZIP region of C/EBP proteins with the p65 homology domain of NF-κB. The cross-coupling of these proteins results in an interaction between distinct TF families and modulation of target gene expression. Furthermore, excess NF-κB might physically interact with C/EBP bound to its site and synergistically activate it. Alternatively, it has been suggested that excess C/EBP could inhibit NF-κB binding to its binding site. These data suggest that although NF-κB has a crucial role in CXCL8 regulation, its activity can be modified by C/EBP (Paz-Priel et al., 2011).

NF-κB and C/EBP family members are functionally and physically interlinked (Paz-Priel et al., 2011). Binding of representatives from both families to cis-DNA elements in the CXCL8 promoter and subsequent physical protein-protein interactions regulate CXCL8 gene expression (Wang et al., 2009a, Dooher et al., 2011). These findings suggest that C/EBP is an important TF for CXCL8 gene expression and is interlinked both functionally and physically with NF-κB.

1.5.2.2 AP-1 and its role in CXCL8 regulation

AP-1 or activator protein-1 is another protein involved in CXCL8 transcription. It belongs to the AP-1 protein family commonly referred to as "immediate-early genes" swiftly induced in response to extracellular stimuli such as pro-inflammatory cytokines, growth factors and bacterial and viral infection (Hess et al., 2004b).

These proteins are responsible for the regulation of the genes accountable for cell proliferation and differentiation, tumorigenic transformation, apoptosis, pulmonary defense, and inflammatory and immune responses (Reddy and Mossman, 2002, Jochum et al., 2001).

AP-1 is a TF composed of proteins belonging to various families, namely Fos (c-Fos, FosB, Fra1, and Fra2), Jun (c-Jun, JunB, and JunD), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and activating transcription factor-1 (ATF) (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2), which form numerous homo- and heterodimers through their leucine zipper regions (Hess et al., 2004b, Rajasekaran et al., 2012). AP-1 homo- or heterodimers generally contain Jun-Jun or Jun-Fos TFs that belong to the basic region-leucine zipper (bZIP) group. AP-1 members can also form heterodimers with other related bZIP families of proteins such as ATFs, C/EBPs, Nrf/Mafs, and helix-loop-helix ZIP proteins including upstream stimulatory factors (USFs) (Chinenov and Kerppola, 2001, Fujioka et al., 2004). Jun-Fos dimers are more stable with greater DNA binding affinity than Jun-Jun homodimers (Reddy and Mossman, 2002). They are involved in the regulation of a range of genes engaged in epithelial injury and repair, and differentiation (Chinenov and Kerppola, 2001, Zenz et al., 2008).

Regulation of AP-1 is complex and can be influenced by interactions of AP-1 components with other TFs in multifactorial complexes (Shaulian and Karin, 2001, Khanjani et al., 2012) and via changes in the transcription of genes encoding AP-1 subunits, post-translational processing and turnover of newly synthesized AP-1 subunits (Hess et al., 2004b, Wagner, 2001).Upon stimulation, phosphorylation of AP-1 occurs via activation of the MAPK family (Hazzalin and Mahadevan, 2002) that,

in turn, phosphorylates Jun via activation of the Jun N-terminal kinase (JNK) cascade (Muselet-Charlier et al., 2007). Once activated by the MAPK cascade, JNK proteins translocate to the nucleus and phosphorylate Jun at serine 63 and 73 within its N-terminal TAD. Though progress has been made in the field of Jun activation and regulation, the enzymes responsible for Fos activation have not been identified yet (Hess et al., 2004a).

AP-1-dependent gene expression is tightly controlled by interaction between subunits within AP-1 as well as via complex formation between AP-1 and other non-ZIP proteins such as nuclear factor of activated T (NFAT) cells, NF-κB, and TATA-binding protein (TBP) (Chinenov and Kerppola, 2001). Furthermore, post-translational modifications such as phosphorylation, acetylation and/or methylation of each component have an important impact on the regulation of AP-1 activity (Lee et al., 2006, Barnes et al., 2005). NF-κB and AP-1 can mutually heighten the response induced by either of the TFs resulting in a greater inflammatory response (John et al., 2009, Hoffmann et al., 2002). Due to an overlap in the signalling pathways (Khalaf et al., 2010), synchronised binding of both AP-1 and NF-κB alongside C/EBP to the CXCL8 promoter suggests contribution of three TFs to the transcriptional activation of CXCL8 in CF (Mukaida et al., 1990, Adcock and Caramori, 2001, Muselet-Charlier et al., 2007, Raia et al., 2005). Despite some progress in the understanding of AP-1 role in the CXCL8 expression, the exact role of this TF in CF lung inflammation is not clear yet.

1.6 Epigenetic regulation of gene transcription

1.6.1 Concept of epigenetics and epigenetic modifications

The term "epigenetics" was introduced around 60 years ago, but only recently chromatin remodelling and inherited changes have been considered influential factors in the control of gene expression (Holliday, 1987). Epigenetic regulation is essential for cell diversity generation, and retention of constancy and continuity of cell expression profiles (Adcock et al., 2006).

Epigenetics is a scientific term used for the inherited changes in gene expression and activity without alteration in DNA sequence (Adcock et al., 2006, Cheung and Lau, 2005) closely controlled by DNA methylation, RNA-associated silencing and chromatin modifications which are often interlinked (Egger et al., 2004, Wilson, 2008, Cheung and Lau, 2005, Urnov and Wolffe, 2001). Abberant alterations in the activities of any of the components lead to abnormal gene transcripiton resulting in a development of an "epigenetic disease" (Cheung and Lau, 2005, Egger et al., 2004). Epigenetic modificaions, apart from DNA methylation and histone tail modifications, also include transient alterations such as histone acetylation and phosphorylation (Bird, 2007, Ospelt et al., 2011).

Epigenetic alterations of activity and/or expression of chromatin remodelling enzymes in cancer and inflammatory airways diseases including CF have been identified emphasising their importance in the development of these pathologies (Adcock et al., 2006, Cheung and Lau, 2005). Thereby, better understanding of epigenetic molecular mechanisms can lead to the development of new drugs to cure severe age-limiting diseases such as CF.

1.6.2 Chromatin remodelling

In mammalian cells, genomic DNA is tightly compressed into chromatin with categorised levels of hierarchy. The basic 7-fold compaction unit of chromatin, a 146-base pair DNA segment firmly wrapped around eight core histone (H) proteins, two of H2A, H2B, H3 and H4, is called the nucleosome (Cheung and Lau, 2005, Shilatifard, 2006) (Figure 1-7). Nucleosomal DNA can be further compacted via association with the linker H1 and other supplementary non-histone proteins (Cheung and Lau, 2005, Urnov and Wolffe, 2001). Whilst, formerly, histones were considered as inert and static structural elements, they are now recognised as essential and dynamic components of the transcriptional machinery (Strahl and Allis, 2000). Nucleosomes continuously pack and unpack the chromosomal DNA exposing it from an inaccessible condensed condition to accessible open chromatin allowing the DNA to be compactly packaged, correctly replicated and organised into daughter cells (Urnov and Wolffe, 2001).

The N-terminal tails of histone molecules protrude through and beyond the DNA coil representing accessible targets for post-translational modifications of selective amino acids (Firestein et al., 2013, Shilatifard, 2006). Lysine (K) residues acetylated at the tails of histone H3 and histone H4 enable coupling of other co-activators such as chromatin modifying enzymes and RNA Polymerase (Pol) II (Urnov and Wolffe, 2001, Roth et al., 2001).

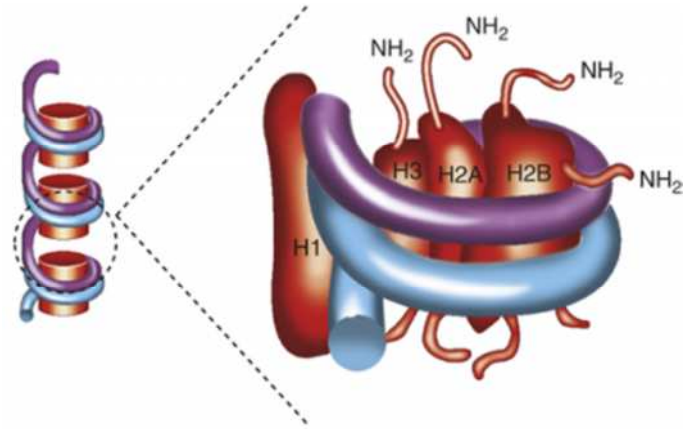


Figure 1-7. The structure of the nucleosome. A 146-base pair DNA segment wrapped around eight core histones (Firestein et al., 2013).

In a non-dividing cell, chromatin is present in two functional states: transcriptionally active euchromatin or inactive heterochromatin (Kouzarides, 2007, Cheung and Lau, 2005). Heterochromatin is a highly packaged condensed form of DNA remaining unchanged during the interphase, inaccessible to TFs and/or chromatin-associated proteins. It is essential in the regulation of chromosomal stability and prevention of mutations and translocations (Talbert and Henikoff, 2006). As opposed to heterochromatin, euchromatin represents a loosened state occurring by the end of mitosis. The genomic regions of euchromatin are flexible and contain transcriptionally active and inactive genes, whilst heterochromatin mostly contains repetitive sequences and silenced genes (Adcock et al., 2006).

Gene transcription is initiated when the chromatin structure is open allowing RNA Pol II and basal transcription complexes to interact with DNA and commence transcription. Activated TFs and chromatin-associated proteins bind to specific DNA recognition sequences and further recruit large co-activator proteins such as CBP,

p300, PCAF and other complexes to the site of gene promoter (Urnov and Wolffe, 2001).

1.6.3 Epigenetic modifications

The molecular mechanisms such as DNA methylation and post-translational modifications of histones such as acetylation, methylation, ubiquitination, sumoylation, and phosphorylation have been implicated in the epigenetic control of the genome (Egger et al., 2004). Histone modifications can occur at several sites, and, thus, can act as signalling bases integrating different pathways to stimulate nuclear responses resulting in a target gene activation or repression (Cheung et al., 2000, Bannister and Kouzarides, 2005). Combinations of different epigenetic alterations serve as epigenetic marks reflecting gene activity and chromatin status (Kouzarides, 2007) constituting a complex "histone code" (Ospelt et al., 2011).

1.6.3.1 Histone acetylation

Acetylation is a reversible modification of lysine (K) residues within the N-terminal tail and/or within the folded core of histones (Grunstein, 1997, Kouzarides, 2007). The positively charged K residues are tightly bound to the negatively charged DNA shaping a closed chromatin structure impenetrable for TFs. Acetylation reduces the affinity between DNA and histones via modification of the charge of K residues, resulting in the decondensation of chromatin and allowing active transcription (Carrozza et al., 2003, Peterson and Laniel, 2004, Ruthenburg et al., 2007). Histone

acetylation has been associated with euchromatin (Kouzarides, 2007), and implicated in metabolism regulation (Guan and Xiong, 2011), DNA recognition and repair (Celic et al., 2006), protein–protein interactions and protein stability (Kouzarides, 2000). The "histone code" suggests that covalent histone modifications serve as epigenetic markers for gene expression (Strahl and Allis, 2000).

Acetylation is associated with gene activation, whilst lack or absence of acetylation is linked to a transcriptional repression (Kuo and Allis, 1998, Waterborg, 2002). Mutation of certain K residues results in an inability of yeast cells to acetylate the histone H4 tail leading to altered GAL1 gene transcription (Durrin et al., 1991). Another study has reported that treatment of mammalian cells with a histone deacetylase (HDAC) inhibitor trichostatin A leads to active transcription of target genes (Yoshida et al., 1995, Lee et al., 2006). Increased histone acetylation has been reported in cancer and different inflammatory diseases (Adcock et al., 2006). Furthermore, it has been recognised to regulate protein-protein interaction and generate a recognition site for bromodomain (BRD) containing proteins and other chromatin remodelling enzymes and co-factors (Filippakopoulos et al., 2010).

1.6.3.2 Histone acetyltransferases and deacetylases

Histone acetylation is carried out by a family of "writer" enzymes named histone acetyltransferases (HATs) that catalyse the transfer of an acetyl group from acetyl-CoA to the lysine 1-amino group on the N-terminal tails of histones (Carrozza et al., 2003). HATs are involved in almost all biologically important cellular processes as

well as DNA repair and replication (Carrozza et al., 2003, Kouzarides, 2000, Yang and Seto, 2007) (Figure 1-8).

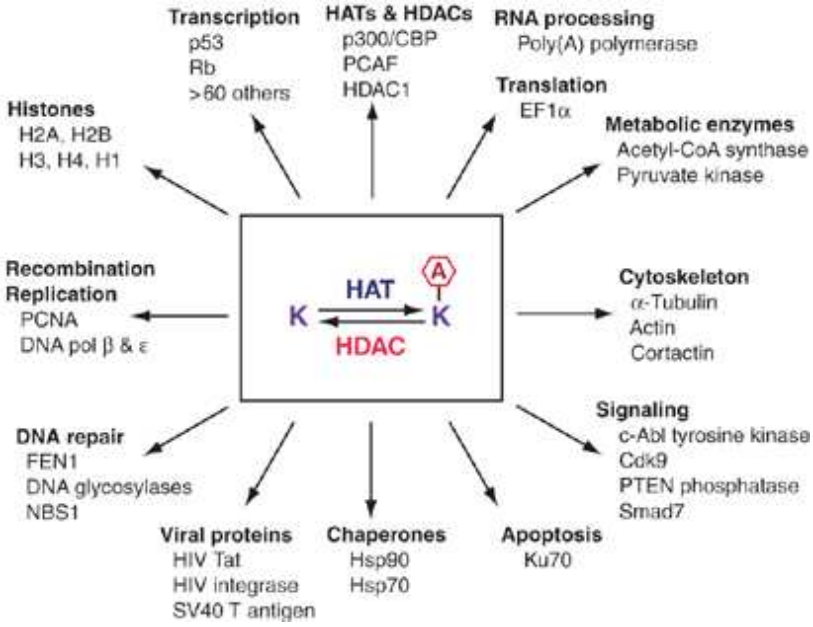


Figure 1-8. Schematic representation of the involvement of reversible lysine acetylation in numerous cellular processes. The letter A refers to an acetylation and for each process representative proteins are presented (Yang and Seto, 2007).

HATs are divided into 5 groups based on sequence homology, structural characteristics and functions. The first group, Gcn5-related N-acetyltransferase (GNAT) family, includes Gcn5, PCAF, HAT1, Elp3, Hpa2, Hpa3, ATF-2, and Nut1 proteins containing a BRD: they have been reported to acetylate K residues on histones H2B, H3, and H4 (Carrozza et al., 2003). The second groups, MYST family, encompasses MOZ, Ybf2 (Sas3), Sas2, and Esa1, MOF, MORF, and HBO1 HATs. These proteins acetylate K residues on histones H2A, H3, and H4 and play an important role in transcriptional activation, DNA repair, and gene silencing (Takechi and Nakayama, 1999, Avvakumov and Cote, 2007). The third group includes p300/CBP HATs: these enzymes have greater HAT domains compared to GNAT and

MYST families (Marmorstein, 2001), a BRD and three other cysteine/histidine-rich domains modulating interactions with other proteins. P300/CBP display no sequence homology with other HATs and are involved in the transcriptional activation. These HATs have been reported to equally acetylate all four core histones (Roth et al., 2001, Wang, 2009). The fourth group is the SRC family or nuclear receptor co-activators; it includes SRC-1, ACTR (RAC3, AIB1 and TRAM-1), TIF-2 and SRC-3. These enzymes include general TF HATs and have been reported to strongly bind histone H4 and be responsible for its modifications. The firth group comprises all other proteins possessing HAT activity and includes TAFII250, TFIIIC (p220, p110 and p90), Rtt109 and CLOCK (Carrozza et al., 2003). Several studies have demonstrated that transcriptional co-activator proteins might also function as molecular switches regulating gene transcription and possess intrinsic HAT activity (Spiegelman and Heinrich, 2004, Roth et al., 2001).

The majority of HATs exist as elements of multicomponent complexes recognising specific modifications of histone tails, directing transcriptional complexes to the gene promoter and modifying target gene transcription (Carrozza et al., 2003, Kouzarides, 2007, Adcock et al., 2006). Acetylation of four core histones differs: whilst H2A and H2B are commonly acetylated at K5 and K12 and K15 respectively, H3 is acetylated mostly at K9, K14, K18, K23, and K56. Histone H4 is characterised by modifications at K5, K8, K12, and K16 (Kouzarides, 2007).

Lysine acetylation is a reversible process regulated by epigenetic "erasers" named histone deacetylases (HDACs). HDACs remove acetyl groups from histone tails and facilitate chromatin condensation and gene silencing (Carrozza et al., 2003). The

superfamily of HDACs is divided into 4 groups based on DNA sequence homology, subcellular localisation, substrates and binding patters as well as regulatory mechanisms. The first group encompasses HDAC1, HDAC2, HDAC3, and HDAC8 which are located in the nucleus, except membrane-associated HDAC3 that can be found both in the nucleus and cytoplasm. The second group is clustered in IIA including HDAC4, HDAC5, HDAC7 and HDAC9, and IIB comprising HDAC6 and HDAC10. Whilst IIA group HDACs are able to shuttle between the nucleus and cytoplasm (de Ruijter et al., 2003, Longworth and Laimins, 2006), HDACs belonging to the IIB group are predominantly located in the cytoplasm. HDAC6 is known to be involved in important biological processes via forming complexes with other proteins (Valenzuela-Fernández et al., 2008). The third group consists of mammalian sirtuins (SIRT1-SIRT7) and Sir2 in the yeast S. cerevisiae, and the fourth family includes HDAC11 located both in the nucleus and cytoplasm (Yang and Seto, 2007).

HDACs are involved in a variety of signalling pathways and cellular processes such as signal transduction, cell cycle and the development of a number of human diseases including cancer and chronic myeloid leukaemia (KEGG, 2014). Studies performed in animal models have demonstrated that absence or deficiency of HDAC1 results in an alteration of the transcription of a specific subset of genes suggesting that HDAC1 might serve as a transcriptional activator (Zupkovitz et al., 2006). Both HATs and HDACs are responsible for the modification of DNA-binding proteins (histones and TFs), non-nuclear proteins (tubulin) and nuclear import factors (Sterner et al., 1979, L'Hernault and Rosenbaum, 1985, Bannister et al.,

2000, Kouzarides, 2000). P65 NF-κB is thought to be regulated via acetylation by PCAF and deacetylation by HDAC3 and HDAC6 (Hasselgren, 2007, Ospelt et al., 2011).

HDACs are regulated by differentially interacting with other non-histone proteins and co-repressor molecules that heighten the repressive effect and ensure greater specificity (de Ruijter et al., 2003). Their effect and functions are controlled by localisation, protein-protein interactions, and post-translational modifications (Adcock et al., 2006). Although some progress has been made in the identification of the acetylation effect on chromatin assembly and the role of HATs and HDACs in the acetylation process, the exact mechanisms remain unclear.

1.6.3.3 Bromodomains and their role in gene transcription

Recently, the bromodomains and extra-terminal (BET) family of proteins have gained increasing attention. These proteins are evolutionary well preserved protein-interaction modules and the principal readers of ε-N-acetyl lysine (K_{ac}) marks (Kouzarides, 2000, Chen et al., 1999, Belkina et al., 2013b). To date, around 61 human BRDs and 40 human BET proteins have been identified (Filippakopoulos et al., 2010). BRDs are present in numerous chromatin-associated proteins such as HATs (GCN5, PCAF) (Nagy and Tora, 2007b), ATP-dependent chromatin-remodelling complexes (BAZ1B) (Trotter and Archer, 2008), helicases (SMARCA) (Filippakopoulos et al., 2010, Sanchez and Zhou, 2009), methyltransferases (MLL, ASH1L) (Malik and Bhaumik, 2010), transcriptional co-activators (TRIM/TIF1, TAFs) (Jacobson et al.,

2000) and mediators (TAF1), nuclear-scaffolding proteins (PB1) and the BET protein family (Muller et al., 2011b).

The human BET family is a group of dual BRD-containing proteins comprising four members: BRD2, BRD3, BRD4 and the testis-specific isoform, BRD-T, proteins (Rhee et al., 1998, Wang et al., 2009b). BRD2, BRD3 and BRD4 are implicated in cell-cycle control (Dey et al., 2003, Mochizuki et al., 2008, Sinha et al., 2005), cell growth (Dey et al., 2000, Maruyama et al., 2002, Houzelstein et al., 2002), transcription elongation (Brès et al., 2008, Yang et al., 2005, Jang et al., 2005), embryogenesis and neurogenesis (Gyuris et al., 2009). Studies in animal models have reported that deletion of BRD2 or BRD4 in mice is fatal; BRD4+/– mice develop severe developmental defects (Houzelstein et al., 2002, Shang et al., 2009). BRD2 acts as a co-activator or co-repressor of transcription in a promoter- and tissue-specific manner (Gyuris et al., 2009). Simulatenous dysfunction of BRD3 and BRD4 is associated with the development of different types of cancer highlighting these proteins as potential targets for drug development (Filippakopoulos et al., 2012). Analysis of 30 BRD containing proteins has shown that these proteins recognise a combination of epigenetic modifications rather than a single acetylated site (Filippakopoulos et al., 2010). They further recruit chromatin remodelling enzymes to distinct epigenetic modifications forming an active platform for co-activator complexes to assure a high level of specificity (Muller et al., 2011b, Kouzarides, 2007).

Studies in mice have demonstrated BRD2, BRD3 and BRD4 involvement in the transcriptional control of CXCL6, TNF-α, MCP-1 and keratinocyte chemoattractant (KC) which is a mouse CXCL8 ortholog (Belkina et al., 2013b). Furthermore, recent studies have shown that IL-1β-induced CXCL8 expression in airway epithelial cells is mediated via increased association of p65 NF-κB and BRD4 with the CXCL8 promoter as well as increased acetylation of histone H3 at the NF-κB binding site (Khan et al., 2014). Although the ability of BRD containing proteins and BET proteins to regulate transcription of pro-inflammatory cytokines has been studied, the exact role is not fully understood.

1.6.3.4 Histone methylation

Histone methylation occurs via transfer of methyl groups from S-Adenosyl methionine onto lysine (K) or arginine (R) residues of histones H3 and/or H4 resulting in either gene activation or repression depending on the target amino acid, extent of methylation and presence/absence of epigenetic modifications in the proximity (Greer and Shi, 2012). Histone methylation is an important process for cell mitosis, gene expression and genomic stability, genetic imprinting, cell maturation and cell lineage development (Sawan and Herceg, 2010). To date, methylation of approximately 17 K and 7 R residues has been identified (Bannister and Kouzarides, 2005).

Histone methylation is catalysed by histone methyltransferases (HMTs) covalently methylating Ks and Rs within histone tails. These enzymes are clustered in two major groups: K-specific and R-specific. K methylation occurs in mono-, di- or tri-

methylation form, whereas R methylation is more likely to be in a mono- or di-form. An additional complexity of R methylation is achieved via symmetrical or asymmetrical positioning of the methylated residues (Bannister and Kouzarides, 2005). K-specific HMTs can be further divided into SET and non-SET-domain containing proteins based on the presence or absence of a Suppressor of variegation-Enhancer of zeste-Trithorax (SET)-domain catalysing the methylation of the K residues on core histones (Sawan and Herceg, 2010).

Methylation marks are recognised by two families of domains in HMTs: PhD finger domains and the Royal family which includes chromo, tudor and malignant brain tumour (MBT) domains (Berger, 2007, Kouzarides, 2007). The domain type determines HMTs' affinity for histones, the ability to recognise the particular methylation marks and catalyse the reaction with binding co-factors (Mosammaparast and Shi, 2010). Thus, the PHD finger recognises trimethylated H3K4 mark, whilst the chromo domain of the Royal family has greater affinity for methylated H3K9 (H3K9me) (Jenuwein and Allis, 2001, Kouzarides, 2007). Methylated marks alone or in combination with other epigenetic modifications such as acetylation create a complex platform functioning as a recognition pattern for the chromatin remodelling either initiating active transcription or silencing it (Bhaumik et al., 2007, Arrowsmith et al., 2012).

Active regions of chromatin are associated with distinct histone methylation modifications. Methylation of histone H3 at the positions of K4, K36 and K79 of the 5' controlling region has been observed in transcriptionally active genes (Bannister and Kouzarides, 2005, Wang and Zhu, 2008). Similarly, H3K4me3 is a characteristic

mark of an actively transcribed gene (Pray-Grant et al., 2005). In contrast, methylation of K9 and K27 on histone H3 and K20 on histone H4 are marks of gene silencing and heterochromatin formation (Strahl and Allis, 2000, Bannister and Kouzarides, 2005). Di- or trimethylation of H3K9 and H3K27me3 lead to transcriptional repression via recruitment of repressive complexes to the target gene promoter (Kouzarides, 2007, Shilatifard, 2006, Berger, 2007).

Arginine methylation is mostly a positive transcriptional regulator catalysed via arginine methyltransferases (PRMTs). The most well-studied PRMT is co-activator-associated PRMT1 (CARM1), that methylates R17 and R26 on histone H3, and PRMT2 that has affinity for H4R3 (Davie and Dent, 2002, Bhaumik et al., 2007). Both of these proteins have been associated with active transcription: CARM1 can act in conjunction with p300 HAT to heighten gene transcription (Daujat et al., 2002). As yet, no R demethylases have been identified (Ehrenhofer-Murray, 2004).

Similarly to acetylation, histone methylation is a reversible process. The removal of methyl groups from histones is catalysed by histone demethylases (HDMs) that are implicated in the regulation of cell development and gene transcription (Cloos et al., 2008). These enzymes are categorised in two groups: the Jumonji (JmjC) family of 2-oxoglutarate dependent HDMs or flavin adenine dinucleotide (FAD)-dependent amine oxidases LSD1 (lysine-specific HDM) and a Fe(II) and α-ketoglutarate-dependent dioxygenase LSD2 (Klose and Zhang, 2007). It is thought that HDMs have substrate specificity for H3K4, H3K9, H3K27 and H3K36: members of LSD1 group specifically target mono- and di-methylated H3K4 and H3K9 (Berger, 2007, Bhaumik et al., 2007). Recognition of epigenetic signatures by HDMs results in the formation

of large complexes with other co-activator/repressor proteins such as HDACs, HMTs and nuclear receptors enhancing each other's activities and altering gene transcription (Kouzarides, 2007).

1.6.3.5 DNA methylation

DNA methylation is a stable epigenetic modification involved in the continuous conservation of some genomic regions (Cheung et al., 2000), stabilisation and maintenance of genomic integrity (Jiang et al., 2004) and expression of tissue specific genes (Ospelt et al., 2011). Dysfunction of this epigenetic modification is present in nearly all types of cancer (Jaenisch and Bird, 2003): studies have demonstrated that loss of methylation in oncogenes and/or abnormal hypermethylation in tumor suppressor genes results in cancer growth and metastasis (Ospelt et al., 2011).

DNA methylation is the covalent transfer of a methyl group from S-adenosyl-L-methionine to cytosines in cytosine-guanine (CG) dinucleotides (CpG sites) catalised by methyltransferases (Ospelt et al., 2011, Cheung and Lau, 2005). In the mammalian genome, around 40% of genes have CpG sites located upstream of their transcriptional start site: 70-80% of these sites are methylated (Bird, 2002, Jiang et al., 2004). Extensive methylation at the regulatory region of the gene results in target gene silencing and alters the DNA-binding affinity of TFs and other regulatory co-factors recruited to the gene promoter (Deng et al., 2001, Ospelt et al., 2011).

DNA methylation is catalysed by DNA methyltransferases (DNMTs). This family of enzymes is divided in four main groups. DNMT1 is responsible for the maintenance of methylation patterns during DNA replication; deletion of DNMT1 in animal models results in death of mice embryos. DNMT2 (TRDMT1) is homologous to other DNMTs, but has limited activity methylating cytosine-38 in transfer RNA rather than DNA. DNMT3a and DNMT3b are involved in de novo DNA methylation and generation of new methylation patterns early in development (Goll et al., 2006). The DNA sequence itself, conformation or secondary structure including specific RNAs targeting homologous regions and specific chromatin proteins, histone modifications and higher-order chromatin structures alone or in combination can serve as potential triggers for the DNA methylation (Freitag and Selker, 2005).

DNA methylation is associated with chromatin condensation and gene silencing (Klose and Bird, 2006, Jones and Liang, 2009). Increased DNA methylation leads to the direct suppression of DNA binding affinity and inability of TFs and regulatory co-activators to bind the gene promoter. Subsequent recruitment of methyl CpG binding proteins (MBPs) to methylated CpG islands in association with HDACs and HMTs form repressor complexes leading to chromatin condensation (Salozhin et al., 2005, Fuks, 2005) and transcriptional silencing (Jaenisch and Bird, 2003).

1.6.3.6 Other histone modifications

Histone phosphorylation is an important mechanism in the cell cycle (Roth and Allis, 1992) and has been implicated in DNA replication control and gene transcription regulation (Happel and Doenecke, 2009). Phosphorylation of histone H1 is involved

in chromatin condensation and decondensation via changes in the affinity for chromatin (Bradbury et al., 1974, Zheng et al., 2010). Phosphorylation of histone H3 at serine 10 regulates transcription of NF-κB-mediated and "immediate-early" genes such as Fos and Jun (Prigent and Dimitrov, 2003, Strahl and Allis, 2000). Further research has acknowledged that transcription of these genes may be regulated cooperatively in conjunction with other epigenetic modifications such as acetylation (Strahl and Allis, 2000).

Another epigenetic modification, ubiquitination, is sequentially catalysed by three E1-activating, E2-conjugating and E3-ligating enzymes resulting in either a repressive or activating effect subject to the site modified (Bhaumik et al., 2007). Monoubiquitination of histone H2A at K119 is a repressive mark, whilst ubiquitination of H2B at K120 is associated with both transcription activation and gene silencing (Berger, 2007, Kouzarides, 2007, Weake and Workman, 2008, Bhaumik et al., 2007). Furthermore, ubiquitination of histones H2A and H2B is interlinked and can have contrary effects: H3K4 methylation as a consequence of H2B ubiquitination is inhibited by H2A ubiquitination (Weake and Workman, 2008).

Sumoylation has been implicated in different biological processes including protein stability, nuclear-cytosolic transport and transcriptional regulation (Gill, 2005, Peters and Schübeler, 2005). Addition of a nearly 100 amino acid long Small Ubiquitin-related MOdifier protein (SUMO) (Shiio and Eisenman, 2003) results in the recruitment of chromatin-modifying enzymes such as HDACs and HP1 protein (Klose and Zhang, 2007). Studies in yeast have described sumoylation of all four core histones: sumoylation of K6/7 and K16/17 on histone H2B, K126 on histone

H2A and all four K tails in the N-terminus of histone H4 correlates with transcriptional repression (Peters and Schübeler, 2005).

1.6.4 Epigenetic regulation of the CXCL8 gene

To date, the exact mechanisms of epigenetic regulation of individual genes including CXCL8 have not been fully understood. One study demonstrated that IL-1ß-induced NF-κB p65 binding to the CXCL8 promoter results in the recruitment of co-activators and chromatin remodelling enzymes such as p300 and CBP resulting in histone H4 acetylation around the NF-κB site (Bartling, 2009). These changes further recruit "readers" of acetylated lysine residues such as BRD containing proteins leading to an increased binding of C/EBP and AP-1 TFs and chromatin remodelling, open chromatin structure and enhanced CXCL8 transcription. Another study has reported similar observations in human airway smooth muscle cells derived from asthmatic patients confirming increased binding of p300, CBP and PCAF to the CXCL8 promoter as a result of increased histone H3 acetylation following TNF-α stimulation (John et al., 2010). A study conducted in bronchial epithelial cells investigating IL-1β-induced CXCL8 transcription has similarly detected pan-acetylation of histone H3 but not H4 associated with increased binding of BRD4 and NF-κB p65 (Khan et al., 2014).

While some progress has been made in the field of cytokine-mediated epigenetic regulation of CXCL8 transcription, the distinct mechanisms of CXCL8 expression in CF have not been fully explored. Thus, it would be interesting to determine

epigenetic modifications affecting cytokine-induced CXCL8 transcription in CF human airway epithelial cells.

1.6.5 Disease epigenetics

In the last few decades, research has highlighted the fundamental role of epigenetic regulation, particularly DNA methylation and histone modifications, in the development and/or progression of cancer, inflammation and metabolic disorders (Sigalotti et al., 2007, Bhaumik et al., 2007, Strahl and Allis, 2000, Klose and Bird, 2006).

Epigenetic modifications at early stages of tumorigenesis alter different signalling pathways involved in cell cycle control, apoptosis, metastasis, angiogenesis and immune recognition (Sigalotti et al., 2007). DNA hypermethylation associated with transcriptional silencing of some tumour repressor genes as a result of "hot spot" mutations in the CpG islands of their promoters has been reported in some aggressive types of cancer (Berger, 2007). Furthermore, studies have acknowledged simultaneous presence of global DNA methylation and individual gene hypermethylation suggesting co-existence of different and sometimes incompatible epigenetic alterations (Weber et al., 2005).

Histone modifications have also been reported to be present at the promoters of individual genes in human tumours. Breast and liver cancer have been characterised by genome-wide loss of H4K20 methylation (Fraga et al., 2005) as well as loss of H3K4 trimethylation (Bhaumik et al., 2007). Increased H3K9

methylation alongside altered H3K27 methylation resulting in gene silencing have been identified as epigenetic signatures of certain forms of cancer (Nguyen et al., 2002, Valk-Lingbeek et al., 2004). Overexpression of chromatin-remodelling enzymes such as HMTs has been reported in some cancers: enhanced transcription of EZH2 which is a H3K27 HMT is associated with silencing of onco suppressor genes in breast and prostate cancer (Valk-Lingbeek et al., 2004). Furthermore, increased levels of G9a, the H3K9 HMT, have been described in liver cancer as a hallmark of malignant phenotype (Kondo et al., 2008, Kondo et al., 2007).

Studies conducted in cancer have reported altered acetylation patterns as a result of a misbalance in the HAT/HDAC equilibrium leading to target gene repression (Halkidou et al., 2004, Song et al., 2005). Lack of H4K16 acetylation is one of the characteristic features of cancer transformation and progression (Fraga et al., 2005). Modifications of HATs and HAT-related genes such as CBP and p300 have been reported to be significantly altered in leukemia (Yang, 2004).

1.6.6 Epigenetics of CF

CF is characterised by profound inflammation in the lungs along with increased transcription of pro-inflammatory cytokines including CXCL8 (Bhattacharyya et al., 2010). Although deficiency of CFTR gene and/or continuous presence of *P. aeruginosa* in the lungs are believed to be responsible for the lung inflammation and altered cytokine profile in CF, little is known about the molecular mechanisms underlying enhanced CXCL8 expression. Altered epigenetic mechanisms regulating

vital cellular processes have been recognised as potential reasons accountable for the altered CXCL8 transcription in CF.

Histone acetylation has been implicated in the altered transcriptional regulation of CXCL8 in CF: CFTR-deficient cells are characterised by greater levels of NF-κB and increased acetylation of histone H4 at the CXCL8 promoter. Furthermore, whilst no changes in the activity and/or expression of chromatin remodelling enzymes such as p300 and/or CBP and HDAC1 have been identified, an intrinsic alteration in HAT/HDAC balance with a particular decrease in HDAC2 protein transcription and activity have been described in CF human airway epithelial cells (Bartling, 2009).

Post-translational modifications are restorable by new "epigenetic drugs" such as inhibitors of HATs, DNMTs and HDACs and inhibitors of BRD proteins. Though they are still in the developmental stage, data, derived from studies using *in vivo* myeloma models, indicate successful application of these drugs and their antiproliferative effect (Delmore et al., 2011). BRD inhibitors have also been applied in the treatment of inflammatory conditions: JQ1 decreased the inflammatory response in animal models via downregulation of the transcription of NF-κB-driven genes. This inhibitor can ablate LPS-induced IL-6 and TNF-α transcription *in vitro* as well as to blunt the "cytokine storm" in endotoxemic mice (Belkina et al., 2013a). Another drug, GSK1210151A (I-BET 151), has successfully been implicated in the treatment of haematological and solid malignancies in both *in vitro* and *in vivo* models (Dawson et al., 2011). Collectively, these studies suggest that BET protein inhibitors can be considered as new targets for the development of new epigenetic drugs for the large number of diseases caused by aberrant histone acetylation.

1.7 Summary

CF is a fatal disease caused by a mutation of CFTR gene and characterised by intensive inflammation in the airways with increased levels of CXCL8 and other pro-inflammatory cytokines. A vicious cycle of continuous inflammation and permanent bacterial colonisation results in irreversible fibrosis, loss of lung function and respiratory failure.

CXCL8 is a powerful pro-inflammatory chemokine, which plays a key role in the pathogenesis of the inflammation in CF. Airway epithelial cells play a fundamental role in the initiation and maintenance of the inflammatory process. Although increased CXCL8 secretion by airway epithelial cells has been described in CF, the exact molecular mechanisms are not completely understood. Work from our group in other airway and parenchymal lung diseases such as idiopathic pulmonary fibrosis (IPF) and asthma has suggested that structural lung cells may be reprogrammed epigenetically proposing that epigenetic processes might have a potential role in the regulation of CXCL8 release from airway epithelial cells in CF. The purpose of the studies in this thesis was to study these processes in greater depth than previously to gain a greater understanding of the molecular mechanisms involved, and to identify the potential targets for therapeutic intervention.

2 HYPOTHESIS AND AIMS

CXCL8 plays an important role in the pathogenesis of CF. Previous studies suggest that CXCL8 release is increased in airway epithelial cells derived from CF patients or engineered CF cell lines *in vitro* suggesting a fundamental relation between CFTR function and CXCL8, independent of the CF airway microenvironment. Previous studies have suggested that this may be partially linked to NF-κB activation, but the exact mechanisms are unclear.

Here we tested the hypothesis, that altered epigenetic regulation of transcription is the key factor increasing CXCL8 production basally and in response to pro-inflammatory cytokines in CF airway epithelial cells.

The specific aims were:

1. To confirm that IL-1β can induce CXCL8 protein secretion from normal and CF airway epithelial cells.

2. To verify that CXCL8 production is increased in CF cells basally and after IL-1β stimulation.

3. To compare the activation and binding pattern of transcription factors to the CXCL8 promoter in normal and CF airway epithelial cells.

4. To analyse the transcriptional mechanisms responsible for the enhanced CXCL8 expression in CF cells.

5. To explore the epigenetic mechanisms regulating the CXCL8 promoter both basally and after stimulation with IL-1ß in normal cells and to determine if they are dysregulated in CF cells.

3 MATERIALS AND METHODS

This chapter provides an outline of general methods used in this thesis with a comprehensive description of cells employed. Full details of all primer sequences, reagents, and buffers are listed in 9.3 of Appendix.

3.1 Cell lines

Two sets of immortalised human bronchial epithelial cells were used for the experiments:

- IB3-1 cells derived from a CF patient with a compound heterozygous mutation (ΔF_{508}/W1282X) in the CFTR gene, and S9 cells (IB3-1 cells, stably transfected with complimentary DNA (cDNA) encoding CFTR protein by the adeno-associated virus type 2 inverted terminal repeat) (Zeitlin et al., 1991, Flotte et al., 1993). IB3-1 and S9 cells were a generous gift from Prof. Pamela L. Zeitlin (Johns Hopkins University, Baltimore, MD). Both cell lines were cultured to passage 15 and 41 respectively in LHC-8 without gentamicin (1X) medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 100U/mL penicillin, 100µg/mL streptomycin, and 2.5µg/mL amphotericin B.
- CFBE41o- cells derived from a CF patient with homozygous mutation Δ508/Δ508, and wild type human airway epithelial cells (parental CFBE41o- cells, stably transfected with cDNA encoding wild type CFTR protein by lentiviral-based transduction). These cell lines were a generous gift from Prof. Bruce Stanton (Dartmouth University, NH). CFBE41o- and wild type cells were cultured to passage 22 and 21 respectively in Minimum Essential Medium Eagle (MEM) medium supplemented with 10% FCS, 100U/mL penicillin, 100µg/mL streptomycin, 2.5µg/mL amphotericin B, and 10µg/mL of puromycin.

For future reference IB3-1 and S9 cells will be referred as IB3-1 normal and IB3-1 CF cells, and CFBE41o- and wild type human airway epithelial cells as CFBE41o- normal and CFBE41o- CF cells.

3.1.1 Cell culture

All cell lines were grown in a humidified incubator at 37°C under 5% CO_2 and 20% O_2. Once the required 80-90% confluence was achieved, cells were growth arrested in serum free medium for 24 hours prior to experiments with further replacement with serum free medium with or without stimuli as per experimental design.

3.1.2 Cell counting

Cells were cultured in T150 or T225cm^2 flasks until 90% confluent, washed once with PBS and trypsinised. After centrifugation at 1000rpm for 5 minutes, the pellet was resuspended in 10mL of medium containing serum, and the number of cells in the cell suspension was determined using a Neubauer haemocytometer and a light microscope: 10µl of the solution was pipetted under the cover slip on the haemocytometer and the cells were observed under the microscope using the x40 magnification lens. The number of cells was counted in each of the 4 large squares in the corners of the grid. Cells lying on the top and left margins of the grid were counted and clusters of cells were counted as one. The total number of cells counted was divided by 4, and finally multiplied by x10^4 to obtain the number of cells per 1mL. To determine the total number of cells in the suspension, the last number was multiplied by the total volume.

3.1.3 Cell freezing

To create frozen stocks, trypsinised and pelleted cells were resuspended in a solution containing 90% FCS and 10% dimethyl sulfoxide (DMSO) to achieve $3x10^6$ cell/mL density. The suspension was aliquoted in 1mL cryovials and placed in a -80°C freezer overnight in a Nunc Cryo 1°C Freezing Container (Mr. Frosty) containing 100% isopropyl alcohol. The following day the cells were transferred to liquid nitrogen and stored until required.

3.2 Human CXCL8 enzyme-linked immunosorbent assay (ELISA)

The human CXCL8/IL-8 DuoSet ELISA kit (R&D Systems, UK) was used to determine the human CXCL8 protein concentration in culture medium. Cells were grown in 24 well plates until confluent, serum deprived for 24 hours prior to experiments, and treated with a stimulant for the required time. Supernatants were collected and stored at -20°C until assayed according to the manufacturer's instructions.

Briefly, 96 well plates were pre-coated with 50 or 100µl (half and full surface area wells respectively) of a capture antibody per well (720µl/mL of mouse anti-human CXCL8 when reconstituted with 1mL of sterile phosphate buffered saline (PBS)), and incubated overnight at room temperature. The following day the plates were washed at least three times with a washing buffer (0.05% Tween-20 in 1L of PBS), and blocked with 150 or 300µl of a block buffer per well (1% bovine serum albumin (BSA) in PBS) for at least an hour. Subsequently, the plates were washed three times and incubated with 50 or 100µl of non-diluted and 1:5 or 1:10 diluted supernatants (in triplicate) and standards (in duplicate) for at least 2 hours at room

temperature. 100ng/mL of recombinant human CXCL8 when reconstituted with 0.5mL distilled water was used as a standard. An eight point standard curve (0, 31.25, 62.5, 125, 250, 500, 1000, 2000pg/mL) was prepared by serial two fold dilutions in reagent diluent (0.1g BSA and 50µl 0.05% Tween-20 in 100mL tris buffered saline).

Following the incubation, the plates were washed at least three times with a wash buffer, and incubated for another two hours with 50 or 100µl of detection antibody per well (3.6µg/mL of biotinylated goat anti-human CXCL8 when reconstituted with 1mL of reagent diluent). Then, the plates were washed again, and 50 or 100µl of streptavidin, conjugated to horseradish-peroxidase (Streptavidin-HRP) diluted in reagent diluent, were added per well with further incubation of 30 minutes away from the direct light. Plates were washed again and incubated with 50 or 100µl of a substrate solution (a 1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (Tetramethylbenzidine)) per well for 20 minutes away from the direct light.

The reaction was terminated by adding 25 or 50µl of stop solution (2N sulphuric acid ($H_2SO_{4)}$)), and the colour intensity was measured using a plate reader (Omega Fluostar, BMG Labtech, UK) at an optical density of 450nm and reference wavelength of 570nm. CXCL8 concentrations in samples were calculated by creating a four parameter logarithmic standard curve fit generated by Omega V2.10R4 software; a multiplication by a relevant dilution factor was applied to calculate the concentration of diluted samples. Readings were expressed as pg/mL and normalised against total cellular protein amount; the final data were presented as pg/mL/µg.

3.3 Bicinchoninic acid (BCA) protein assay

Colorimetric bicinchoninic acid (BCA) protein assay was used to determine protein concentration in the experimental samples using kits supplied by Pierce, UK according to the manufacturer's instructions.

Having removed the supernatants to be assayed by ELISA, cells were washed with PBS once, lysed with a lysis buffer (100µl of TritonTM X-100 diluted in 1mL of double-distilled water) and either stored at -80°C until required or processed immediately. 25µl of samples diluted 1:5 when required (in triplicate) alongside standards (in duplicate) were added to 96 well plates. Known concentrations of BSA were used to create a nine point standard curve (0, 25, 125, 250, 500, 750, 1000, 1500, 2000µg/mL) by means of serial dilutions in the lysis buffer. Reagent B (4% cupric sulphate) and Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 sodium hydroxide) were mixed in a 50:1 ratio to create the working reagent. 200µl of the working reagent was added per well followed by incubation at 37°C for 30 minutes. Samples were left to cool to room temperature, and the absorbance measured at a 590nm wavelength using a plate reader. The best-fit standard curve was generated by Omega V2.10R4 software; a multiplication by the relevant dilution factor was applied to calculate the protein concentrations of diluted samples.

3.4 Real-time polymerase chain reaction (qPCR)

Real-time polymerase chain reaction (qPCR) is a technique used to assess qualitative gene expression within a cell through measuring messenger RNA (mRNA) levels by creating cDNA transcripts from RNA. It is a multistage process

including total RNA extraction from the cells followed by reverse transcription into cDNA and qPCR analysis using gene specific primers.

3.4.1 Total RNA isolation

Total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Cells were grown in 6 well plates until confluent, growth arrested for 24 hours prior to experiments and treated with a stimulant as described in the experimental design.

After removing media, cells were lysed in 350µl of lysis buffer RA1 supplemented with 10µl/mL ß-mercaptoethanol. Cell lysates were loaded onto a NucleoSpin® Filter column containing a silica membrane in 350µl of 70% ethanol and centrifuged at 11000xg for 30 seconds. Subsequently, having discarded the eluate, 350µl of membrane desalting buffer (MDB) was added to each tube followed by centrifugation at 11000xg for 1 minute. Next, 95µl of recombinant deoxyribonuclease (rDNase) reaction mixture (10µl of reconstituted rDNase in 90µl of a reaction buffer for rDNase) was directly applied onto the center of the silica membrane of each column, followed by 15 minutes incubation at room temperature. Then, silica membranes were washed with 200µl of wash buffer RAW2 and centrifuged at 11000xg for 30 seconds. The first wash was followed by a second one with 600µl of buffer RA3 and subsequent centrifugation at 11000xg for 30 seconds. The supernatants were discarded, samples washed a final time with 250µl of buffer RA3, and centrifuged for 2 minutes at 11000xg. The columns were then placed into a nuclease-free collection tube, and centrifuged at 11000xg for 2 minutes to ensure all ethanol residue was removed from the column. Finally, RNA

was eluted in 40µl of nuclease-free water (supplied with a kit) by centrifugation at 11000xg for 1 minute with further storage at -80°C until required.

3.4.2 Reverse transcription

Reverse transcription (RT) was used to synthesise cDNA from an RNA template using a reverse transcriptase enzyme.

For each reaction, 1µl of extracted RNA was heated for 5 minutes at 72°C in 13.7µl of a master mix 1 containing 200µg/mL oligodeoxythymidylic acid (OligoDT) primer, 10mM of deoxynucleoside triphosphates (dNTPs) mix, and 5.7µl of nuclease-free water in a thermo cycler (Bio-Rad, PTC 100, UK). After 5 minutes incubation on ice, RNA was reverse transcribed in 25µl of master mix 2 containing 132 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 26.4 units of recombinant RNasin ribonuclease (RNAase) inhibitor, 5µl of 1xM-MLV RT buffer, and nuclease-free water at 42°C for 90 minutes in a thermal cycler. Resulting cDNA samples were stored at -20°C until required or used for the quantitative qPCR immediately.

3.4.3 Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) was used to amplify a specific region of CXCL8 DNA using a fluorescence detecting thermal cycler.

1µl of reverse transcribed cDNA was subjected to qPCR in a master mix containing 10µl of SYBR® Premix Ex Taq™ II (Tli RNase H Plus), and 0.4µl (40nM) of human CXCL8 forward and reverse primers; the final volume was adjusted to 20µl with nuclease-free water. Human CXCL8 expression was determined using an

Mx3000P® QPCR System thermal cycler (Stratagene, California, USA) (primer sequences and cycling conditions are described in 9.5 of the Appendix). All samples were assayed in triplicate and successful amplification of a single product was confirmed by presence of a single dissociation peak in melting curve analysis. CXCL8 expression was normalised to β_2-microglobulin expression; data were presented as relative expression compared to control non-treated samples.

3.5 Transfections

CXCL8 promoter activity and identification of TFs that influence CXCL8 promoter activity were determined by transient transfection of luciferase reporter constructs. Plasmids were grown on ampicillin selective LB agar plates at 37°C in a humidified atmosphere overnight. The following day, the largest single colony was selected and grown in ampicillin selective LB broth for further 24 hours at 37°C in an Excella™ E24/E24R temperature-controlled benchtop shaker (New Brunswick Scientific, UK). Plasmid DNA was isolated and purified using a plasmid purification midi kit (Qiagen, UK) according to the manufacturer's instructions.

A wild type CXCL8 promoter-driven luciferase construct (-162/+44), and constructs containing mutations in the binding sites for AP-1, C/EBPβ or NF-κB in the CXCL8 promoter region were a kind gift of Dr. A. R. Braiser (Department of Medicine, Sealy Center for Molecular Science, Galveston, TX). Cells were seeded in 24 well plates at 50000 cells per well density, grown to 60% of confluence and serum deprived for 16 hours prior to experiments. Master mixes containing 0.8μg of wild type or mutant CXCL8 promoter constructs DNA, 0.8μg pRL-SV40 plasmid containing Renilla

luciferase gene as an internal control, and Lipofectamine™ 2000 transfection reagent at a 1:2 DNA:LF2000 ratio were made in antibiotic and serum free medium and incubated at room temperature for 15 minutes to form complexes. After aspirating growth media, 100µl of media containing DNA: transfection reagent complexes was added to specific wells and incubated for 3 hours. Then, cells were stimulated with 1ng/mL IL-1β for 3 hours or left unstimulated without changing the media at 37°C in a humidified atmosphere. After stimulation, cells were washed in PBS, lysed in 100µl of 1x passive lysis buffer (supplied with the kit), and stored at -80°C until required. Firefly and Renilla luciferase activities were measured using a Dual-Luciferase reporter™ assay system kit (Promega, UK) first by adding 50µl of luciferase assay reagent II (LAR II) to the samples. This step was followed by Renilla luciferase activity quantification through addition of 50µl of Stop&Glo® reagent to the same sample on a plate reader (Omega Fluostar, BMG Labtech, UK). Data were normalised by dividing Firefly readings by Renilla and expressed as a ratio of Firefly: Renilla (fold change over control).

3.6 Cell viability and proliferation assay

The toxicity of drug compounds used during the study was determined using a colorimetric assay identifying the reduction of a yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reagent.

Cells were grown as per the experimental design; at the end of the experiment, media was removed, and 250µl of 1mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, diluted in serum free medium was added per well.

Samples were incubated at 37°C for 30 minutes, the medium was removed and plates left to air dry overnight. The following day, 250µl of DMSO was added to each well to solubilise purple-colored tetrazolium crystals, and 100µl of each sample was loaded in 96 well plates in duplicate.

The color intensity in the experimental samples was measured using a plate reader (Omega Fluostar, BMG Labtech, UK) at an optical density of 550nm. The color change was directly proportional to the levels of viable cells; data were expressed as percent viability relative to control.

3.7 Chromatin immunoprecipitation (ChIP)

ChIP was used to identify histone modifications and transcription factor binding to the CXCL8 promoter. The assay can be divided into 4 major phases: cell fixation, sonication, immunoprecipitation and qPCR analysis. The assay was performed using the ChIP-IT® Express kit (Active Motif, UK) according to the manufacturer's instructions.

3.7.1 Cell fixation

Cells were grown in T225cm^2 flasks, serum deprived for 24 hours when confluent, and either incubated with 1ng/mL IL-1β for 0, 0.5, 1 and 2 hours or left in fresh serum free medium without any treatment to be used as control samples. Cells were fixed in 20mL of fixative solution (1% formaldehyde solution in serum free media to preserve DNA/protein interactions) for 10 minutes on a shaking platform at room temperature. Next, the cells were washed with 10mL of ice-cold PBS and incubated in 10mL 1x glycine stop solution for a further 5 minutes on a shaking

platform at room temperature. This step was followed by another wash with 10mL of ice-cold PBS. Then, cells were scraped in 2mL of PBS containing 5µl/mL 100mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged for 10 minutes at 720rcf at 4°C. The supernatants were discarded and 1µl of 100mM PMSF and proteinase inhibitor cocktail (PIC) added to the cell pellet. The pellets were either processed the same day or stored at -80°C until required.

3.7.2 Sonication

On the day of sonication, cells were lysed in 2mL (IB3-1 CF) or 3mL (IB3 normal) 1x lysis buffer (supplied with the kit) supplemented with 10µl and 15µl of PMSF and PIC respectively on ice for 30 minutes. The cells were pelleted by centrifugation at 2400rcf for 10 minutes at 4°C, the supernatants discarded, and the pellet resuspended in 1mL shearing buffer (supplied with the kit) supplemented by 5µl of PMSF and PIC each. The total volume was divided into 333µl aliquots for sonication. Sonication was performed using an EpiShear™ Multi-Sample sonicator (Active Motif, UK). Cycles of 59 seconds of sonication were followed by 59 seconds of rest for 15 minutes at 35% of power; the total duration was 15 minutes. Aliquots of the same time points were combined together and centrifuged at 13000rpm at 4°C for 10 minutes. The samples were either processed the same day or stored at -80°C until required. 25µg of chromatin was isolated to be processed as control Input DNA later on.

3.7.3 Immunoprecipitation

For ChIP reactions 25µg of chromatin was incubated in siliconised tubes overnight on a roller at 4°C in 200µl solution containing 25µl of protein G magnetic beads,

20µl of ChIP buffer 1 (supplied with the kit), 2µl of PIC, 3-5µg of antibody of interest or IgG antibody or normal rabbit/goat serum used as a negative control (as determined by titration experiments for each antibody), and nuclease-free water. The next day, the magnetic beads were washed once with 800µl of ChIP buffer 1 and twice with ChIP buffer 2. The immunoprecipitated complexes were eluted in 50µl of elution buffer by 15 minutes incubation on a roller at room temperature. Then, crosslinks were reversed by incubation in 50µl reverse cross-linking buffer at 95°C for 15 minutes. Immunoprecipitated samples and Input DNA were further incubated with 2µl of proteinase K at 37°C for 1 hour to remove remaining protein. Input DNA was processed in 100µl solution containing 25µg of chromatin, 5mM NaCl and ChIP buffer 2. To terminate the reactions, 2µl of proteinase K stop solution was added.

Input DNA underwent further purification by incubation at 37°C for 30 minutes with 1µl RNase A. Subsequently, 200µl of nuclease-free water and 300µl of phenol/chloroform were added to Input DNA samples followed by vortexing and centrifugation at 13000rpm for 5 minutes. Afterwards, the aqueous part located on the top was transferred to a new eppendorf tube, and 750µl of 100% ethanol, 35µl of 3M sodium acetate (pH=5.2) and 1µl of glycogen (20mg/mL) were added to the samples. The samples were vortexed and left at –70°C overnight. The following day, the samples were centrifuged at 13000rpm for 10 minutes, and supernatants discarded. The samples were further washed with 500µl of 70% ethanol and pelleted at 13000rpm for 10 minutes. The pellet was left to air dry, and resuspended in 500µl of nuclease-free water.

3.7.4 QPCR

4µl of samples including Input DNA were subject to qPCR in 20µl of a master mix containing 12.5µl of SYBR® Premix Ex Taq™ II (Tli RNase H Plus), 50nM of human CXCL8 promoter forward and reverse primers and nuclease-free water. Human CXCL8 promoter levels were determined using a thermal cycler, the details of primer sequences and cycling conditions (John et al., 2009) are provided in the 9.5 of Appendix.

Successful amplification of a single product was confirmed by the presence of a single dissociation peak in melting curve analysis. ChIP assay was considered as successful when CXCL8 promoter DNA levels in the experimental samples incubated with antibodies of interest were lower compared to samples incubated with IgG antibody used as a negative control. CXCL8 promoter DNA levels were normalised to the Input DNA; data were presented as relative association with the CXCL8 promoter compared to control non-treated samples.

3.8 Co-Immunoprecipitation (Co-IP)

Co-Immunoprecipitation (Co-IP) is a method designed to study protein/protein interactions within a cell by precipitation of a target protein and identification of any bound interacting proteins using specific antibodies.

3.8.1 Isolation of nuclear and cytoplasmic proteins

Isolation of nuclear proteins for Co-IP was performed using Nuclear Extract kit (Active Motif, UK) according to the manufacturer's instructions.

Briefly, cells were grown in T225cm^2 flasks until confluent, growth arrested 24 hours prior an experiment and incubated in media with or without 1ng/mL IL-1β as per the experimental design. Following stimulation, cells were washed with 5mL ice-cold PBS supplemented with phosphatase and deacetylase inhibitors, scraped in 3mL of the same solution and transferred to pre-chilled 15mL conical tubes. Cells were centrifuged at 1500rpm for 5 minutes at 4°C, supernatants discarded, and pellets resuspended in 500µl complete hypotonic buffer supplemented with phosphatase and deacetylases inhibitors, PIC and PMSF. This was followed by 15 minutes incubation on ice and addition of 25µl detergent. Then, cell suspensions were centrifuged at 14000xg for 30 seconds at 4°C, and supernatants containing cytoplasmic fractions discarded.

The remaining nuclear pellets were resuspended in 100µl complete digestion buffer supplemented with phosphatase and deacetylases inhibitors, PIC, and PMSF. This step was followed by addition of 0.5µl enzymatic shearing cocktail and vortexed gently for 2 seconds. Suspensions were then incubated in a water bath for 10 minutes at 37°C and vortexed every 2-3 minutes during the incubation. To stop the reactions, 2µl of 0.5M ethylenediaminetetraacetic acid (EDTA) was added and samples were gently vortexed at the lowest setting for 2 seconds. This was followed by 5 minutes incubation on ice and centrifugation at 14000xg for 10 minutes at 4°C. Resulting supernatants were transferred to a fresh microcentrifuge tube and stored at -80°C until required or processed immediately.

3.8.2 Co-immunoprecipitation (Co-IP)

250µg of nuclear extract was incubated with either 5µg of antibody of interest or IgG used as a negative control and complete Co-IP/Wash buffer in a total volume of 500µl for 4 hours at 4°C on an end-to-end rotator. Subsequently, antibody/extract complexes were centrifuged at 4000rpm for 30 seconds at 4°C and further incubated with 25µl of Protein G magnetic beads for 1 hour at 4°C on an end-to-end rotator. Following 30 seconds centrifugation at 4000rpm at 4°C, the supernatants were discarded and pellets washed in 500µl complete Co-IP/Wash buffer four times. Then, each bead pellet was resuspended in 20µl of 2x Reducing buffer, vortexed and stored at -20°C until required or run in western blotting immediately. BCA assay was performed to quantify total protein in the cell lysates before proceeding further.

3.8.3 Western blotting

To identify specific proteins within samples, western blotting, a semi-quantitative technique using gel electrophoresis to separate proteins according to their molecular weight, was used.

3.8.3.1 Protein sample preparation

20µl of nuclear extract samples isolated as described in 3.8.1 were denatured at 100°C for 15 minutes on a heated block and run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

3.8.3.2 Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins within the cell sample were separated by SDS-PAGE. The gel casting system (Protean 3, Bio-Rad, UK) was set up according to the manufacturer's instructions. A 10% resolving gel was prepared and poured into the gel casting system (for the recipe see 9.4 of Appendix); the gel was allowed to set for 40 minutes at room temperature. Once set, a stacking gel was prepared and poured on top of the resolving gel (for the recipe see 9.4 of Appendix); a comb was inserted and the gel was allowed to set at room temperature for 30 minutes. Having removed the comb, the apparatus was carefully placed in the tank and filled with 1x running buffer (for the recipe see 9.4 of Appendix). The first lane was loaded with 5µl of Rainbow™ coloured protein marker and 20µl of samples and relevant controls were loaded into the subsequent wells. Due to a large number of samples, they were split in 3 gels. The samples were subjected to electrophoresis at 150V constant voltage for approximately 1 hour.

3.8.3.3 Protein transfer

Proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) Immunblott™ membrane (Bio-Rad, UK) in a Transblott apparatus (Bio-Rad, UK). A piece of PVDF membrane was soaked in 100% methanol for 30 seconds and then washed in 1x transfer buffer. Subsequently, two pieces of filter paper, two sponges, the PVDF membrane and the gel were soaked in 1x transfer buffer (for recipe see 9.4 of Appendix) for 5 minutes on a rocker. Next, one of the sponges was laid on to the transfer cassette, followed by filter paper and then the PDVF membrane. The gel was placed on top of the membrane and carefully laid on to the cassette

between two pieces of filter paper and two sponges. The transfer cassette was placed in the transfer tank filled with 1x transfer buffer (for recipe see 9.4 of Appendix) and the system run at 100V for approximately 90 minutes on ice. Once the transfer was completed, the membrane was removed and washed twice for 5 minutes in 1x TBS-T buffer (for recipe see 9.4 of the Appendix).

3.8.3.4 Protein detection

The membrane was incubated in a blocking buffer (1x TBS-T containing 5% non-fat milk) at room temperature for 1 hour to reduce the non-specific binding of antibodies. The membrane was then washed three times 10 minutes each in 1x TBS-T; and incubated with the primary antibody of interest diluted in 5% non-fat milk at the correct ratio on a rotator at 4°C overnight. The following day, the membrane was washed three times 10 minutes each in 1x TBS-T on a rocker and incubated in the secondary antibody diluted at 1:2000 in 5% non-fat milk on a rocker for 1 hour at room temperature. Once completed, another three washes 10 minutes each were applied to the membrane and the membrane was blotted dry. Equal volumes of ECL™ Western blotting detection kit (GE Healthcare Life Sciences, UK) reagents were mixed in 1:1 ratio and poured over the membrane surface and left to incubate for 2 minutes. Then the membrane was blotted dry to remove the excess of ECL™ detection reagent and placed face down in a plastic wallet. The membrane then was developed in a dark room using hyper film ECL™ (GE Healthcare Life Sciences, UK); presence of a single strong band at the relevant size was identified using the Rainbow™ protein molecular weight markers (GE healthcare, UK).

3.9 Bisulphite sequencing

Pyrosequencing was used to identify the methylation levels of CpG sites within the CXCL8 promoter region. This method allows sequencing of a single strand of DNA by synthesising the complementary strand alongside and determining the base added at each step. It is a complex technique consisting of genomic DNA extraction, bisulphite conversion followed by PCR of the converted DNA and pyrosequencing.

3.9.1 Genomic DNA extraction

Cells were grown to confluence in T75cm^2 flasks, serum deprived 24 hours prior to any experiment, and either treated with 1ng/mL IL-1β for 2 hours or left unstimulated. Cells were washed with sterile PBS, scraped and centrifuged at 1000rpm for 5 minutes. Genomic DNA was isolated using the QIAamp DNA blood mini kit (50) (Qiagen, UK) according to the manufacturer's instructions.

Briefly, cell pellets were resuspended in 400µl of sterile PBS supplemented with 40µl of protease, and lysed in 400µl lysis buffer AL followed by pulse-vortexing for 15 seconds and 10 minutes incubation at 56°C. Subsequently, 200µl of 100% ethanol was added to the samples and mixed by pulse-vortexing for 15 seconds. The mixture was carefully applied to the QIAamp mini spin column, and centrifuged at 6000xg for 1 minute. Then, samples were washed with 500µl wash buffer AW1 and centrifuged at 6000xg for 1 minute. Samples underwent another wash in 500µl wash buffer AW2 and centrifugation at 20000xg for 3 minutes and 1 minute additionally. Genomic DNA was eluted in 200µl elution buffer AE by incubation for 5 minutes at room temperature, and centrifugation at 6000xg for 1 minute.

3.9.2 Bisulphite conversion

2µg of genomic DNA was bisulphite converted using the EpiTect® Bisulfite kit (Qiagen, UK) in accordance with the manufacturer's protocol.

DNA solution underwent bisulphite conversion in 140µl of a master mix containing 85µl bisulphite mix, 35µl DNA protect buffer and nuclease-free water using a thermal cycler (for cycling conditions see 9.5 of Appendix). When completed, bisulphite converted DNA underwent further clean up by addition of 560µl loading buffer BL with consequent vortexing and brief centrifugation. The mixture was transferred to EpiTect spin columns and centrifuged at 20000xg for 1 minute. Then, the samples were washed with 500µl wash buffer BW and centrifuged at 20000xg for 1 minute. 500µl desulfonation buffer BD was added to each spin column and incubated for 15 minutes at room temperature with subsequent centrifugation at maximum speed for 1 minute. DNA samples were washed twice with 500µl wash buffer BW and centrifuged at the maximum speed for 1 minute. Samples were incubated on a heating block at 56°C for 5 minutes. Finally, 40µl elution buffer EB was dispensed into the center of each column, and purified DNA samples were centrifuged at 15000xg for 1 minute.

3.9.3 PCR of bisulphite converted DNA

Eluted and purified DNA was subjected to PCR (Bio-Rad, PTC 100, UK) to amplify regions of interest within the CXCL8 promoter using primers designed by the PyroMark assay design software. DNA templates were amplified in 50µl reactions containing 1x CoralLoad PCR buffer, 200µM of dNTPs, 0.2µM of primer A and primer B, 2.5 units/reaction of HotStarTaq Plus DNA polymerase, and nuclease-free

water (for cycling conditions see 9.5 of Appendix). Once accomplished, 10µl of PCR products were run on 2% agarose gel at 100V for 30 minutes using Bio-Rad PowerPac 300 supply (Bio-Rad, UK). Presence of a single strong band at the relevant size was considered as successful, and samples underwent further pyrosequencing.

3.9.4 Pyrosequencing

5-20µl of biotinylated PCR products were immobilised on streptavidin-coated sepharose beads in a master mix containing 1µl agarose beads, 40µl binding buffer and nuclease-free water with total volume of 80µl. The reactions containing PCR products were dispersed into PCR strips, sealed and agitated constantly for at least 5-10 minutes at 1400rpm using a thermo-shaker (Grant-bio PCMT, UK). Primers used for sequencing were designed by PyroMark assay design software (for primer sequences see 9.5 of Appendix). 25µl 0.3µM primers diluted in annealing buffer were added to required wells of a PyroMark Q24 plate.

Immediately after immobilisation, the PCR and the PyroMark Q24 plates were placed on the PyroMark Q24 work station. Vacuum was applied and filters were lowered into the PCR plate for 15 seconds to capture the beads containing immobilised template. The filter probes with samples were washed in 50mL of 70% ethanol for 5 seconds, then in 40mL of denaturation solution for 5 seconds, and finally in 50mL of 1x wash buffer for 10 seconds. Then, beads were released in the PyroMark Q24 plate containing sequencing primers, the filter probes were washed in 50mL of nuclease-free water for 10 seconds twice and left to be drained of the residual liquid. Subsequently the PyroMark Q24 plate was heated at 80°C for 2 minutes, and allowed to cool for at least 5 minutes before being processed. A

PyroMark Q24 cartridge was filled with enzyme and substrate mixtures and dNTPs; required volumes as well as a run design were calculated using PyroMark Q24 2.0.6 software. Data were expressed as a percentage of CpG sites methylated in the CXCL8 promoter region.

3.10 Statistics

The data were presented as mean ± SEM (standard error of the mean). Analysis for statistical significance was performed using one-way ANOVA and unpaired Student's t-test and GraphPad Prism software versions 5.0 and 6.0. A p value <0.05 was considered as statistically significant.

4 DIFFERENCES IN EXPRESSION AND PRODUCTION OF CXCL8 IN CF AND NON-CF AIRWAY EPITHELIAL CELLS

4.1 Introduction

CF is characterised by severe inflammation affecting the lungs from infancy (Armstrong et al., 2005, Nixon et al., 2002). Several studies have shown that the CFTR defect results in bacterial overload and enhanced inflammation characterised by a continuous influx of immunocompetent cells and altered expression of pro-inflammatory mediators in the airways (Elizur et al., 2008, Cloutier et al., 2009). Although progress has been made in understanding how the basic defect in CF leads to the lung pathology, there are still many unanswered questions.

The bronchoalveolar lavage (BAL) fluid of CF patients contains increased quantities of pro-inflammatory mediators such as CXCL8, IL-6, IL-1, and TNF-α in early infancy prior to any symptoms of altered lung function and/or signs of bacterial presence (Ranganathan et al., 2011, Stecenko et al., 2001). Furthermore, studies using animal models have showed that the BAL fluid of CFTR-deficient mice contains increased levels of inflammatory mediators which correlate with higher mortality rates compared to control mice (Heeckeren et al., 1997).

CXCL8 is a powerful neutrophil chemoattractant which coordinates the inflammatory response in CF airways. It is produced by several cell types including bronchial epithelial cells (Strieter, 2002). While little CXCL8 is expressed basally, it is induced by a range of stimuli including pro-inflammatory mediators such as IL-1ß (Bonfield et al., 1995, Cao et al., 2005). The molecular mechanisms responsible for the increased CXCL8 expression in CFTR-deficient cells are still not fully understood.

In this chapter, we performed largely confirmatory experiments to verify that IL-1ß would enhance CXCL8 release via an increase in CXCL8 mRNA levels in two different

CF cell lines. This was a necessary prelude to performing mechanistic studies in later chapters.

4.2 Aims

The aims of this chapter were to determine:

- whether CXCL8 secretion is increased in two different CFTR-deficient cell lines compared to normal cells both basally and after stimulation with IL-1ß;
- whether CXCL8 mRNA expression is increased in CFTR-deficient cell lines compared to normal cells both basally and after stimulation with IL-1ß;
- whether mRNA stability experiments show any evidence of altered post-transcriptional regulation.

4.3 Methods

4.3.1 Concentration response and time course experiments

To detect the amount of secreted CXCL8, IB3-1 normal, IB3-1 CF, CFBE41o- normal and CFBE41o- CF cells were cultured until confluent in 24 well plates. 24 hours prior to each experiment, cells were serum starved in 1mL serum free medium; subsequently, medium was replaced with fresh medium containing specified concentrations of IL-1ß. For concentration response experiments supernatants were collected after 24 hours; for time course experiments stimulated and unstimulated supernatants were collected at the time points stated. CXCL8 levels were measured either immediately by ELISA or stored at -80°C until required.

Concentrations were normalised against the amount of total cellular protein as described in 3.3 of Chapter 3 Materials and Methods.

4.3.2 CXCL8 mRNA expression

To identify the effect of IL-1β on CXCL8 expression, cells were grown in 6 well plates. Confluent cells were serum starved for 24 hours before the experiments, and media was replaced with serum free media with or without 1ng/mL IL-1β for 0, 2, 4, 6, 8, and 24 hours. Total RNA was extracted and stored at –80°C until required. CXCL8 mRNA was quantified by qPCR as described in 3.4.3 of Chapter 3 Materials and Methods. Data were normalised to $ß_2$-microglobulin which was used as a housekeeping gene.

4.3.3 CXCL8 mRNA stability experiments

To determine mRNA stability, confluent and growth arrested cells were incubated with 5µg/mL of transcription inhibitor Actinomycin D for 0, 1, 2, 4, 8, and 24 hours. Total RNA was collected at each time point, extracted and quantified for CXCL8 mRNA by qPCR as described in 3.4.3 of Chapter 3 Materials and Methods. Data were normalised to $ß_2$-microglobulin used as a housekeeping gene.

4.4 Results

4.4.1 IL-1ß stimulates increased CXCL8 protein production from CF airway epithelial cells

We determined the ability of normal and CF airway epithelial cells to express CXCL8 and the effect of IL-1ß on CXCL8 secretion. IL-1β was chosen as a stimulus due to its potent pro-inflammatory properties and implications in the pathophysiology of CF. All four (two normal and two CF) cell lines were stimulated with IL-1ß at concentrations of 0.1, 1, 2, 5, and 10ng/mL for 24 hours. Basal CXCL8 expression was observed in all experiments, and stimulation with IL-1ß significantly increased CXCL8 expression at the concentration 0.1ng/mL and above (Figure 4-1).

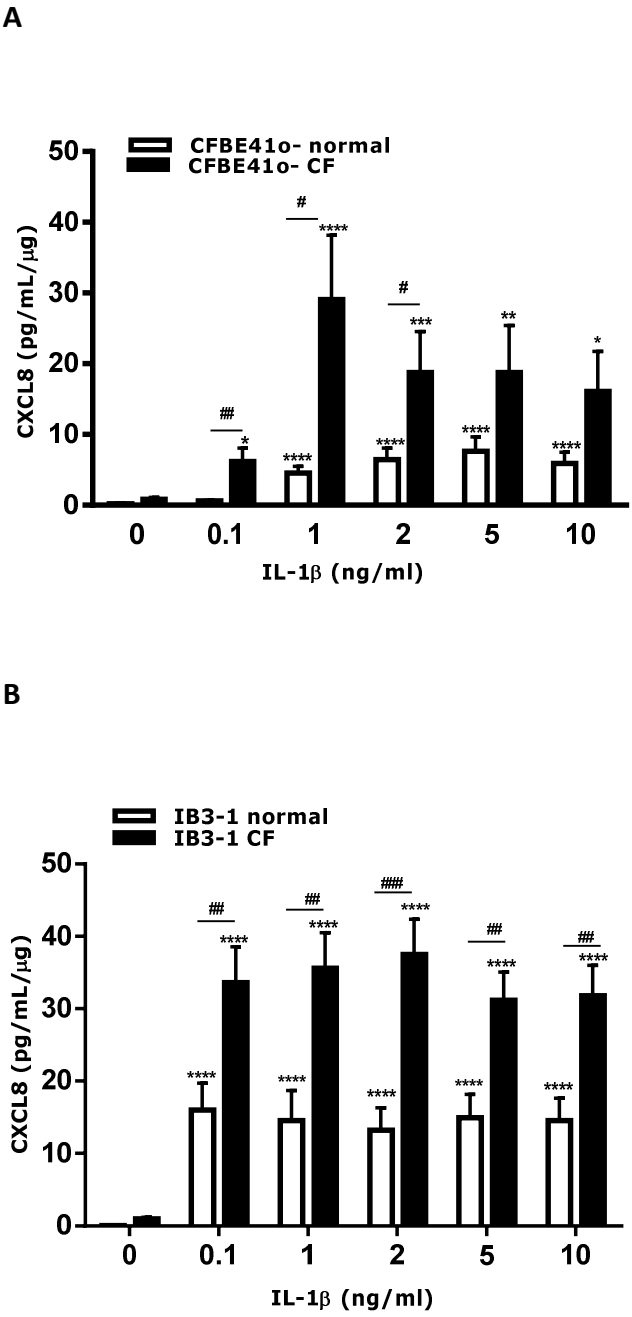
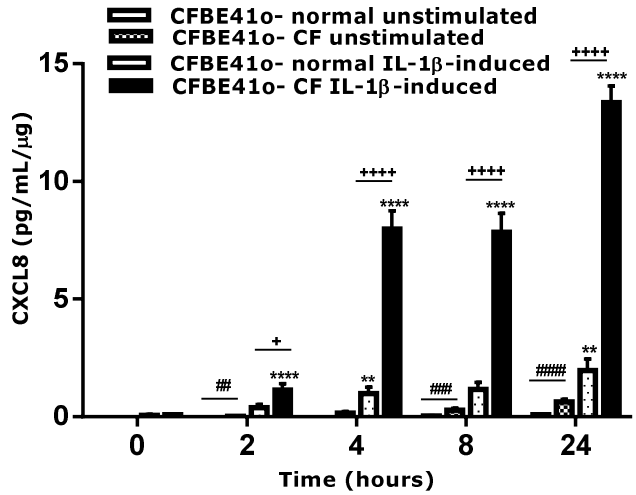


Figure 4-1. Concentration response of IL-1ß on CXCL8 expression from normal and CF airway epithelial cells. CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines, confluent and growth-arrested 24 hours prior to an experiment, were incubated with increasing concentrations of IL-1ß for 24 hours. CXCL8 protein levels were measured by ELISA, and values normalised to total amount of cellular protein. Each bar represents means ± SEM from triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis, $^{*,\#}$p value <0.05, $^{**,\#\#}$p<0.01, $^{***,\#\#\#}$p<0.001 and ****p<0.0001 (* indicates the difference between unstimulated and IL-1ß-induced samples within the same cell line, $^{\#}$ is the difference between normal and CF cells).

Following the concentration response experiments, time course experiments were performed to determine the optimal time point for IL-1ß-induced CXCL8 production. 1ng/mL IL-1ß induced CXCL8 expression in a time-dependent manner with the greatest increase at 24h (Figure 4-2).

A



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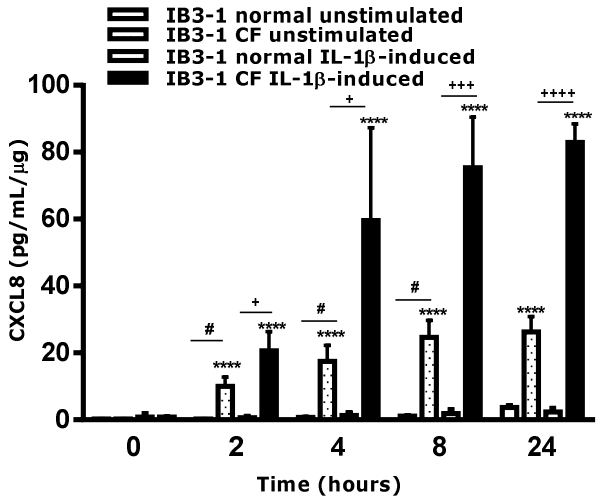


Figure 4-2. Time course of IL-1ß-induced CXCL8 expression in normal and CF airway epithelial cells. CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines, confluent and growth-arrested 24 hours prior an experiment, were incubated with 1ng/mL of IL-1ß over 24 hours. CXCL8 protein levels were measured by ELISA, and values normalised to total amount of cellular protein. Each bar represents means ± SEM from triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis, $^{\#,+}$p value <0.05, $^{**,\#\#}$p<0.01, $^{\#\#\#,+++}$p<0.001 and $^{****,\#\#\#\#,++++}$p<0.0001 (* indicates the difference between unstimulated and IL-1ß-induced samples within the same cell line, $^{\#}$ is the difference between unstimulated normal and CF cells, $^{+}$ is the difference between IL-1ß-induced normal and CF cells).

CXCL8 was constitutively expressed in both normal airway epithelial cell lines. IL-1β stimulation resulted in an induction of CXCL8 secretion in a time and concentration dependent manner. Both CF cell lines produced statistically significantly higher basal levels of CXCL8 ($p<0.05$): a fourfold increase in CFBE41o- CF and seven fold increase in IB3-1 CF cells compared to the corresponding normal cells (Figure 4-3). Likewise, IL-1ß stimulation resulted in greater CXCL8 production in CFTR-deficient than normal cell lines (six fold increase in CFBE41o- CF versus seven fold increase in IB3-1 CF cells) in comparison to the analogous normal cells ($p<0.05$) (Figure 4-3).

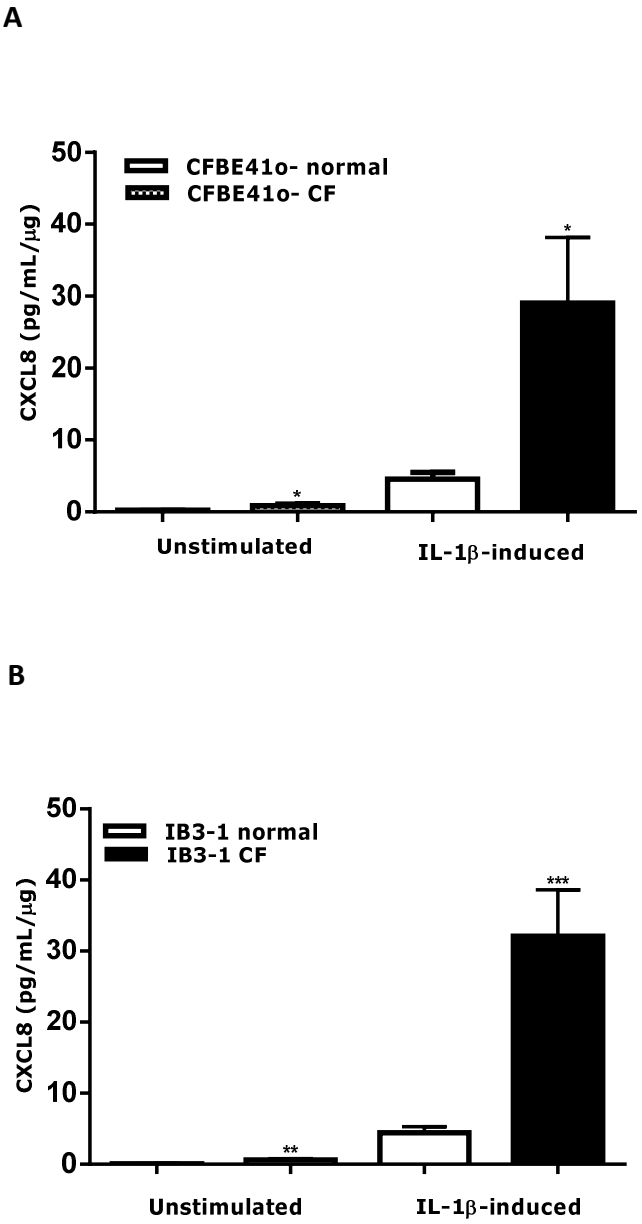


Figure 4-3. IL-1β–induced CXCL8 secretion in normal and CF airway epithelial cells. CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines, confluent and growth-arrested 24 hours prior an experiment, were incubated with 1ng/mL of IL-1ß for 24 hours. CXCL8 protein levels were measured by ELISA, and values normalised to total amount of cellular protein. Each bar represents means ± SEM from triplicate determinants of at least 3 independent experiments. Data were analysed using Student t-test, *p value <0.05, **p<0.01, ***p<0.001 were considered as significant (* indicates the difference between normal and CF cells).

4.4.2 IL-1ß induces increased CXCL8 mRNA expression in CF airway epithelial cells

To identify the effects of IL-1ß on CXCL8 mRNA expression in normal and CF airway epithelial cells, time course experiments were carried out. Total RNA was collected at 0, 2, 4, 6, 8, and 24 hours from cells treated with and without 1ng/mL IL-1ß and then reverse transcribed. qPCR was performed on the resulting cDNA. Representative graphs are shown due to variability in the relative expression levels across individual experiments (Figure 4-4).

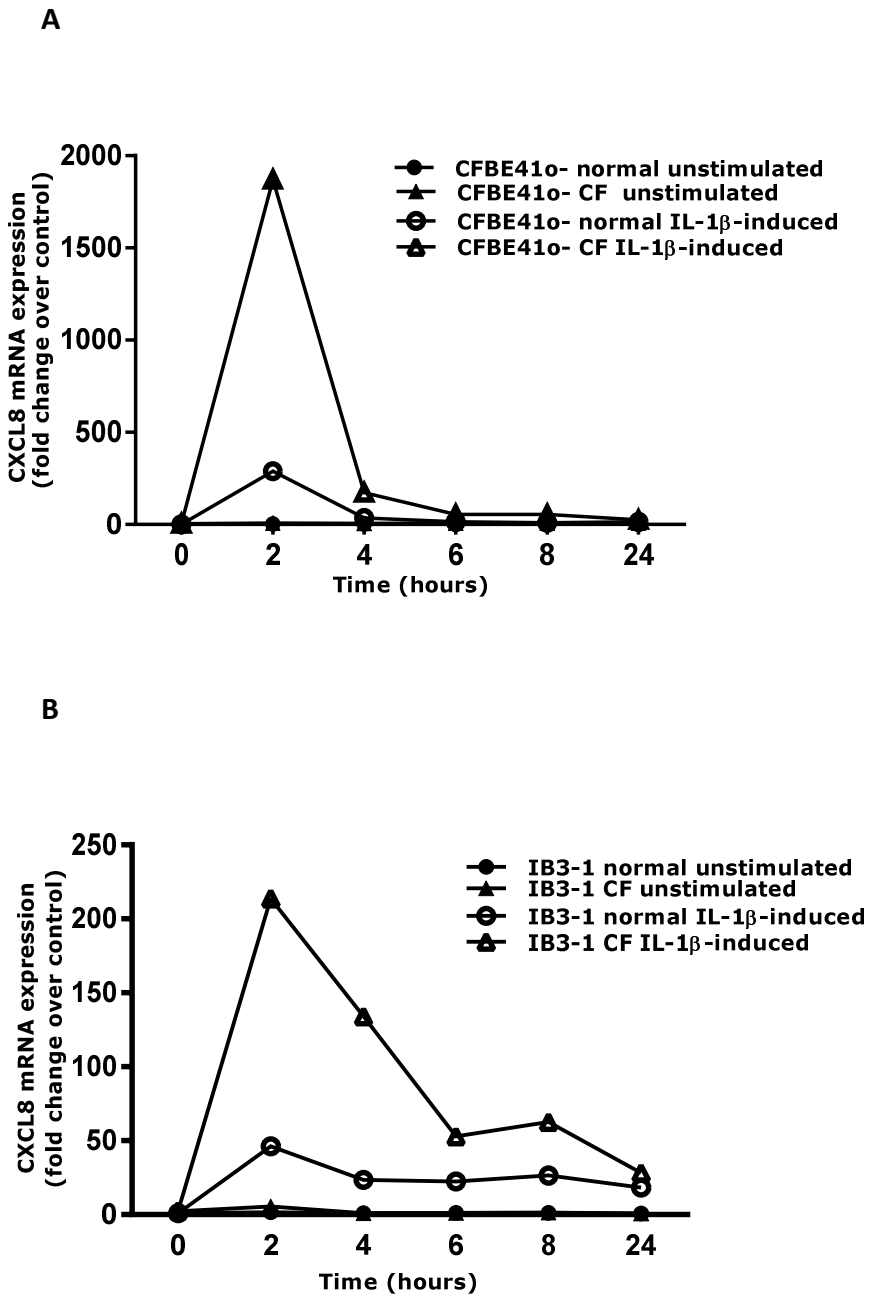


Figure 4-4. The effect of IL-1β on CXCL8 mRNA expression. CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines were growth arrested for 24 hours and incubated in media with or without 1ng/mL IL-1ß for the stated times. Isolated total RNA was reverse transcribed and resulting cDNA was analysed by qPCR; the data were normalised to $ß_2$-microglobulin which was used as a housekeeping gene. The graphs shown are the representative of analogous results obtained in three independent experiments.

Normal cells showed an increase in the mRNA levels after stimulation with IL-1β. Both CFBE41o- CF and IB3-1 CF cell lines had higher basal levels of CXCL8 mRNA in comparison to corresponding normal cells: nearly five fold increase in both CF cell lines compared to normal cells. IL-1ß stimulation increased CXCL8 mRNA expression in both CF cell lines. Both CF cell lines expressed higher IL-1β-stimulated levels compared to the matched controls: 400 fold increase in CFBE41o- CF versus 200 fold increase in IB3-1 CF cell lines (Figure 4-5).

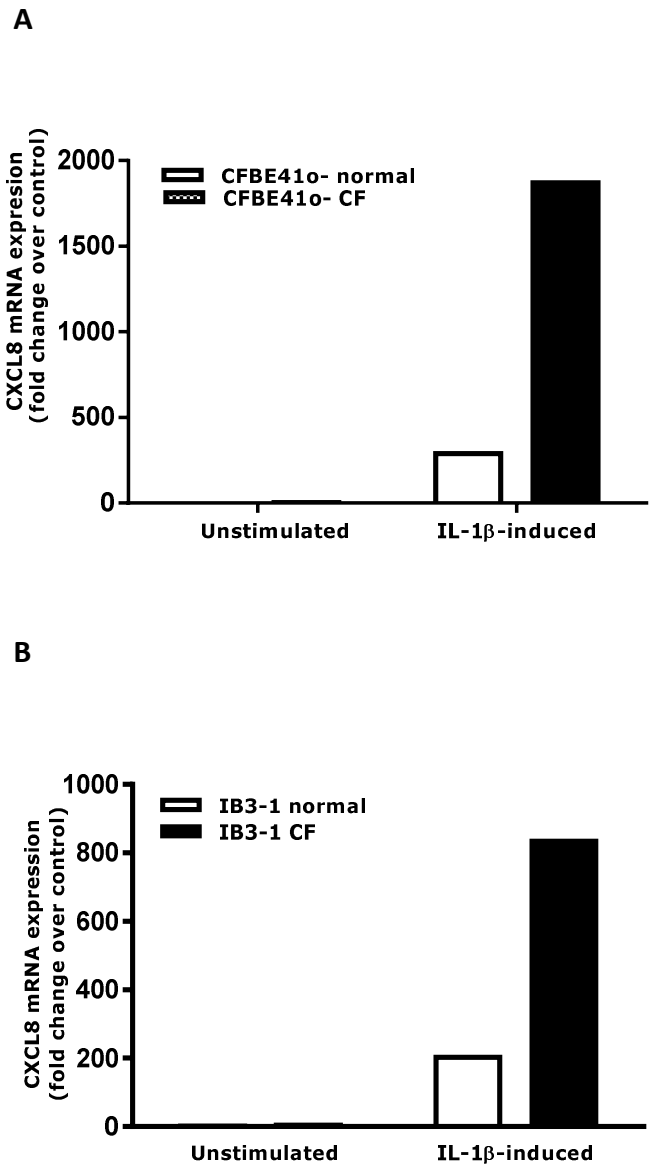


Figure 4-5. Comparison of the effect of IL-1β on CXCL8 mRNA expression in normal and CF airway epithelial cells. CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines were growth arrested for 24 hours and incubated with 1ng/mL IL-1ß for 2 hours. Isolated total RNA was reverse transcribed and quantified by qPCR; the data were normalised to $ß_2$-microglobulin which used as a housekeeping gene. The graphs shown are the representative of comparable results obtained in three independent experiments.

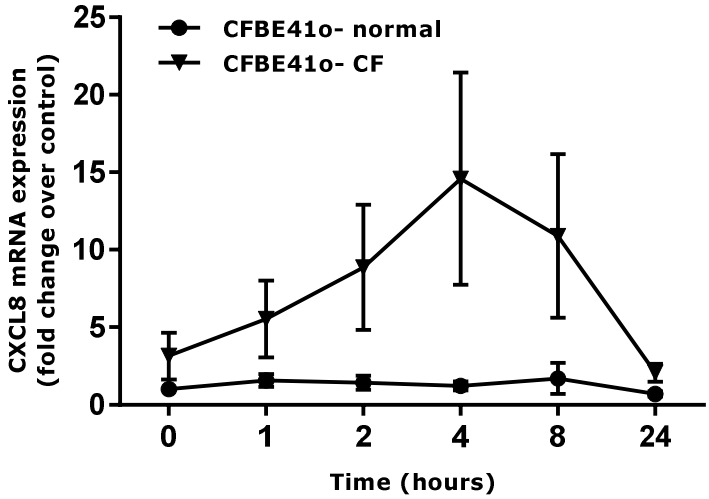
Overall, the data demonstrated that IL-1ß-induced CXCL8 mRNA expression with a peak at 2 hours followed by a decline (Figure 4-4). The 2 hour time point was, therefore, chosen for future experiments (Figure 4-5).

4.4.3 The effect of transcription inhibitor Actinomycin D on basal CXCL8 mRNA expression

Experiments using a trancriptional inhibitor Actinomycin D were performed to assess possible alterations of CXCL8 mRNA stability in CF cells. All four cell lines were grown to confluence and serum starved 24 hours before experiments. Cells were incubated with 5μg/mL Actinomycin D for 0, 1, 2, 4, 8 and 24 hours, and the extracted mRNA underwent qPCR analysis. We chose to analyse mRNA breakdown levels under basal conditions as large basal differences in CXCL8 protein production were seen between CF and normal cells. The four fold difference between normal and CF cells observed basally was not increased further by IL-1β suggesting that basal changes in CXCL8 production may be driving the induced release.

There was no difference in the rate of decay in mRNA levels between CF and normal cell lines suggesting that greater mRNA stability was not responsible for the increase in CXCL8 in CF cells (Figure 4-6). The differences in the CXCL8 expression levels are, therefore, more likely to be due to altered transcriptional regulation and this is addressed in the next chapter.

A



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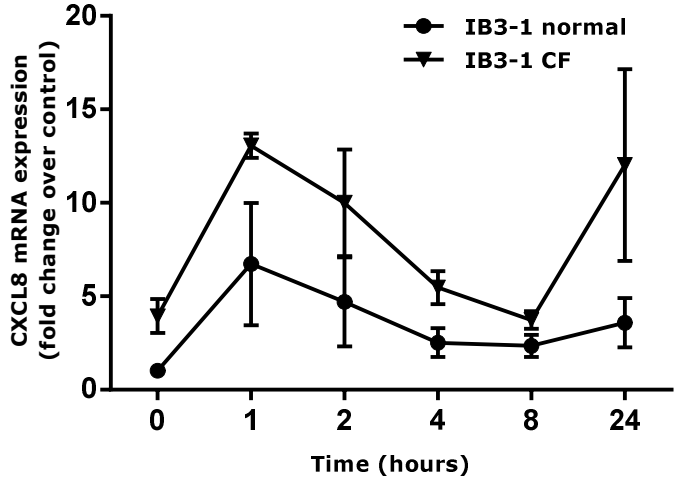


Figure 4-6. mRNA stability in normal and CF airway epithelial cells. Confluent and growth arrested CFBE41o- normal and CFBE41o- CF ***(A)*** and IB3-1 normal and IB3-1 CF ***(B)*** cell lines were incubated with 5µg/mL Actinomycin D for the stated time points. Isolated total RNA was assayed for CXCL8 mRNA via qPCR; the data were normalised to ß$_2$-microglobulin which was used as a housekeeping gene. Each bar represents mean ± SE of at least 3 independent experiments.

4.5 Discussion

The principal findings of the experiments in this chapter are that CFTR-deficient airway epithelial cells express significantly higher levels of CXCL8 protein and mRNA transcripts both constitutively and in response to IL-1ß stimulation. We also showed that IL-1ß induces increased CXCL8 secretion in a concentration- and time-dependent manner.

The principal aim of this chapter was to measure the production of CXCL8 protein from normal and CF cells and to determine any differences in the expression. We showed that CF cells had greater levels of CXCL8 protein as compared to normal cells under basal conditions, and this was the same in both CF and matched normal cell lines used. Stimulation with IL-1ß resulted in a significant increase in CXCL8 production in all four cell lines. IL-1ß-induced CXCL8 expression was significantly higher in CF cells as compared to the corresponding normal cell lines.

We chose to study two published CF and corresponding normal cell lines to determine whether the difference between normal and CF cells is consistently seen. The fact that we obtained similar results in both cell lines suggests, that the difference between normal and CF cells is real. The IB3-1 normal and IB3-1 CF cell lines were used at different passage numbers (15 and 41 respectively), and we considered that altered passage number might have affected the results. However, the CFBE41o- normal and CFBE41o- CF cells were used at a similar passage number (21 and 22 respectively) and had similar findings to the IB3-1 cells, suggesting that the difference between normal and CF cells is not an artefact produced by differences in passage number.

Our data in airway epithelial cells confirm the findings of other studies conducted in macrophages derived from CF patients (Khan et al., 1995b, Conese et al., 2009) and airway epithelial cells (Saadane et al., 2011) showing that CF cells secrete greater levels of CXCL8 compared to healthy subjects (Khan et al., 1995b, Saadane et al., 2011). BAL fluid of infants with CF has been reported to have significantly higher levels of CXCL8 even though culture for common bacterial pathogens is negative (Khan et al., 1995b, Nixon et al., 2002). Collectively, these observations suggest that CF cells have altered CXCL8 release as a result of a fundemental abnormality of the cells themselves rather than as a result of an altered airway environment.

In contrast to our studies, Massengale *et al* using normal human bronchial (HBE4-E6/E7-C1) and airway epithelial (CF/T43), CFTR-deficient (CFT1, CFT1-LC3) and CFTR-corrected (CFT1-LCFSN) cell lines showed lower secretion of CXCL8 protein from CF cells compared to the control cell lines under basal conditions and following induction with IL-1ß (Massengale et al., 1999). Differences in the results between this study and our findings are unclear, but partially reflect differences in the cell lines used and the lower concentration of IL-1ß used (100pg/mL) versus 1ng/mL used in our study.

Consistent with the protein results we found that CXCL8 mRNA levels were increased in both CF cell lines compared to the normal cells basally and after induction with IL-1ß. These findings suggest that overexpression of CXCL8 in CF cells might be due to upregulated transcription of CXCL8 mRNA or, conversely, a reduced breakdown of mRNA through an increase in its stability.

To examine whether changes in mRNA expression were due to altered post-transcriptional mechanisms, we analysed CXCL8 mRNA stability by performing experiments using transcriptional inhibitor Actinomycin D. Our results showed no increase in mRNA stability in CF cells basally, suggesting, that the mechanism of increased CXCL8 production was probably transcriptional, which we address further in the next chapter. In retrospect, we should, perhaps, have also studied mRNA stability after IL-1ß treatment, but our observations of higher basal CXCL8 protein secretion and mRNA levels, and the similar four fold increase between normal and CF cells both basally and upon IL-1ß stimulation suggested, that altered basal expression may be more important. Previous studies on CXCL8 mRNA stability show conflicting results with a study showing no alteration like ours and another showing an increase in mRNA stability in CF (Balakathiresan et al., 2009, Bhattacharyya et al., 2010). Again, the variability may reflect the differences between the cell lines used in their experiments and ours.

Collectively, our findings suggest that the enhanced CXCL8 production in CF cells is likely to be due to an increase in mRNA levels possibly reflecting altered transcriptional regulation. We decided to use IB3-1 normal and IB3-1 CF cells for further studies due to the stronger signal. In the next chapter, we studied CXCL8 trancription using CXCL8 promoter luciferase reporter assays.

5 NF-κB, AP-1 AND C/EBPß TRANSCRIPTION FACTORS ARE INVOLVED IN CXCL8 EXPRESSION IN CF AIRWAY EPITHELIAL CELLS

5.1 Introduction

In the studies in Chapter 4, we showed that CF cells express greater levels of both CXCL8 protein and mRNA basally and following IL-1ß stimulation as compared to normal cells. In this chapter we performed mechanistic studies looking at the potential transcriptional mechanisms involved in CXCL8 upregulation in CF.

Transcriptional regulation of CXCL8 expression is tightly controlled by a range of transcription factors (TFs) through their coordinated binding to cis-acting DNA elements in the promoter region. The nucleotide sequence from -1 to -133 within the 5'-flanking region of the CXCL8 gene containing CCAAT/enhancer binding protein (C/EBP)β, nuclear factor (NF)-κB, activator protein (AP)-1, and octamer (Oct)-1 binding sites is crucial for the transcriptional regulation of the gene (Mukaida et al., 1994, John et al., 2009) particularly for IL-1ß-induced CXCL8 expression (Mukaida et al., 1990, Carroll et al., 2005). Studies performed in gastric cancer cells (Yasumoto et al., 1992) and fibrosarcoma cell lines (Mukaida et al., 1990) have reported, that while IL-1β or TNF-α-induced CXCL8 transcription is initially NF-κB-driven, synchronised binding of NF-κB, AP-1, and C/EBPß is required for maximum activation of CXCL8 transcription (Verhaeghe et al., 2007b, Li et al., 2002, Holtmann et al., 1999, Hoffmann et al., 2002).

Although the mechanisms involved in transcriptional activation of CXCL8 in response to inflammatory stimuli have been well characterised, the mechanisms regulating basal CXCL8 production have not been studied in as much detail. Sequence analysis suggested that the presence of the negative regulatory element (NRE) partially overlapping with NF-κB response element at the CXCL8 promoter in combination with NF-κB-repressing factor (NRF) protein bound to the NF-κB site

results in transcriptional repression of the CXCL8 promoter in unstimulated cells (Hoffmann et al., 2002, O'Dea and Hoffmann, 2010). Collectively, these findings suggest that basal CXCL8 transcription is closely controlled via a complex and multilevel hierarchical system.

The mechanisms implicated in the altered CXCL8 transcription in CF cells have not been fully characterised: the available evidence implicates both NF-κB and AP-1 (Hoffmann et al., 2002). In this chapter we characterised the TFs involved in basal and IL-1ß-induced CXCL8 expression in normal and CF cells to determine if there were any differences.

5.2 Aims

The aims of this chapter were:

- to determine the TFs involved in CXCL8 transcription in normal and CF airway epithelial cells basally and after IL-1ß stimulation using CXCL8 promoter reporter constructs with mutated TF binding sites;
- to characterise IL-1ß-induced activation and binding of TFs to the corresponding cis-elements in the CXCL8 promoter in normal and CF airway epithelial cells.

5.3 Methods

Transient transfections

To identify the TFs involved in CXCL8 expression and determine the effect of IL-1ß on their activity, dual luciferase reporter assays were performed as described in 3.5 of Chapter 3 Materials and Methods. Normal and CF cells were transiently transfected with vectors encoding the wild type (wt)CXCL8 promoter (-162/+44), site mutations of one of the binding sites (NF-κB, AP-1, and C/EBPβ) within the CXCL8 promoter as well as control luciferase reporter pRL-TK. Vectors were a generous gift of Dr. A. R. Braiser (Department of Medicine, Sealy Center for Molecular Science, Galveston, TX).

Chromatin immunoprecipitation

To identify the TFs binding to the CXCL8 promoter region, chromatin immunoprecipitation (ChIP) was performed as described in 3.7 of Chapter 3 Materials and Methods. Binding of TFs was measured in normal and CF cells basally and following IL-1β stimulation for up to 2 hours.

5.4 Results

5.4.1 IL-1β-induced CXCL8 promoter activation requires C/EBPß, NF-κB and AP-1 transcription factors

5.4.1.1 Basal and IL-1ß-induced stimulation of the wild type CXCL8 promoter

Unstimulated CF cells transfected with wild type (wt)CXCL8 promoter reporter expressed significantly lower levels (three fold decrease) of luciferase activity compared to normal cells. IL-1β stimulation resulted in a significant increase in luciferase activity in both normal (eight fold increase) and CF cells (nearly 15 fold increase) with no significant difference between these two cell lines (Figure 5-1).

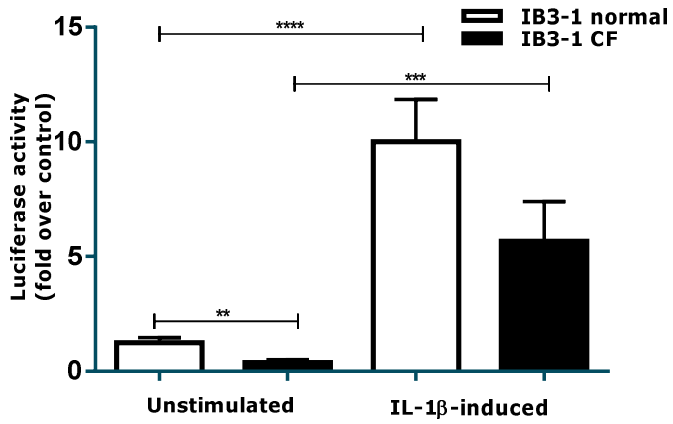


Figure 5-1. The effect of IL-1ß on the CXCL8 promoter luciferase reporter activity. IB3-1 normal and IB3-1 CF cells were growth arrested 16 hours prior to an experiment, were transiently transfected with (wt)CXCL8 firefly vectors along with pRL-TK renilla luciferase reporter plasmid DNA at 1:2 ratio with LF2000 for 3 hours. This was followed by incubation for another 3 hours in the presence or absence of 1ng/mL IL-1β. Activities of firefly and renilla luciferase reporters were measured using an Omega Fluostar luminometer; data were normalised by dividing firefly readings by renilla expressed as relative luciferase activity (fold change over control). Each column represents means ± SEM from at least triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis; **p value<0.01, ***p<0.001 and ****p<0.0001.

5.4.1.2 Mutation of the NF-κB binding site

Mutation of the NF-κB site reduced basal and IL-1ß-induced luciferase activity in both normal and CF cell lines compared to (wt)CXCL8 promoter transfections (Figure 5-2).

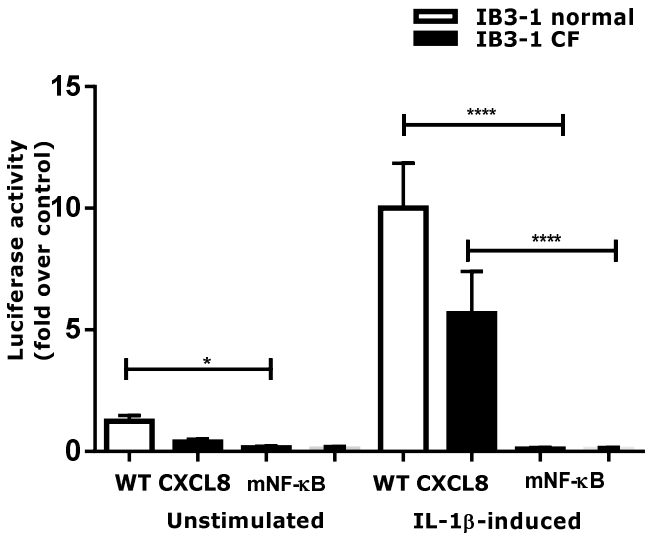


Figure 5-2. The effect of IL-1ß on the NF-κB mutant CXCL8 luciferase reporter activity. IB3-1 normal and IB3-1 CF cells were growth arrested 16 hours prior to an experiment, were transiently transfected with a CXCL8 promoter firefly luciferase reporter containing a mutated NF-κB site along with pRL-TK renilla luciferase reporter plasmid DNA at 1:2 ratio with LF2000 for 3 hours. This was followed by incubation for another 3 hours in the presence or absence of 1ng/mL IL-1β. Activities of firefly and renilla luciferase reporters were measured using an Omega Fluostar luminometer; data were normalised by dividing firefly readings by renilla expressed as relative luciferase activity (fold over control). Each column represents means ± SEM from at least triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis; *p value <0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

5.4.1.3 Mutation of the C/EBPß binding site

Mutation of the C/EBPβ binding site also reduced luciferase activity in both normal and CF cells compared to the transfections with (wt)CXCL8 promoter basally and after IL-1ß stimulation (Figure 5-3).

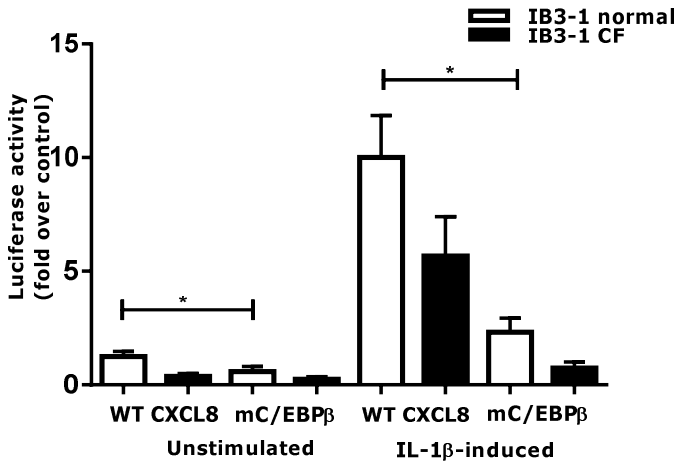


Figure 5-3. The effect of IL-1ß on the C/EBPß mutant CXCL8 luciferase reporter activity. IB3-1 normal and IB3-1 CF cells were growth arrested 16 hours prior to an experiment, were transiently transfected with a CXCL8 promoter firefly luciferase reporter containing a mutated C/EBPβ site along with pRL-TK renilla luciferase reporter plasmid DNA at 1:2 ratio with LF2000 for 3 hours. This was followed by incubation for another 3 hours in the presence or absence of 1ng/mL IL-1β. Activities of firefly and renilla luciferase reporters were measured using an Omega Fluostar luminometer; data were normalised by dividing firefly readings by renilla expressed as relative luciferase activity (fold over control). Each column represents means ± SEM from at least triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis; *p value <0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

5.4.1.4 Mutation of the AP-1 binding site

Mutation of the AP-1 binding site reduced luciferase reporter activity in normal and CF cells under both basal and IL-1ß stimulated conditions (Figure 5-4).

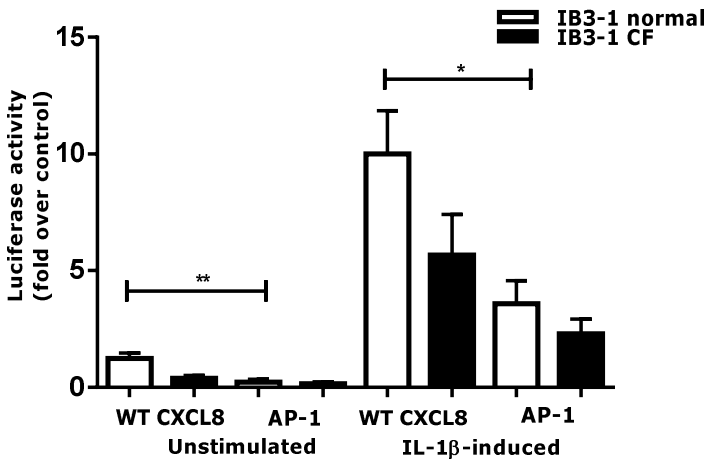


Figure 5-4. The effect of IL-1ß on the AP-1 mutant CXCL8 luciferase reporter activity. IB3-1 normal and IB3-1 CF cells were growth arrested 16 hours prior to an experiment, were transiently transfected with a CXCL8 promoter firefly luciferase reporter containing a mutated AP-1 site along with pRL-TK renilla luciferase reporter plasmid DNA at 1:2 ratio with LF2000 for 3 hours. This was followed by incubation for another 3 hours in the presence or absence of 1ng/mL IL-1β. Activities of firefly and renilla luciferase reporters were measured using an Omega Fluostar luminometer; data were normalised by dividing firefly readings by renilla expressed as relative luciferase activity (fold over control). Each column represents means ± SEM from at least triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis; *p value <0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Collectively these studies suggest that all three TFs are involved in basal and IL-1β-induced release of CXCL8 in both normal and CF cells. The fact, that CF cells did not show an increase in the (wt)CXCL8 promoter luciferase activity compared to normal cells was surprising considering the results of the previous chapter showing greater levels of CXCL8 protein production and mRNA expression in CF cells as compared to the normal cells. However, the reporter assays do not measure binding of a TF to a gene in the chromatin environment - a key regulator of TF access. Thus, hypothesising, that abnormal CXCL8 production could be due to increased binding of TFs to the CXCL8 promoter as a result of an altered chromatin environment

facilitating TF binding, we went on to perform ChIP which have the advantage of studying TF binding in the chromatin environment.

5.4.2 Increased basal binding of NF-κB p65 transcription factor to the CXCL8 promoter

CF cells displayed greater levels of NF-κB p65 associated basally with the CXCL8 promoter compared to normal cells. Whilst IL-1ß stimulation resulted in an increase in NF-κB p65 binding in normal cells, CF cells did not show any further rise in NF-κB p65 binding levels over the already elevated basal levels. However, levels of NF-κB p65 association with the CXCL8 promoter were greater in CF compared to normal cells both basally and following IL-1ß stimulation (Figure 5-5).

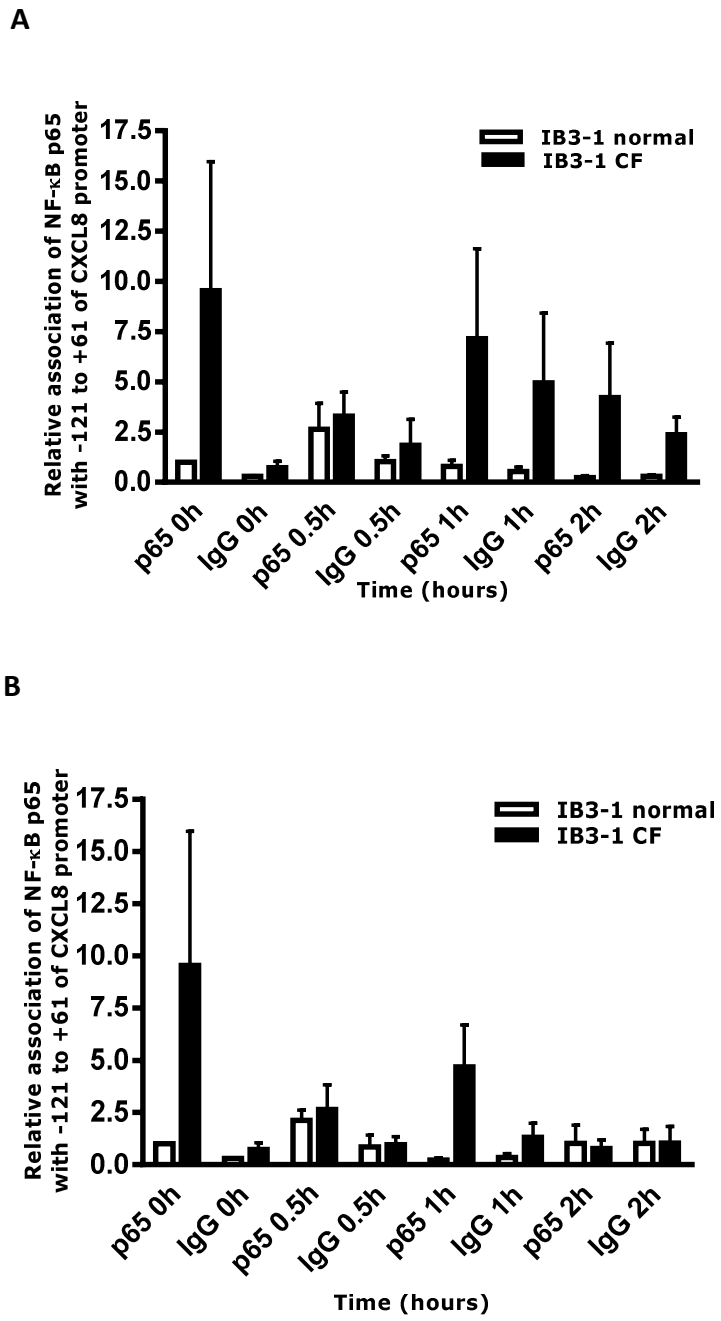
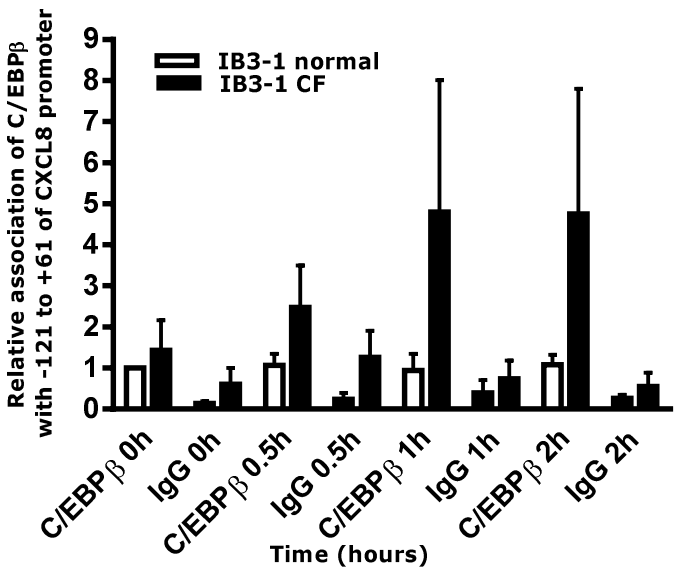


Figure 5-5. NF-κB p65 binding to the human CXCL8 promoter. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells were incubated without ***(A)*** or with 1ng/mL IL-1ß ***(B)*** for 0, 0.5, 1 and 2 hours. Chromatin, extracted from cells, was sheared and incubated with anti-NF-κB p65 antibody; the binding levels were assessed through the analysis of isolated DNA by qPCR using CXCL8 promoter-specific primers. DNA, resulting from chromatin immunoprecipitation using normal rabbit IgG, was used as a negative control. Data are expressed as fold change relative to the 0h mean of normal cells first normalised to Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

In summary, the greater binding of NF-κB p65 to the CXCL8 promoter under basal conditions might in part explain the increased basal CXCL8 transcription in CF cells.

There was no difference in basal C/EBPß binding to the CXCL8 promoter between normal and CF cells. IL-1ß caused a rapid increase in C/EBPß binding greatest at 0.5 hour (four fold increase) that was not different between CF and normal cells (Figure 5-6).

A



B

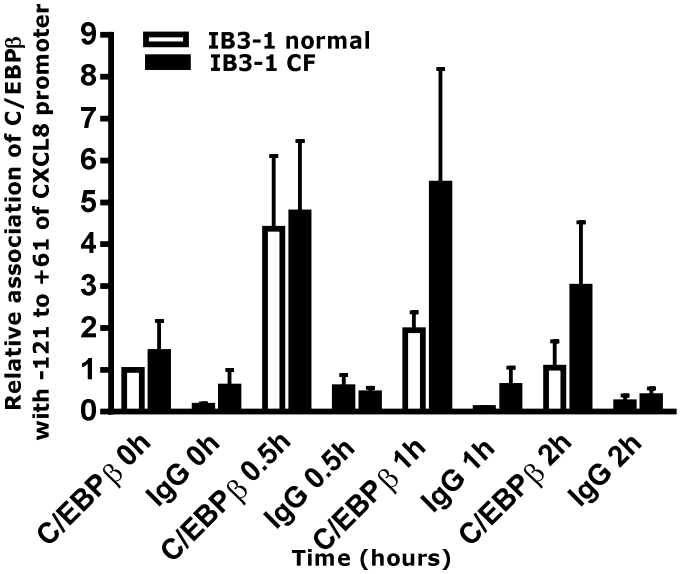


Figure 5-6. C/EBPß binding to the human CXCL8 promoter. IB3-1 normal and IB3-1 CF cells, confluent and growth arrested, were incubated without ***(A)*** or with 1ng/mL IL-1ß ***(B)*** for 0, 0.5, 1 and 2 hours. Extracted chromatin was sheared and immunoprecipitated with antibody against C/EBPβ; binding of C/EBPβ transcription factor was assessed through the analysis of isolated DNA by qPCR using CXCL8 promoter-specific primers. Samples, immunoprecipitated with normal rabbit IgG, were used as a negative control. Data are expressed as fold change relative to the 0h mean of normal cells first normalised to Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

Finally, ChIP analysis of AP-1 binding to the CXCL8 promoter was performed. Unfortunately, numerous attempts to measure binding of AP-1 to the CXCL8 promoter were unsuccessful due to technical difficulties in obtaining a single product during qPCR.

5.5 Discussion

The key findings in this chapter are that NF-κB, C/EBPß and AP-1 TFs are involved in basal and IL-1ß-induced CXCL8 expression in both normal and CF cells and that basal binding of NF-κB to the CXCL8 reporter was increased in CF cells.

Our promoter mutation transfection studies showed that under both basal and IL-1ß stimulated conditions all three TFs were required for optimum CXCL8 expression both in normal and CF cells. In contrast to the increased CXCL8 protein and mRNA levels in CF cells described in the previous chapter, there was no increase in (wt)CXCL8 promoter luciferase activity in CF versus normal cells. The studies showing involvement of all three TFs in CXCL8 transcription are consistent with other studies in the literature performed in several cell types and suggesting, that all three TFs are necessary for the full activation of the CXCL8 promoter both basally and following IL-1ß stimulation (John et al., 2009, Holtmann et al., 1999, Cloutier et al., 2009).

A disadvantage of transient transfections is that the plasmid is not incorporated into the genome and, therefore, epigenetic chromatin-based regulatory mechanisms do not influence plasmid expression. In previous studies carried out in our department in idiopathic pulmonary fibrosis (IPF) and bronchial asthma (Clarke

et al., 2010, John et al., 2010), our group showed that whilst promoter mutation experiments are effective for identifying the TFs involved in a target gene regulation, they can give misleading results when compared with ChIP assays in terms of alterations in disease-related binding of TFs.

In view of the discrepancy in our transfection study results, we performed ChIP assays to look at binding of TFs to the CXCL8 promoter in the chromatin environment. The latter is influenced by histone modifications and/or DNA methylation which alter DNA structure and TFs access. In contrast to our transfection studies, ChIP analysis showed higher NF-κB p65 binding basally but no difference in C/EBPß binding to the CXCL8 promoter in unstimulated CF cells compared to normal cells. This suggests that greater NF-κB p65 association with the CXCL8 promoter in CF cells may be responsible for the increased CXCL8 production in CF under basal conditions.

Our data are consistent with studies reporting greater levels of NF-κB, but not C/EBPß in the nucleus of unstimulated CF human bronchial epithelial cells as compared to normal cell lines (DiMango et al., 1998, Chan et al., 2006, Joseph et al., 2005). Other studies have reported increased basal NF-κB activity and higher nuclear localisation in CF nasal epithelial cells as compared to non-CF cells (Carrabino et al., 2006, Raia V et al., 2005). However, we did not find any increase in the binding levels of NF-κB in CF cells after induction with IL-1ß in contrast to studies that have identified greater levels of NF-κB in the nucleus following stimulation with IL-1β or PAO1 of *P. aeruginosa* in CF cell lines (DiMango et al., 1998, Joseph et al., 2005). The difference in findings could be a result of failure of

different cell lines to respond to the cytokine challenge due to impairment in the basal nuclear NF-κB activity and/or depleted ability of cells to synthetise CXCL8.

In contrast to NF-κB, there was no difference in binding of C/EBPβ to the CXCL8 promoter in CF cells compared to normal cells under basal and/or IL-1β-induced conditions. Other studies in human bronchial epithelial cells showed that C/EBPβ was not involved in basal regulation of CXCL8 transcription, yet, stimulation with IL-1β, TNF-α and/or *P. aeruginosa* resulted in the activation of C/EBPβ (DiMango et al., 1998, Hoffmann et al., 2002) suggesting an important role of this TF in CXCL8 regulation. No other studies have investigated the role of C/EBPß in the regulation of CXCL8 in CF.

We were unable to reliably detect AP-1 binding to the CXCL8 promoter and so cannot comment as to whether it is abnormal in CF cells. The inability to identify a single product during qPCR might be due to a difference in the primers covering different regions of the CXCL8 promoter. In future, it would be interesting to carry out a more detailed ChIP analysis of AP-1 binding to identify the presence of this protein at the CXCL8 promoter, its' role in the enhanced CXCL8 transcription and the interaction between AP-1 and other proteins in CF airway epithelial cells.

In summary, our data suggest that CF cells have increased CXCL8 protein and mRNA levels due to enhanced CXCL8 transcription involving increased binding of NF-κB. As the enhanced binding of this TF was observed in ChIP experiments which have the contribution of a chromatin environment, we hypothesised that other chromatin-based mechanisms such as histone acetylation and/or DNA methylation might

potentially play a key role in CXCL8 expression in CF. This is addressed in the next chapter.

6 NF-κB, HISTONE ACETYLATION/METHYLATION AND DNA METHYLATION AT THE CXCL8 PROMOTER IN CF AIRWAY EPITHELIAL CELLS

6.1 Introduction

The studies in chapter 2 and 3 suggest that enhanced CXCL8 expression in CF cells is caused by abnormal transcription through increased binding of NF-κB which is only observed in the chromatin environment. The possible mechanisms responsible for the chromatin-dependent increase in CXCL8 transcription including altered histone modifications and/or DNA methylation have not been identified and are the focus of this chapter.

Gene expression is influenced by several covalent modifications of histone proteins or DNA itself. Acetylation, methylation, phosphorylation, and ubiquitination of lysine (K) residues and/or core domains of histones can alter chromatin structure and/or binding of non-histone proteins to chromatin. Histone methylation can be a marker of both active and inactive chromatin (Plath et al., 2003, Lachner and Jenuwein, 2002). Di- and trimethylation of histone H3 at K4 is generally associated with transcriptional activation, whereas di- and trimethylation of histone H3 at K9 and K27 as well as trimethylation of histone H4 at K20 are linked to transcriptional repression (Rice et al., 2003).

Another histone modification, histone acetylation, is a hallmark of a transcriptionally active chromatin. Studies in different mammalian cells have demonstrated that actively transcribed chromatin has more acetylated sites on histones in comparison to inactive chromatin that is mostly hypoacetylated (Barnes et al., 2005, Tsaprouni et al., 2011). Studies in yeast and mammalian cells have demonstrated that K9 and K14 acetylation on histone H3 as well as pan-acetylation of histone H4 are conserved hallmarks of transcriptionally active promoters (Pokholok et al., 2005, Bernstein et al., 2005).

Increased histone acetylation enables access of transcription factors (TFs) to the DNA facilitating active transcription through structural changes in the nucleosomes (Lee et al., 1993, Adcock et al., 2006). Acetylation of K residues on histones directly influences gene transcription levels (Mizuguchi et al., 2001) serving as binding sites for special domains and co-activators through creation of a platform recognised by other proteins and, consequently, facilitating downstream signalling (Strahl and Allis, 2000). Histones are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs). HATs can also directly acetylate TFs to influence target gene transcription. There is some evidence that p65 NF-κB acetylation can regulate CXCL8 expression. Cigarette smoke exposure in macrophages and rat lungs results in p65 NF-κB acetylation and enhanced CXCL8 transcription (Yang et al., 2007). Studies have shown that NF-κB-mediated CXCL8 release is influenced by reversible acetylation of p65 at K310 (Chen et al., 2002, Yeung et al., 2004, Huang et al., 2009). Finally, DNA methylation also affects gene transcription. Studies in human cancer cells have shown that the presence of unmethylated CGI regions (CpG islands) initiates active gene transcription, while methylation of the same sites represses promoter activity (Herman and Baylin, 2003).

DNA methylation and histone modifications are tightly controlled events in eukaryotes and often interlinked (Cheng and Blumenthal, 2010). The potential mechanism involves binding of methyl-CpG-binding domain proteins to the methylated CpG islands at the target gene promoter with further recruitment of HDACs resulting in histone deacetylation and consequently leading to nucleosome

condensation and structural changes followed by inhibition of gene expression (Urnov and Wolffe, 2001).

There are few studies looking at the epigenetic modifications affecting the CXCL8 promoter in CF cells. A recent study has reported that increased acetylation of histone H4, caused by existing oxidative stress in CF, might be responsible for the dysregulated transcription of several inflammatory genes such as CXCL8, IL-6, CXCL1, CXCL2, and CXCL3 in airway epithelial cells (Bartling, 2009), although the exact mechanism was not probed in depth. To the best of our knowledge, there are no studies addressing the effect of histone methylation on CXCL8 expression in CF. Furthermore, there are no studies exploring the role of DNA methylation in the increased expression of CXCL8 in airway cells in CF, although a study in buccal epithelial cells has shown hypermethylation of the CXCL8 gene promoter in CF patients with periodontitis (Andia et al., 2010).

In summary, histone modifications, TF acetylation and DNA methylation can all modify gene transcription. In this chapter we studied whether any of these alterations are responsible for the increased CXCL8 transcription in CF epithelial cells.

6.2 Aims

The aims of this chapter were:

– to measure the H3K4 methylation status of the CXCL8 promoter in normal and CF airway epithelial cells basally and after IL-1β stimulation;

– to measure histone acetylation levels at the CXCL8 promoter in normal and CF airway epithelial cells basally and after IL-1β stimulation;

– to measure NF-κB acetylation levels at the CXCL8 promoter in normal and CF airway epithelial cells basally and after IL-1β stimulation;

– to measure the methylation status of CpG sites within the CXCL8 promoter in normal and CF cells basally and after IL-1β stimulation and, thereby, to determine whether altered DNA methylation contributes to aberrant CXCL8 transcription in CF cells.

6.3 Methods

Chromatin immunoprecipitation (ChIP)

To identify H3K4 methylation, histone H3 and H4 acetylation and NF-κB p65 K310 acetylation at the CXCL8 promoter, ChIP was performed as described in 3.7 of Chapter 3 Materials and Methods.

Bisulphite sequencing

To identify the methylation status of CpG sites within the CXCL8 promoter, bisulphite sequencing was performed as described in 3.9 of Chapter 3 Materials and Methods.

6.4 Results

6.4.1 Increased histone H3 lysine 4 trimethylation (H3K4me3) at the CXCL8 promoter in CF airway epithelial cells

Unstimulated normal cells had low basal levels of H3K4 trimethylation which was doubled following IL-1β stimulation. CF cells had higher basal levels of trimethylated H3K4 at the CXCL8 promoter compared to normal cell lines; no further increase was observed after IL-1β stimulation (Figure 6-1).

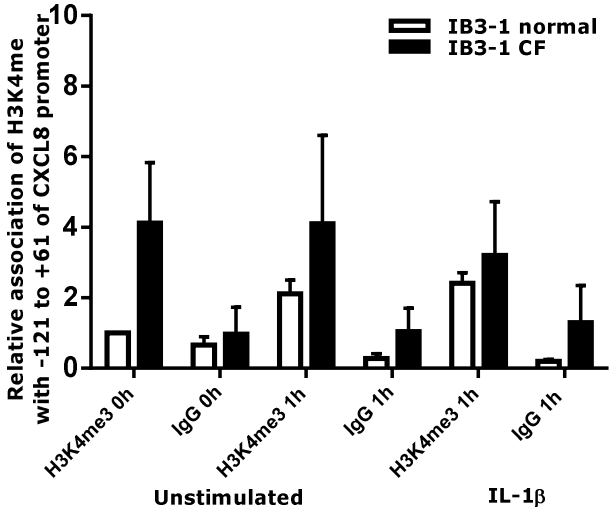


Figure 6-1. H3 lysine 4 trimethylation (H3K4me3) at the human CXCL8 promoter. Association of trimethylated lysine 4 on histone 3 (H3K4me3) was measured in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0 and 1 hour. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against H3K4me3 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

6.4.2 Histone acetylation at the CXCL8 promoter in CF airway epithelial cells

No difference between acetylated histone H3 and IgG negative control levels was identified in either normal or CF cell lines basally or following IL-1β stimulation (Figure 6-2).

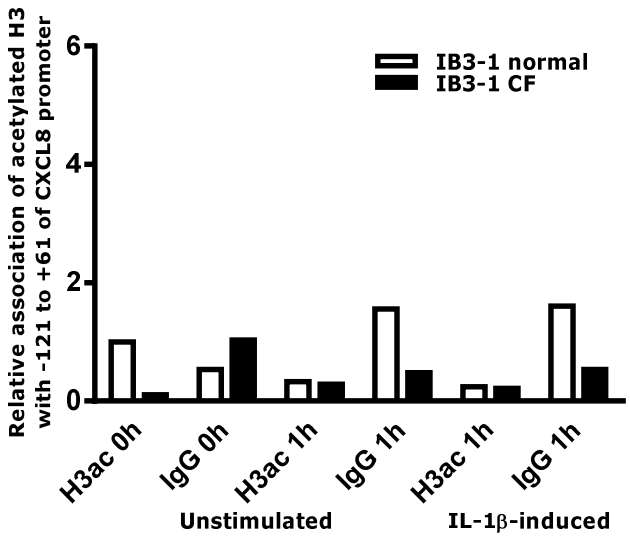


Figure 6-2. Histone H3 acetylation (H3ac) at the human CXCL8 promoter (a representative graph). Association of acetylated histone H3 with the CXCL8 promoter was determined in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0 and 1 hour. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against pan-acetylated H3 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. The graph is a representative of three independent experiments.

Normal cells showed a 56 fold increase in acetylated histone H4 association following IL-1ß induction, whilst there was no difference between acetylated histone H4 and IgG negative control levels at the CXCL8 promoter in CF cells both basally and after IL-1β stimulation (Figure 6-3).

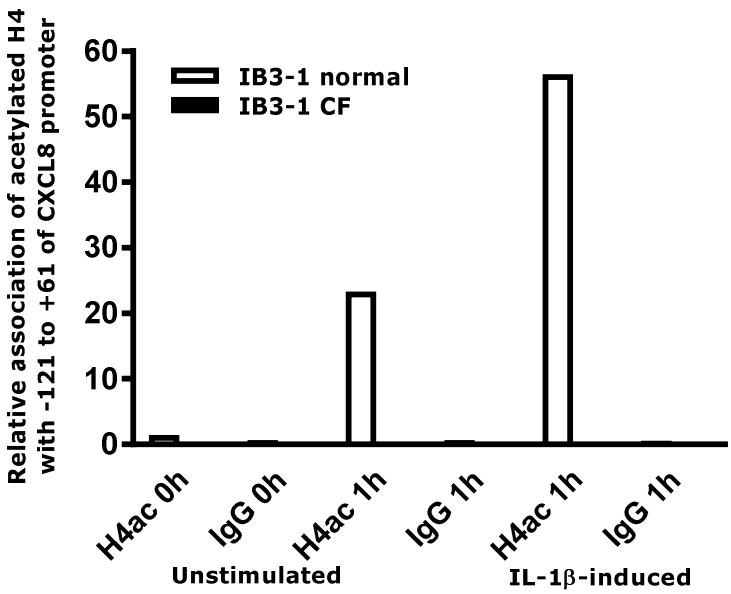


Figure 6-3. Histone H4 acetylation (H4ac) at the human CXCL8 promoter (a representative graph). Association of acetylated H4 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0 and 1 hour. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against pan-acetylated H4 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. The graph is a representative of three independent experiments.

Collectively, these findings suggest that neither increased histone H3 nor H4 acetylation at the CXCL8 promoter explain the differences in CXCL8 release between normal and CF cell lines.

6.4.3 NF-κB acetylation at the CXCL8 promoter in CF airway epithelial cells

Little p65 K310 acetylation was measured over IgG control in either normal or CF cells under basal or IL-1β stimulated conditions. No difference in basal NF-κB p65 K310 acetylation levels was observed between normal and CF cells. IL-1ß

stimulation did not result in any increase in NF-κB p65 K310 acetylation levels in either cell line (Figure 6-4).

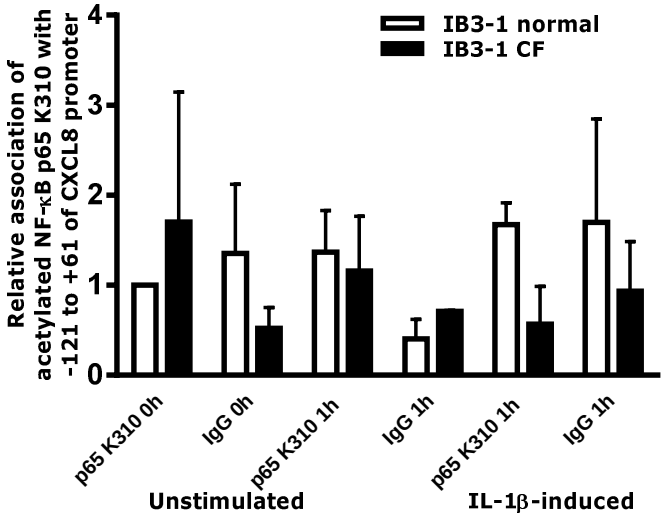


Figure 6-4. NF-κB p65 K310 acetylation (p65 K310) at the human CXCL8 promoter. Association of acetylated NF-κB p65 K310 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0 and 1 hour. Extracted chromatin was sheared and immunoprecipitated with 4μg of antibody against anti-NF-κB p65 acetyl-K310 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

These findings suggest that altered acetylation of NF-κB p65 at K310 is not responsible for the increased CXCL8 production in CF cells.

6.4.4 P300 binding to the CXCL8 promoter in CF airway epithelial cells

Although we failed to show either histone H3, H4 or NF-κB p65 K310 acetylation, we considered the fact that HATs can sometimes have transcriptional co-activator

properties that are independent of their HAT activities (Clarke et al., 2010). We looked at the recruitment and binding of HATs that potentially could be involved in the altered CXCL8 expression in CF cells. No binding of p300 above IgG control levels was obtained in either cell line under any condition (Figure 6-5).

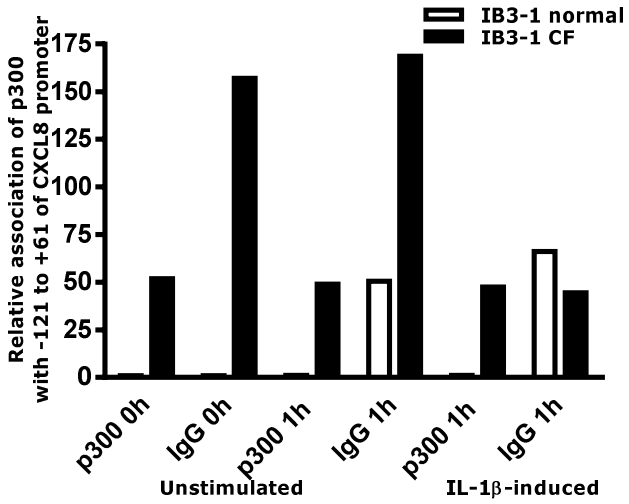


Figure 6-5. p300 binding to the human CXCL8 promoter (a representative graph). Association of p300 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0 and 1 hour. Extracted chromatin was sheared and immunoprecipitated with 1µg of antibody against p300 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. A representative graph of two independent experiments is shown.

This suggests that there was no increased p300 binding to the CXCL8 promoter that could explain the abnormal CXCL8 expression in CF cells.

6.4.5 DNA methylation at the CXCL8 promoter in CF airway epithelial cells

Finally, we examined methylation patterns of CpG sites within the CXCL8 promoter as well as global DNA methylation.

The CXCL8 promoter is not a classic "CpG island" promoter as it contains sparse CpG dinucleotide sequences. Although, the four CpG sites located within close proximity of the promoter do not form a distinctive "CpG island", they still represent potential targets for methylation. The CpG sites are situated within -136 to +43 region of the CXCL8 promoter, namely at -7, -83, -158 and -168; two more sites are positioned at -1241 and - 1311 upstream of the TATA box. This region contains sequences of four cis-elements of NF-κB (-82 to -70), C/EBP (-94 to -84), and AP-1 protein (-126 to –120) binding sites. The fourth cis-regulator element is activated by Oct-1 factor down regulating CXCL8 transcription and is located between -90 and -83. Location of the CpG sites near TATA and CCAAT boxes suggests that they might play a crucial role in transcription initiation (De Larco et al., 2003).

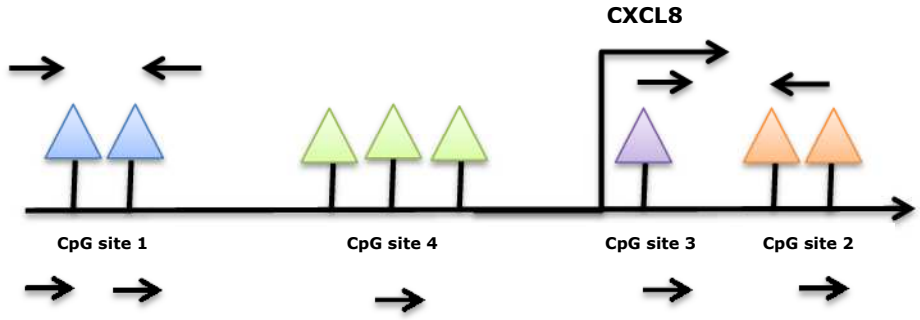


Figure 6-6. A schematic diagram of the CXCL8 gene showing the approximate location of CpG sites 1, 2, 3, and 4 within CXCL8 gene.

Considering the close proximity of the CpG sites and primer sequences designed by PyroMark Assay Design SW 2.0 software, CpG sites were clustered as follows: CpG site 1 containing CpG clusters 1.1 and 1.2 (blue triangles,

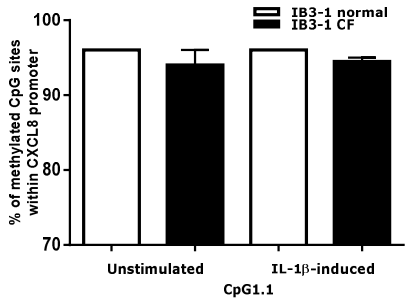
Figure **6-6**), CpG site 4 encompassing CpG clusters 3.1, CpG 3.2 and CpG 3.3 (green triangles,

Figure **6-6**) and CpG site 2 including CpG clusters 2.1 and 2.2 (orange triangles,

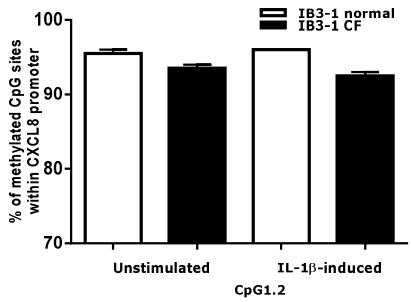
Figure **6-6**).

Data analysis showed that unstimulated normal cells were severely methylated at CpG clusters 1.1, 1.2 and less methylated at 2.1, 2.2, 2.3, 3.1, 3.2 and 3.3. Similarly, untreated CF cells were methylated at CpG sites 1.1, 1.2, 2.2, 3.3 and highly methylated at CpG site 3.1 with DNA methylation levels being slightly lower at CpG sites 1.1, 1.2, 3.3 and 3.1 as compared to normal cells. This suggests that altered methylation of CpG 1.1, 1.2, 2.2, 3.1 and 3.3 might contribute to the abnormal CXCL8 production in CF cells under basal conditions. IL-1ß stimulation resulted in a decrease in methylation levels of CpG clusters 2.2, 2.3 and 3.1 in normal cells and at CpG clusters 1.2, 2.3, 3.3 but not 3.1 in CF cells consistent with it activating transcription. Global DNA methylation (LINE-1 assay) was no different between normal and CF cells both under basal conditions and following IL-1β stimulation (Figure 6-7).

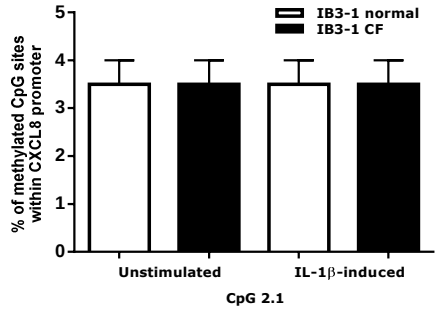
A


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
100
90
80
70
Unstimulated
IL-1β-induced
CpG1.1

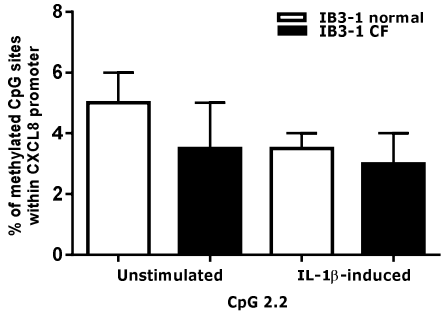
B


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
100
90
80
70
Unstimulated
IL-1β-induced
CpG1.2

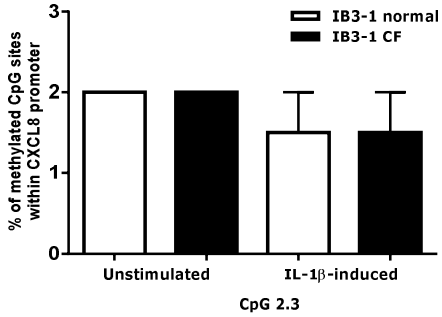
C


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
5
4
3
2
1
0
Unstimulated
IL-1β-induced
CpG 2.1

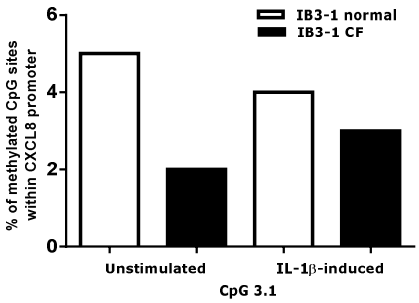
D


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
8
6
4
2
0
Unstimulated
IL-1β-induced
CpG 2.2

E


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
3
2
1
0
Unstimulated
IL-1β-induced
CpG 2.3

F


IB3-1 normal
IB3-1 CF
% of methylated CpG sites within CXCL8 promoter
6
4
2
0
Unstimulated
IL-1β-induced
CpG 3.1

G

H

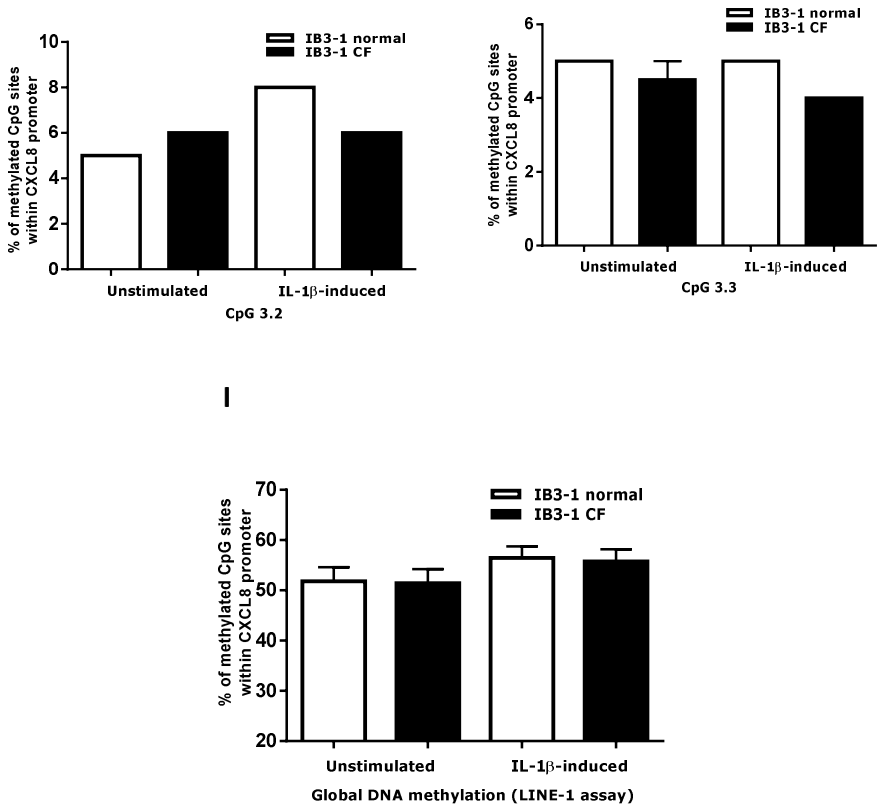


Figure 6-7. Methylation status of CpG sites within the CXCL8 promoter. Methylation levels of CpG sites 1 ***(A, B)***, 2 ***(C, D)***, 3 ***(E)***, and 4 ***(F, G, H)*** as well as global DNA methylation (LINE-1 assay) ***(I)*** in IB3-1 normal and IB3-1 CF cells were analysed. 2µg of genomic DNA was subjected to bisulphite conversion and purified DNA underwent PCR analysis using specific primers to amplify DNA regions containing CpG sites and to identify global DNA methylation pattern. PCR products were run on 2% agarose gel to identify the correct band size. Data are expressed as a percentage of methylated CpG sites; each column represents means ± SEM from at least 2 or 3 independent experiments.

Collectively, the DNA methylation data suggests that the CXCL8 promoter is slightly hypomethylated in CF cells in comparison to the normal cells.

6.5 Discussion

The major findings in this chapter are increased histone H3K4 trimethylation and hypomethylation of CpG site 3.1 under basal conditions at the CXCL8 promoter in CF cells compared to normal cells. IL-1ß stimulation did not result in any changes in

H3K4me3 levels but caused hypomethylation of CpG sites in both normal and CF cell lines. There was no difference in histone H3, H4 or NF-κB p65 K310 acetylation at the promoter either basally or following IL-1β stimulation.

Our study showed that CF cells had greater levels of trimethylated H3K4 under basal conditions as compared to normal cells. As IL-1β stimulation failed to induce any increase in H3K4me3 levels in CF cell line, it is less likely that H3K4me3 is responsible for altered IL-1β-induced CXCL8 expression in CF cells. However, the basal increase in trimethylated H3K4 together with the increased association of NF-κB p65 with the CXCL8 promoter reported in the previous chapter suggests the existence of a hyperactive transcription complex associated with the CXCL8 promoter basally in CF cells.

Previous work has shown a strong correlation between H3K4 trimethylation levels and gene transcription activity (Pokholok et al., 2005). This epigenetic modification can function as an initiating point for the increased recruitment of chromatin specific remodelling complexes/enzymes such as p300 HAT followed by further histone hyperacetylation and enhanced gene transcription (Li et al., 2007). There is a positive correlation between H3K4 trimethylation and transcriptional activity of NF-κB-induced genes (Saccani et al., 2001). Though this modification is well described in other cells, our study is the first to show altered H3K4me3 at the CXCL8 promoter in CF airway epithelial cells.

Several studies have shown increased levels of histone H3 and/or H4 acetylation alongside increased H3K4me3 levels at the promoters of transcriptionally active NF-κB-regulated genes (Saccani et al., 2001, Natoli, 2009). In contrast, we showed no

difference in histone H3 or H4 acetylation levels at the CXCL8 promoter between normal and CF cells either basally or after IL-1β stimulation. One previous study measured the acetylation status of the CXCL8 promoter in CF epithelial cells and in contrast to our observations reported increased histone H4 acetylation at NF-κB binding sites at the CXCL8 promoter (Bartling, 2009). The disagreement between our findings and results reported by Bartling *et al* could potentially be due to the difference in the cell lines used and variance in their properties. However, only one out of three CF cell lines and corresponding controls used in Bartling's study showed increased histone H4 acetylation at the CXCL8 promoter. Variations in the experimental design might also be an influential factor: cells were stimulated with a combination of potent pro-inflammatory cytokines (1ng/mL TNFα and 0.5ng/mL IL-1β) for 2 hours versus a single stimulus (1ng/mL IL-1β) and a different time point used in our study (1 hour). Furthermore, the inconsistency in the results might be partly explained by a greater CXCL8 promoter coverage of the PCR primers used in the study conducted by Bartling as compared to the ones used in our study. Finally, the presence of other specific epigenetic modifications involved in the control of CXCL8 expression, that either of the studies investigated, might possibly influence the CXCL8 release from CF cells.

We found no difference in the acetylation levels of the p65 subunit of NF-κB at K310 between normal and CF cells. Thus, NF-κB p65 K310 acetylation is not responsible for the increased CXCL8 expression in CF cells. Likewise, we found no increase in the recruitment of p300 to the CXCL8 promoter suggesting that increased recruitment of this HAT is not implicated in CF epithelial cells. In future, it

would be interesting to determine any changes in the levels of other HATs recruited to the CXCL8 promoter in CF epithelial cells.

Lastly, DNA methylation showed hypomethylation of CpG site 3.1 at the CXCL8 promoter under basal conditions, suggesting that this may contribute to the increased basal transcription in CF cells. There was a further reduction in methylation levels of several CpG sites after stimulation with IL-1ß which was greater in CF cells, suggesting that this might contribute to IL-1ß-induced activation of CXCL8. There was no difference in global DNA methylation levels between normal and CF cells, suggesting, that any changes are likely to be promoter-specific. This is the first time that the methylation status of CpG sites within the CXCL8 promoter has been described in CF cells.

Almost 50% of all CpG sites within the genome overlap transcription initiation sites and commonly lack DNA methylation (Clifford et al., 2013, Illingworth et al., 2010). DNA methylation in the regulatory region results in gene silencing via prevention of TFs access to the gene promoter (Deng et al., 2001). Recent genome-scale analysis has shown that DNA methylation also negatively correlates with H3K4 methylation levels at the target gene promoter (Cheng and Blumenthal, 2010, Laurent et al., 2010). These findings are consistent with our findings of both H3K4 hypermethylation and DNA hypomethylation under basal conditions at the CXCL8 promoter in CF cells.

To conclude, the results in this chapter showed increased H3K4 trimethylation in CF cells under basal conditions, but no alteration in histone H3, H4 or NF-κB acetylation or p300 recruitment. Furthermore, there was hypomethylation of CpG

sites at the CXCL8 promoter in CF cells compared to normal cell lines that were further hypomethylated by IL-1ß. Collectively, these abnormalities are likely to contribute to the altered CXCL8 transcription in CF epithelial cells. The next chapter will focus on identifying potential co-factors recruited to the CXCL8 promoter in CF cells as well as studying drugs which modify epigenetic signatures of CXCL8 secretion.

7 BET PROTEIN INHIBITORS ABOLISH CXCL8 EXPRESSION IN CF AIRWAY EPITHELIAL CELLS

7.1 Introduction

In Chapter 6 we showed that enhanced CXCL8 release from CF airway epithelial cells might be due to increased H3K4 trimethylation and hypomethylation of CpG site 3.1 at the CXCL8 promoter further hypomethylated following IL-1ß stimulation. This chapter will aim to identify co-factors recruited to the CXCL8 promoter as well as to study compounds influencing CXCL8 secretion in CF airway epithelial cells.

Epigenetic alterations such as DNA methylation and/or histone modifications affect gene expression not only through a direct effect on protein-protein interactions, but via recruitment of regulatory molecules targeting gene transcription. Recently, bromodomain and extra-terminal (BET) proteins (BRD2, 3, 4 and BRD-T) have been shown to play an important role in gene activation. Studies in animal models have shown that deletion of either BRD2 or BRD4 in mice is lethal: BRD4-deficient mice develop severe developmental defects (Houzelstein et al., 2002, Shang et al., 2009). BRD2 and BRD3 are associated with increased acetylation of histone H3 at K14 and histone H4 at K5 and K12 respectively. These BET proteins have been reported to activate RNA Pol II-driven transcription of target genes (LeRoy et al., 2008). BRD3 has been implicated in the activation and interaction with acetylated TF GATA1 targeting it to chromatin (Gamsjaeger et al., 2011). BRD4 has been involved in basal NF-κB activation in cancer suggesting an interaction between acetylated NF-κB and BRD4 at the promoter of target genes (Zou et al., 2014).

Bromodomains (BRDs) are also present in some chromatin-remodelling nuclear proteins such as HATs (Nagy and Tora, 2007a), methyltransferases (Malik and Bhaumik, 2010) and transcriptional activators (Brès et al., 2008). Studies in LPS- or IL-1ß-stimulated macrophages (Nicodeme et al., 2010, Hargreaves et al., 2009) and

different cancer cell lines (Filippakopoulos et al., 2010) have reported BRD2 and BRD4 to control target genes via recruitment of protein interaction and activator molecules including positive transcription elongation factor complex (P-TEFb) (Muller et al., 2011a, Nicodeme et al., 2010).

Recently, BET protein inhibitors have been shown to have a potential role in the treatment of several cancers. Treatment of midline carcinoma cells with selective BET protein inhibitors JQ1 or PFI-1 resulted in substantial cell growth arrest and apoptosis alongside with substantial reduction in tumour growth (Barbieri I et al., 2013). Treatment with JQ1 resulted in a reduced cell viability and osteoblastic differentiation via transcriptional silencing of MYC and RUNX2 genes in both *in vitro* and *in vivo* models (Lamoureux et al., 2014). Another inhibitor, I-BET-151, was effective in pre-clinical models of multiple myeloma (Chaidos et al., 2014). I-BET762 is being evaluated in a phase I clinical trial for treatment of human testis midline carcinoma (Zhao et al., 2013).

BET proteins may also be a target in inflammatory diseases, although this has been studied to a lesser extent. I-BET-151 inhibits transcription of NF-κB-mediated inflammatory genes and reduces production of IL-6 and CXCL8 via downregulation of NF-κB p105/p50 interaction (Gallagher et al., 2014). Similar results have been observed in mouse macrophages following treatment with JQ1. Furthermore, JQ1 and PFI-1 inhibit NF-κB-driven transcription of IL-6 and CXCL8 cytokines in primary and immortalised airway epithelial cells. BRD4 deficiency and/or treatment with JQ1 results in the ubiquitination and degradation of the active nuclear form of NF-

κB p65 (Khan et al., 2014), suggesting, that BET proteins play an important role in the transcription of individual NF-κB-mediated genes (Belkina et al., 2013b).

As the role of BET protein inhibitors in regulating CXCL8 in CF airway epithelial cells is unknown, the present chapter examines this.

7.2 Aims

The aims of this chapter were:

- to determine the effect of BET protein inhibitors on CXCL8 release from normal and CF airway epithelial cells basally and after IL-1ß stimulation;
- to measure binding of BET proteins to the CXCL8 promoter in normal and CF airway epithelial cells basally and after IL-1ß stimulation;
- to study the interaction between BET proteins and TFs at the CXCL8 promoter in normal and CF airway epithelial cells basally and after IL-1β stimulation.

7.3 Methods

Inhibitor studies

To identify the effect of BET protein inhibitors on CXCL8 protein secretion in normal and CF cells, confluent and growth arrested cells were pre-treated with 10^{-5}, 10^{-6}, 10^{-7}M of PFI-1, 10^{-6}, 10^{-7}, 10^{-8}M of JQ1 or DMSO used as a vehicle control for 30 min and stimulated with 1ng/mL IL-1ß for 24 hours. Supernatants were collected and assayed for CXCL8 by ELISA as described in 3.2 of Chapter 3 Materials and Methods.

Cell viability (MTT) assay

To measure the toxicity of drug compounds used during the study, cell viability assay was performed as described in 3.6 of Chapter 3 Materials and Methods.

Chromatin immunoprecipitation (ChIP)

To measure binding of BRD2, BRD3 and BRD4 to the CXCL8 promoter and the effect of BET protein inhibitors on their binding, ChIP assay was performed as described in 3.7 of Chapter 3 Materials and Methods.

Nuclear Co-Immunoprecipitation (Co-IP)

To study the interaction between NF-κB p65 and BRD4, co-immunoprecipitation (Co-IP) assay followed by western blotting analysis was performed as described in 3.8 of Chapter 3 Materials and Methods.

7.4 Results

7.4.1 BET protein inhibitors PFI-1 and JQ1 reduce CXCL8 protein release from normal and CF airway epithelial cells

Both PFI-1 and JQ1 reduced basal CXCL8 and IL-1β-induced CXCL8 production in both normal and CF cells in a concentration dependent manner (Figure 7-8).

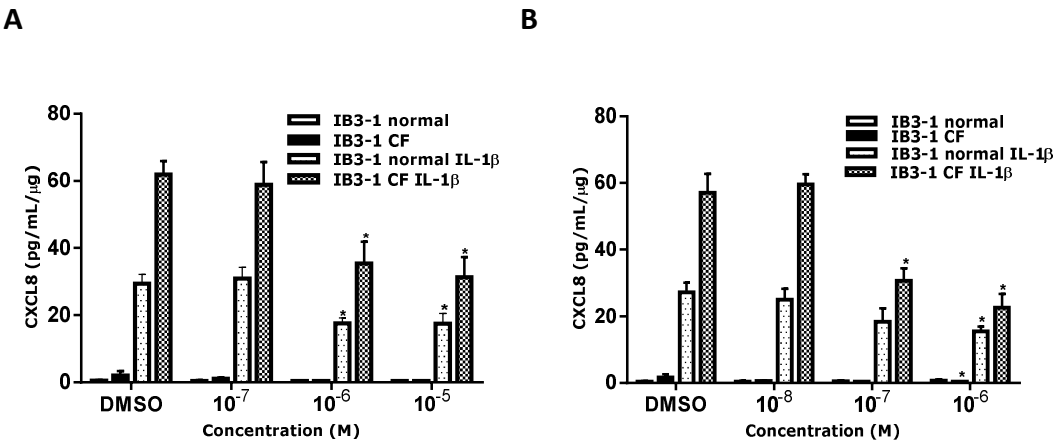


Figure 7-8. The effect of BET protein inhibitors, PFI-1 and JQ1, on CXCL8 protein release from normal and CF cells. IB3-1 normal and IB3-1 CF cells, confluent and growth arrested 24 hours prior to an experiment, were pre-incubated for 30 min with stated concentrations of PFI-1 ***(A)*** and (+)/- JQ1 ***(B)*** followed by stimulation with or without 1ng/mL IL-1β. Supernatants were assayed for CXCL8 protein by ELISA; the raw data were normalised against total cellular protein. Each bar represents means ± SEM from triplicate determinants of at least 3 independent experiments. Student t-test was used for the analysis, *p value <0.05 compared to corresponding cells without PFI-1 or JQ1 treatment was considered as significant.

No toxicity was observed under any experimental condition (see 9.1 of Appendix for the cytotoxicity data). As both compounds had similar effects, we used JQ1 in further experiments.

Our results suggest that BET proteins and/or BRD containing regulatory proteins might be involved in the CXCL8 dysregulation in CF.

7.4.2 Increased binding of BRD3 and BRD4 to the CXCL8 promoter in CF airway epithelial cells

Next, binding levels of BET proteins to the CXCL8 promoter were determined by ChIP.

There was no difference in basal BRD2 binding to the CXCL8 promoter between normal and CF cells. Stimulation with IL-1ß resulted in a slight or no increase in BRD2 binding levels in both cell lines (Figure 7-9, ***A, B***). BRD3 binding levels were higher in unstimulated CF cells under basal conditions compared to normal cells (nearly two and half fold difference). IL-1β stimulation did not alter the binding levels (Figure 7-9, ***C, D***). There was a two fold increase in basal BRD4 binding to the CXCL8 promoter in unstimulated CF cells compared to normal cells. IL-1β stimulation resulted in a fourfold increase in bound BRD4 levels after 2 hours upon IL-1ß stimulation (Figure 7-9, ***E, F***).

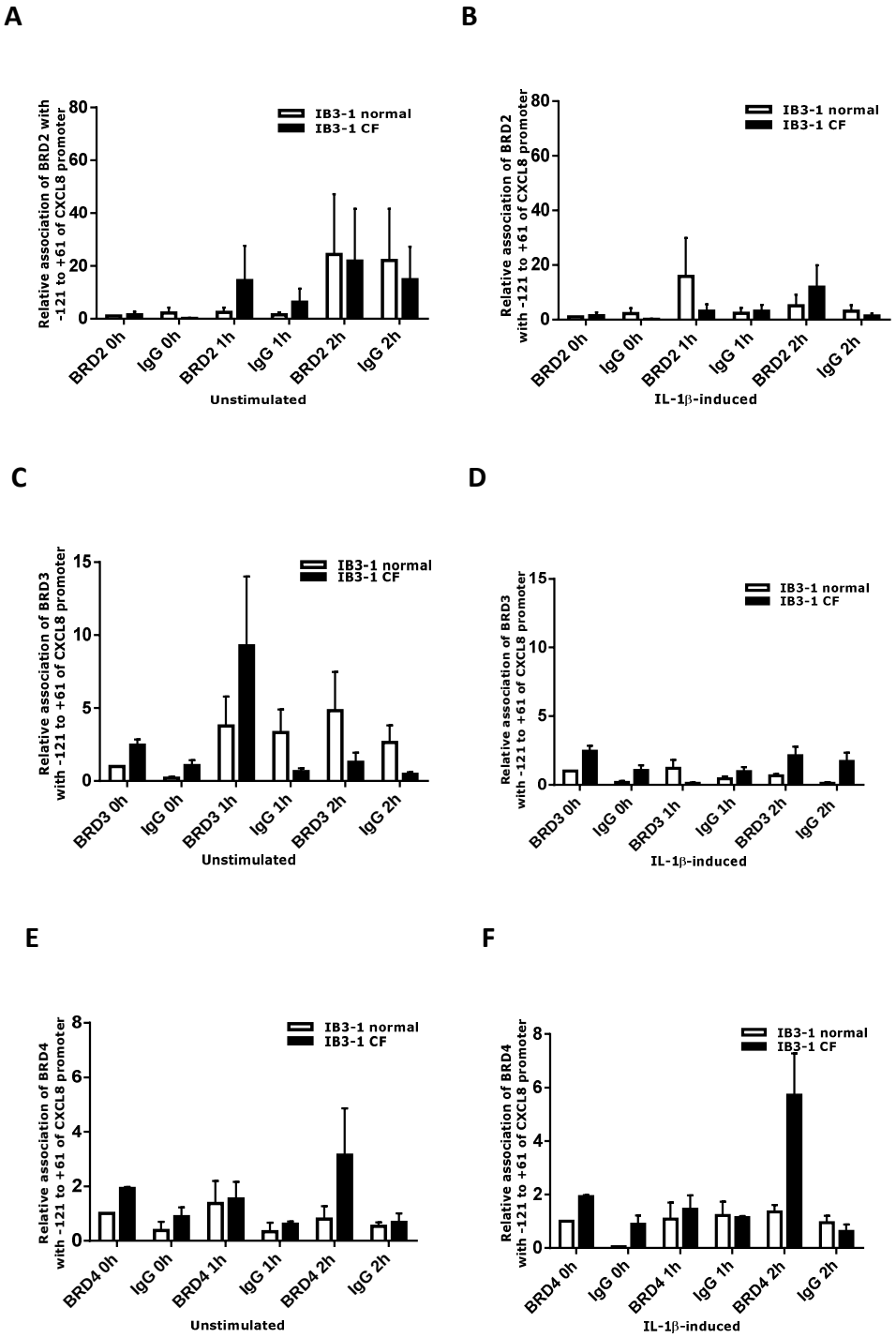


Figure 7-9. Association of BRD2, BRD3, and BRD4 with the human CXCL8 promoter in normal and CF airway epithelial cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells, serum starved 24 hour prior to an experiment, were incubated without ***(A, C, E)*** or with 1ng/mL IL-1ß ***(B, D, F)*** for 0, 1 and 2 hour. Extracted chromatin was sheared and immunoprecipitated with 3µg of BRD2 ***(A, B)***, BRD3 ***(C, D)*** and BRD4 ***(E, F)*** or IgG used as a negative control. Isolated DNA underwent qPCR analysis with the CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

Collectively, these results suggest that BRD3 and BRD4 are both important for basal CXCL8 expression in CF, but BRD4 is involved in IL-1β-induced CXCL8 secretion in CF cells. Thus, further studies in this chapter were concentrated on BRD4.

7.4.3 The effect of JQ1 on NF-κB recruitment to the CXCL8 promoter in CF airway epithelial cells

The effect of JQ1 on NF-κB p65 binding to the CXCL8 promoter in normal and CF cells was determined by ChIP.

There was modest binding of NF-κB p65 to the CXCL8 promoter in unstimulated normal cells with no reduction following treatment with JQ1. IL-1β stimulation increased NF-κB p65 binding at 2 hours, but JQ1 did not cause any decrease in binding levels (Figure 7-10). JQ1 resulted in reduced NF-κB p65 levels in unstimulated CF cells at 1 hour. Stimulation with IL-1ß increased NF-κB p65 binding at 2 hours, but JQ1 did not reduce the binding levels (Figure 7-10).

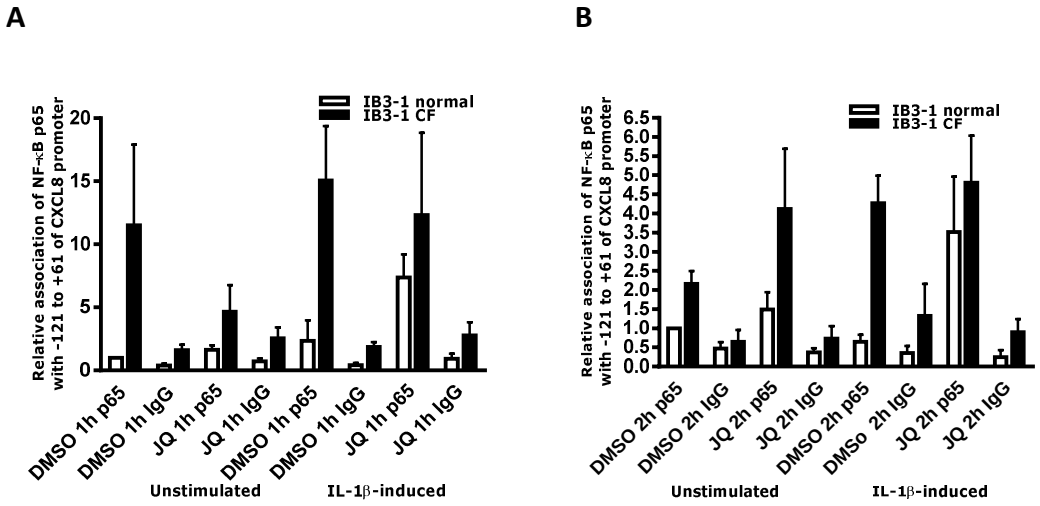


Figure 7-10. The effect of JQ1 on NF-κB p65 recruitment to the human CXCL8 promoter in normal and CF cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF airway epithelial cells were serum starved 24 hour prior to an experiment, pre-incubated with 10^{-6} M of JQ1 for 30 min and incubated with/without 1ng/mL IL-1ß for 1 ***(A)*** and 2 ***(B)*** hours. Extracted chromatin was sheared and immunoprecipitated with 3µg of NF-κB p65 or IgG used as a negative control. Isolated DNA underwent qPCR analysis with the CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. Each column represents means ± SEM from at least four independent experiments.

These results did not identify any major effect of JQ1 on NF-κB p65 association with the CXCL8 promoter in CF cells.

7.4.4 The effect of TPCA-1 on BRD4 recruitment to the CXCL8 promoter in CF airway epithelial cells

To determine the effect of TPCA-1, a selective IKK2 inhibitor, on the BRD4 binding to the CXCL8 promoter, ChIP assays were performed.

There was insignificant binding of BRD4 to the CXCL8 promoter in unstimulated normal cells with no reduction in cells following TPCA-1 treatment. IL-1ß stimulation increased BRD4 binding at 2 hours, but TPCA-1 did not have any effect on binding levels (Figure 7-11). TPCA-1 reduced bound BRD4 levels in unstimulated

CF cells at 1 hour. IL-1β stimulation led to an increase in the binding levels of BRD4 at 2 hours, and TPCA-1 reduced binding levels of BRD4 to the CXCL8 promoter at 1 hour (Figure 7-11).

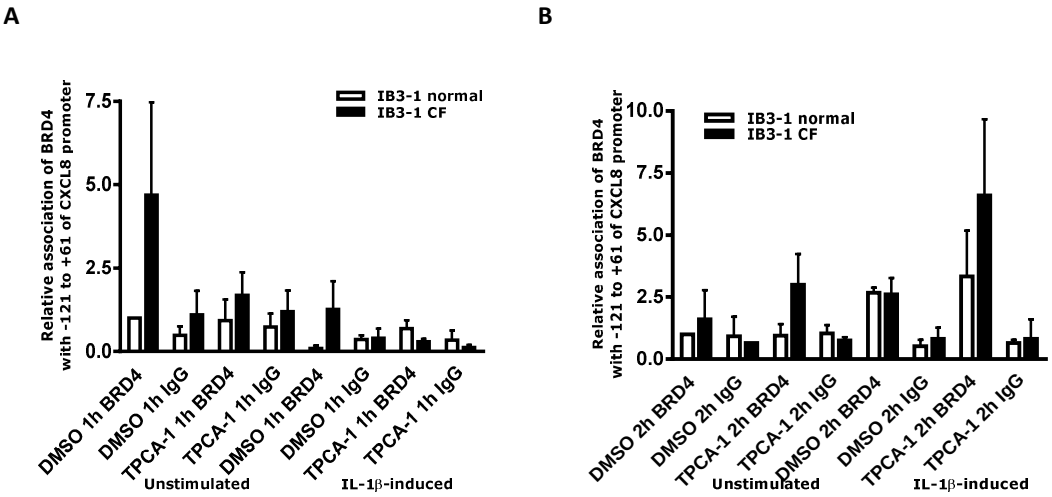


Figure 7-11. The effect of TPCA-1 on BRD4 recruitment to the human CXCL8 promoter in normal and CF cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells were serum starved 24 hour prior to an experiment, pre-incubated with 10^{-5}M of TPCA-1 for 30 min and then incubated with/without 1ng/mL IL-1ß for 1 ***(A)*** and 2 ***(B)*** hours. Extracted chromatin was sheared and immunoprecipitated with 3µg of BRD4 or IgG used as a negative control. Isolated DNA underwent qPCR analysis with the CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA. Each column represents means ± SEM from at least 3 independent experiments.

Collectively, our findings showed an effect of TPCA-1 on BRD4 association with CXCL8 promoter in CF cells both basally and following IL-1β stimulation suggesting an interaction between BRD4 and NF-κB.

7.4.5 Direct protein interaction between NF-κB p65 and BRD4 in CF airway epithelial cells

To determine the presence of a protein complex containing BRD4 and NF-κB p65, we performed co-immunoprecipitation (Co-IP) assays on nuclear extracts of

unstimulated and IL-1β-stimulated IB3-1 normal and IB3-1 CF cells (0, 1 and 2 hours).

Initial immunoprecipitation was performed with an anti-NF-κB p65 antibody followed by immunoblotting with BRD4 (Figure 7-12). We did not identify any difference between bands of Co-IP and IgG samples at the expected molecular weight for BRD4. No protein was present in the loading samples in both cell lines either basally or following IL-1ß stimulation.

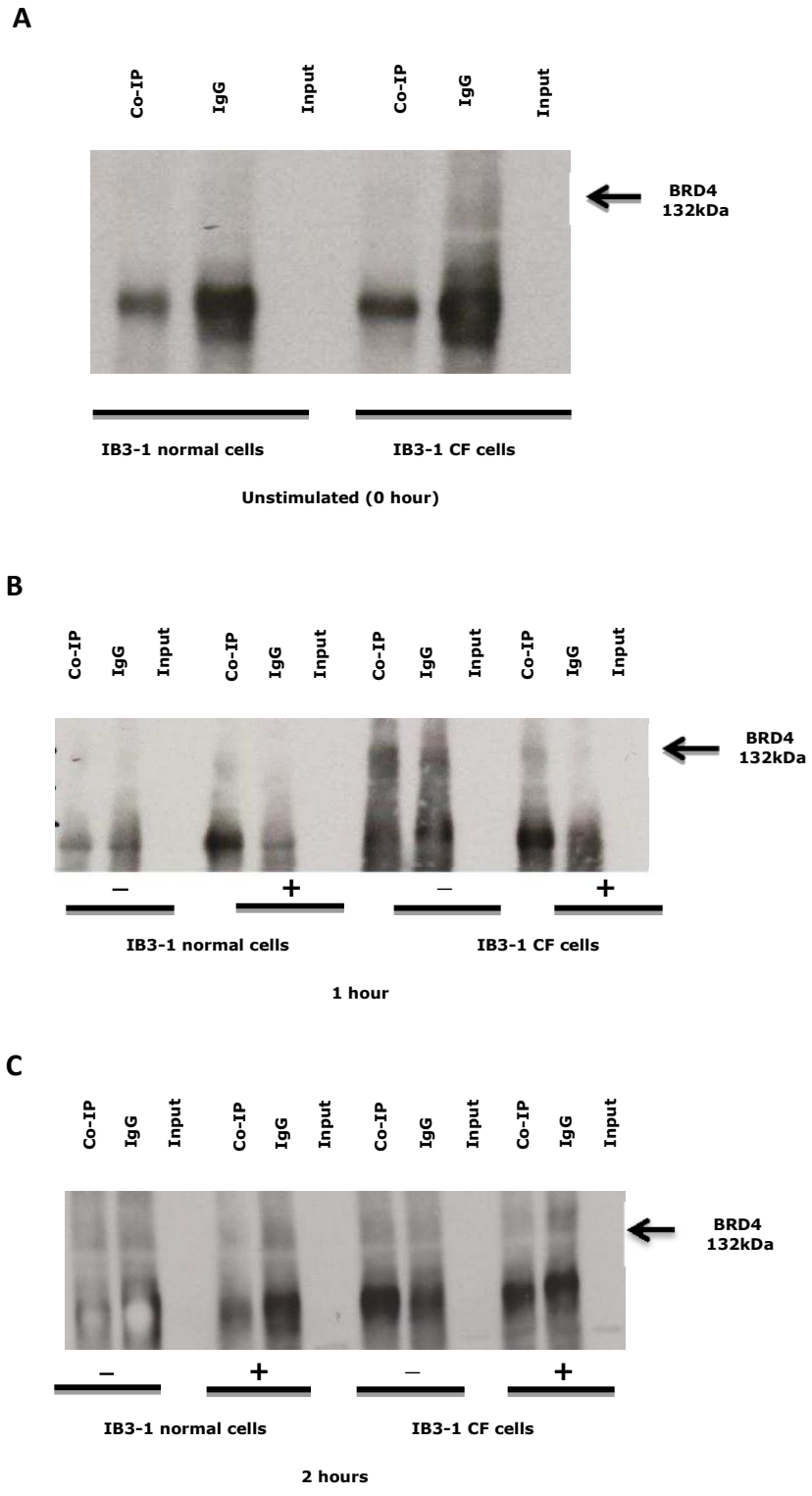


Figure 7-12. The effect of IL-1ß on association of NF-κB p65 and BRD4 in normal and CF cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells were serum starved 24 hour prior to an experiment and incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Nuclear extracts were co-immunoprecipitated with magnetic IgG beads in the presence of NF-κB p65 or IgG control antibody. 10µg of total cell protein prior to co-immunoprecipitation was loaded and used as a loading control (Input). The complexes were then separated by SDS-PAGE and immunoblotted for BRD4. The figure shown is a representative image of two independent experiments.

To further investigate whether a nuclear NF-κB p65 association with BRD4 was present, the Co-IP assay was repeated in reverse: nuclear extract protein was co-immunoprecipitated with BRD4 and immunoblotted with an anti-NF-κB p65 antibody.

We did not observe any obvious difference between bands of Co-IP and IgG samples at the expected molecular weight for NF-κB p65 in both normal and CF cells. Although NF-κB p65 protein was present in samples prior to immunoprecipitation, however, loading was not equal between the samples (see Input protein bands, Figure 7-13).

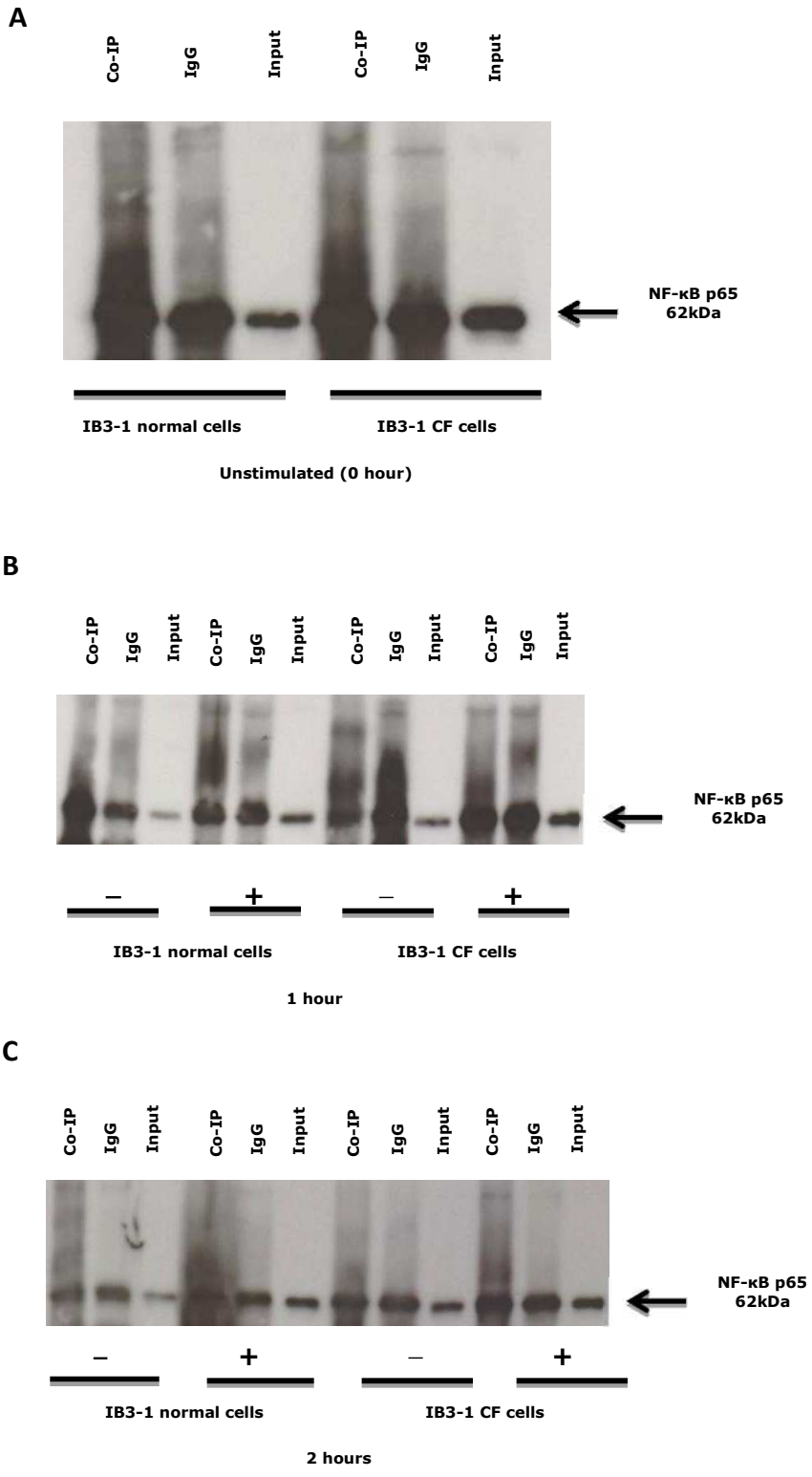


Figure 7-13. The effect of IL-1ß on association of NF-κB p65 and BRD4 in normal and CF cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells were serum starved 24 hour prior to an experiment and incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Nuclear extracts were co-immunoprecipitated with magnetic IgG beads in the presence of BRD4 or IgG control antibody. 10µg of total cell protein prior to co-immunoprecipitation was loaded and used as a loading control. The complexes were then separated by SDS-PAGE and immunoblotted for NF-κB p65. The blot shown is a representative image of one experiment.

Due to technical difficulties, at this stage we cannot conclusively state whether a protein complex containing associated NF-κB 65 and BRD4 proteins exists under basal or IL-1ß-induced conditions in normal or CF cells that could explain increased CXCL8 transcription.

7.5 Discussion

The main findings of this chapter are that BET protein inhibitors JQ1 and PFI-1 reduce CXCL8 protein release from CF airway epithelial cells both basally and following IL-1ß stimulation. We also identified increased binding of BRD4 and BRD3 to the CXCL8 promoter under basal and IL-1β-induced conditions in CF cells.

We found, that BET protein inhibitors, JQ1 and PFI-1, reduced basal and IL-1ß-induced CXCL8 release from normal and CF cells in a concentration dependent manner. No previous studies have explored the effect of BET protein inhibitors on CXCL8 secretion in CF airway epithelial cells, although our findings are similar to a recent study showing that JQ1 and PFI-1 inhibit CXCL8 production in BEAS-2B cells (Khan et al., 2014). Likewise, treatment of bone marrow-derived macrophages with I-BET-151 following LPS stimulation results in the downregulation of several pro-inflammatory cytokines such as IL-6, IL-1β, and IFN-γ (Nicodeme et al., 2010). Similar observations have been made in cancer: application of JQ1 in glioblastoma cells of different backgrounds leads to significant cell growth arrest and apoptosis due to displacement of individual BET proteins from their targets resulting in altered gene expression of p21CIP1/WAF1, hTERT, Bcl-2 and Bcl-xL (Cheng et al., 2013).

Subsequently, we performed ChIP and showed that BRD3 and BRD4, but not BRD2 are important for basal CXCL8 expression, but it is only BRD4 that is involved in IL-1β-induced CXCL8 expression CF cells. Taken alongside the inhibitor studies, our data suggest that BRD4 plays an essential role in CXCL8 transcription in CF airway epithelial cells. Similar observations have been made in BEAS-2B cells where knockdown of BRD4, but not BRD2, resulted in inhibition of IL-1β/H_2O_2-induced secretion of CXCL8 (Khan et al., 2014).

The studies in Chapter 5 showed increased basal binding of NF-κB p65 to the CXCL8 promoter in CF cells, thus, we looked at the interaction between BRD4 and NF-κB at the CXCL8 promoter using selective inhibitors of each protein and ChIP. We found that selective inhibition of NF-κB p65 did not affect BRD4 binding under basal or IL-1β-induced conditions in CF cells. However, treatment with JQ1 reduced NF-κB p65 binding to the CXCL8 promoter basally and following IL-1β stimulation in CF cells. This suggests that both proteins are present at the promoter complex, and inhibition of one of them alters the binding of the other one. Although our results showed a reduction in binding levels of NF-κB p65 in CF cells, there was a variability observed throughout all experiments. This could also suggest that the effect of BET protein inhibitors on CXCL8 secretion is not mediated by alteration of NF-κB properties, but by an effect on BRDs.

Next, to decisively confirm our data, we went to look at a complex formation between NF-κB p65 and BRD4 by performing Co-IP. However, due to technical problems we were unable to confirm a direct association between BRD4 and NF-κB p65 in nuclear protein extracts in unstimulated and IL-1β-induced CF cells. Our

results differ from those reported by Khan *et al* in BEAS-2B cells showing that IL-1β increases recruitment of NF-κB and BRD4 to the CXCL8 promoter in airway epithelial cells (Khan et al., 2014).

We have considered reasons that could explain the contradiction between our results and Khan's study findings. Technically challenging, Co-IP is not designed to determine weak and transient interactions such as IL-1ß-induced association between NF-κB p65 and BRD4. Another major limitation is that antibodies used for the Co-IP can cross-react with other nuclear proteins affecting the results. Although we started to optimise the Co-IP method, the results might be influenced by low levels of immunoprecipitated protein in samples. Another disadvantage of this technique is that although it shows the interaction between proteins within the cell, it does not allow speculations about where the interaction occurs and whether it is direct or not. In our studies we used polyclonal rabbit IgG as a negative control. Presence of both heavy and light chains increases the cross-reactivity with other immunoglobulins and antigens of similar weight present in the sample. Using monoclonal light chain of native IgG antibody possessing higher purity and better specificity in future experiments could significantly improve detection of protein-protein interaction by Co-IP. However, data in previous chapters as well as findings reported in the current chapter suggest that both BRD4 and NF-κB p65 are required for transcriptional activation of CXCL8 in CF airway epithelial cells.

In summary, the results in this chapter suggest that BRD4 is involved in the increased CXCL8 transcription in CF epithelial cells and targetting BET proteins may have therapeutic potential in CF.

8 GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

The main findings of this thesis are that there is increased recruitment of BRD3 and BRD4 associated with enhanced binding of NF-κB to the CXCL8 promoter due to increased H3K4me3 and DNA hypomethylation in CF cells basally. IL-1β stimulation leads to a further increase in BRD4 binding and DNA hypomethylation resulting in the formation of an active complex responsible for the upregulation of CXCL8 transcription in CF airway epithelial cells.

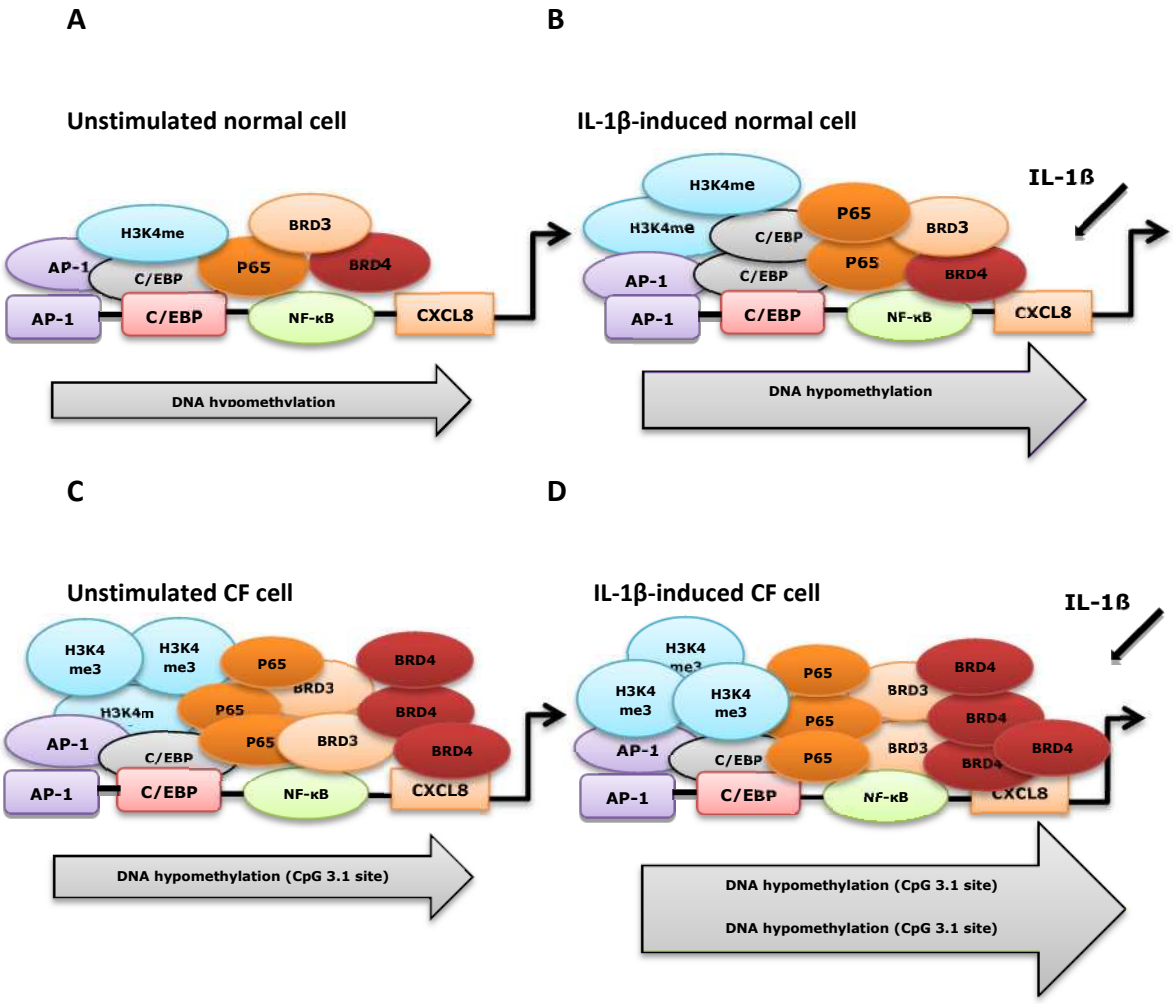


Figure 8-14. Overview of a proposed molecular mechanism of basal CXCL8 transcription in normal and CF airway epithelial cells. NF-κB, AP-1 and C/EBPß are involved in the CXCL8 transcription in both normal and CF cells. An active transcription complex associated with the normal CXCL8 promoter contains low levels of NF-κB p65, BRD3 and BRD4 accompanied by insignificant levels of H3K4 trimethylation and DNA methylation basally ***(A)***. IL-1β stimulation results in an increased binding of C/EBPβ, NF-κB and further H3K4 trimethylation and DNA hypomethylation of the CXCL8 promoter ***(B)***. In contrast, the CXCL8 promoter in CF cells contains a transcriptionally active complex containing increased levels of NF-κB p65, BRD3 and BRD4 along with increased

H3K4me3 and DNA hypomethylation at CpG site 3.1 ***(C)***. Stimulation with IL-1β results in an increased binding of BRD4 and further DNA hypomethylation of the CXCL8 promoter in CF cells ***(D)***.

The initial work in this thesis was largely confirmatory to make sure that we were able to see the same increase in CXCL8 in CF that others had described and to study the TFs regulating CXCL8 expression in our cells. We confirmed that CF cells expressed higher levels of CXCL8 than normal cells and that the TFs regulating CXCL8 release under both basal and IL-1beta stimulated conditions namely, NF-κB, AP-1 and C/EBP, were similar to those reported in a number of other studies in a range of cell types (Verhaeghe et al., 2007b, Li et al., 2002, Hoffmann et al., 2002, John et al., 2009).

We used ChIP assays to analyse any alteration in the binding of TFs to the CXCL8 promoter in CF cells in the chromatin environment and found enhanced binding of NF-κB p65, but not C/EBPβ to the CXCL8 promoter in CF cells under basal conditions that was not further increased following IL-1β stimulation. Although our transfection studies showed a role for AP-1 in CXCL8 transcription, we failed to detect c-jun binding to the CXCL8 promoter either in normal or CF cells. In future, it would be interesting to further explore the role of AP-1 in basal and IL-1β-induced transcription of CXCL8 possibly by designing region-specific primers and further optimising the ChIP technique used. Future studies could also look at the involvement and interaction between other TFs at the CXCL8 promoter as well as identification of other signalling pathways involved.

We found an increase in basal H3K4 trimethylation that was not further increased by IL-1β stimulation. We also did not identify any alteration in histone H3 or H4 acetylation at the CXCL8 promoter in CF airway epithelial cells. It would be interesting in future studies to look at other histone modifications such as acetylation of specific lysines including H3K14, H3K18 and H4K5 (Heintzman et al., 2007, Barrera and Ren, 2006, Strahl and Allis, 2000) and other methylation sites such as H3K9me1, H3K27me1, H4K20me1, H3K4me1,2 and H3K36me3. An interesting area for further research might also be to explore the presence and effect of other post-translational modifications, such as phosphorylation, ubiquitination and sumoylation, on the transcriptional regulation of CXCL8 in CF.

We did not study the enzymes responsible for regulating H3K4 trimethylation in our studies and identification of the methyltransferases and demethylases regulating this mark at the CXCL8 promoter might potentially provide novel molecular targets for future theraputic strategies.

In our experiments we showed no alteration in the recruitment of p300 HAT to the CXCL8 promoter. However, we did not investigate other HATs that could be involved in CXCL8 transcription in CF cells. In future, it would be interesting to look at the changes in the activity and function of CBP, PCAF and other chromatin-remodelling enzymes that have been reported to affect CXCL8 transcription (Huang and McCance, 2002).

Our study showed hypomethylation of a CpG site within the CXCL8 promoter in unstimulated CF cells which was further hypomethylated upon IL-β stimulation.

Future studies could look at the DNA methyltransferases regulating CpG site 3.1 in the promoter and whether these are abnormal in CF.

We did not study ways in which the changes in DNA methylation and the histone modifications might be linked. Studies in cancer cells and fibroblasts have identified an inverse correlation between DNA methylation and levels of H3K4me3 at the promoters of target genes (Balasubramanian et al., 2012). H3K4me3 can influence DNA methylation via recruitment of the Dnmt3-associated protein Dnmt3L that specifically interacts with unmodified H3K4 (Ooi et al., 2007). The presence of H3K4me3 also results in the association of RNA Pol II with the promoters of target genes via facilitation of global recruitment of TFIID (Lauberth et al., 2013, Vermeulen et al., 2007) acting as a platform for H3K4me3 lysine methyltransferase complexes (Horton et al., 2010). Furthermore, H3K9me3 and H3K4me3 can interact leading to the recruitment of DNA methylases and altered TFs binding to the promoters of associated genes (Przybilla et al., 2013). Future studies using optimised sequencing and ChIP-on-chip/ChIP-seq analysis might be helpful to explore the distribution of histone modifications and their localisation at the CXCL8 promoter as well as to investigate DNA/histone protein interactions on a genome wide basis.

Our data suggests that BET protein inhibitors might reduce airway inflammation in CF by reducing CXCL8 secretion. A number of pharmaceutical companies are developing these agents for the treatment of inflammatory and neoplastic diseases, and it would be useful to test them in mouse models of CF inflammation and if suitable, perhaps, as agents in clinical trials. The mechanistic studies in Chapter 6

suggest a possible interaction between BRD4 and NF-κB p65 at the CXCL8 promoter. Although we found that BET protein inhibitors seemed to have no obvious effect on the binding levels of NF-κB p65 to the CXCL8 promoter, it would be interesting to further explore the effects of BET protein inhibitors and determine if they alter the binding of any other TFs to the CXCL8 promoter in CF cells using ChIP assays. Further optimisation of the Co-IP technique as well as application of sensitive and accurate methods such as Re-ChIP, tandem affinity purification and pull down assays detecting protein in the correct cellular and chromatin environment could decisively confirm a direct interaction between NF-κB and BRD4 in CF cells. Furthermore, other techniques including crosslinking protein interaction and label transfer protein interaction analysis designed to identify transient and weak interactions could be used for future studies.

Our studies used immortalised cell lines. In future it would be interesting to strengthen and confirm the CXCL8 data by using primary cells and BAL fluid obtained from CF patients as this would more reflect the environment in the airways of CF patients.

In our study we investigated the effect of one cytokine, IL-1β, on the release of CXCL8 from CF airway epithelial cells. LPS, TNF-α and IFN-γ have been shown to be present in abundance in the CF airways and stimulate CXCL8 expression in different cells including epithelial cells, endothelial cells, neutrophils, fibroblasts, smooth muscle cells and others (Conese et al., 2009, Carrabino et al., 2006, Stecenko et al., 2001). Future experiments could determine the effect of these stimuli on CXCL8

production from CF airway epithelial cells as well as identify other signal transduction pathways involved in the secretion of CXCL8 in CF.

9 APPENDIX

9.1 PFI-1 and JQ1 MTT (cell viability) assay

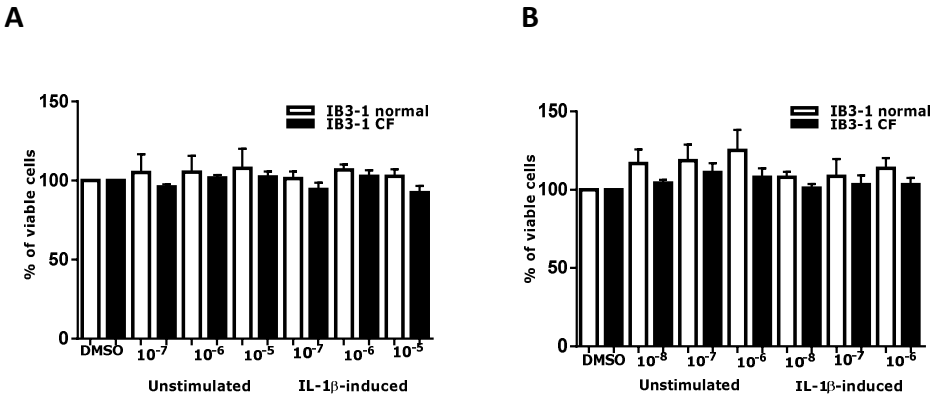
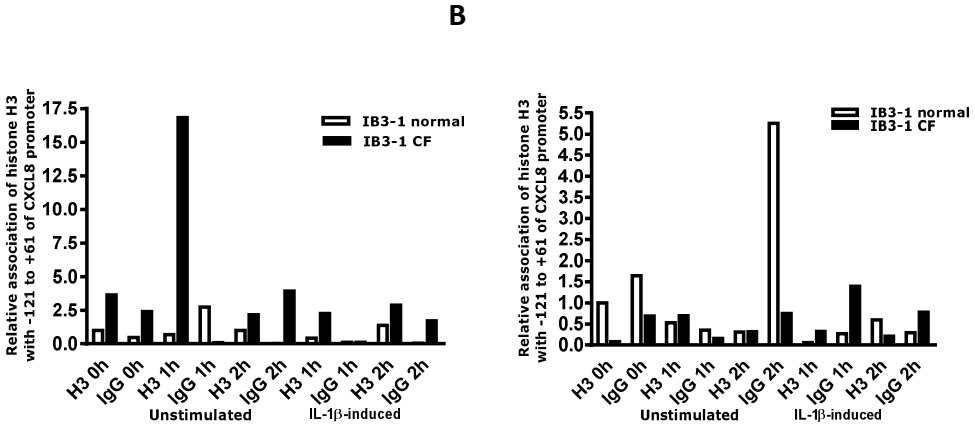


Figure 9-1. The toxicity of PFI-1 and JQ1 compounds in unstimulated and IL-1β-induced normal and CF airway epithelial cells. Confluent and growth arrested IB3-1 normal and IB3-1 CF cells were pre-treated with stated concentrations of PFI-1 ***(A)*** and JQ1 ***(B)*** and stimulated with 1ng/mL IL-β for 24 hours. At the end of the experiment, MTT assay was performed as described in 3.6 of Chapter 3 Materials and Methods. Each bar represents mean ± SEM of at least three independent experiments.

9.2 Additional figures



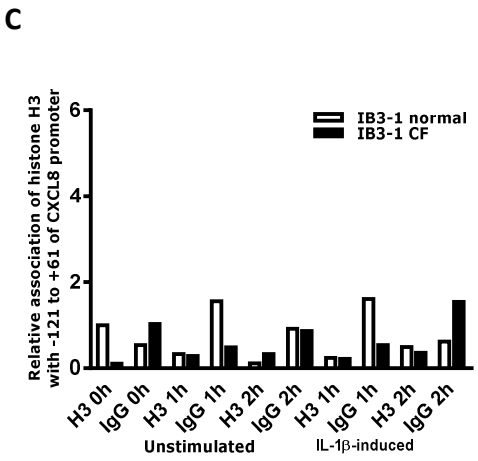
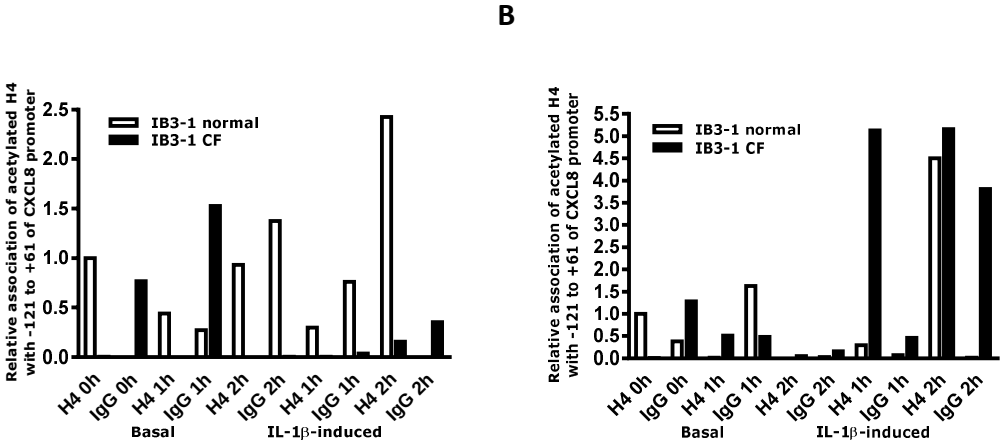


Figure 9-2. Histone H3 acetylation (H3ac) at the human CXCL8 promoter. Association of acetylated histone H3 with the CXCL8 promoter was determined in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against pan-acetylated H3 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA.



C

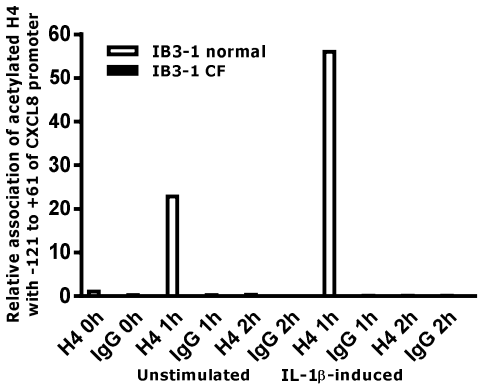
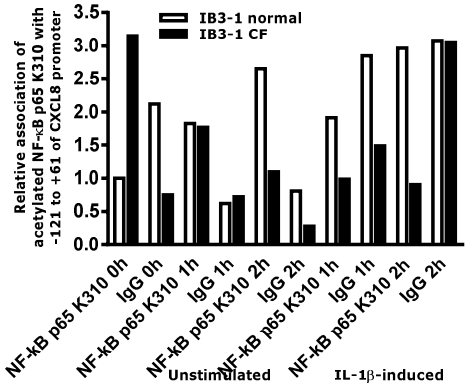
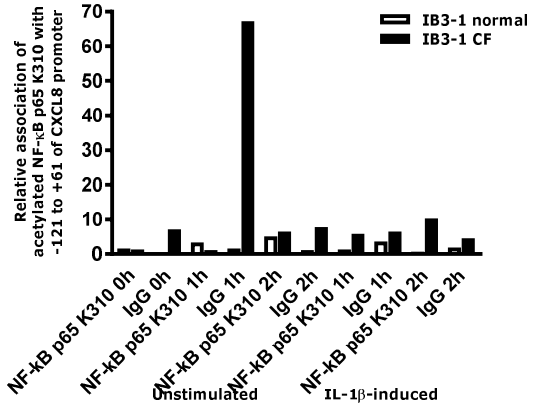


Figure 9-3. Histone H4 acetylation (H4ac) at the human CXCL8 promoter. Association of acetylated H4 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against pan-acetylated H4 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA.

A



B



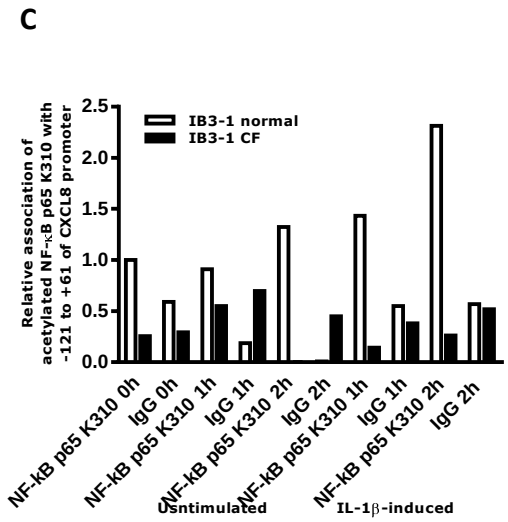


Figure 9-4. NF-κB p65 K310 acetylation (p65 K310) at the human CXCL8 promoter. Association of acetylated NF-κB p65 K310 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Extracted chromatin was sheared and immunoprecipitated with 4µg of antibody against anti-NF-κB p65 acetyl-K310 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA.

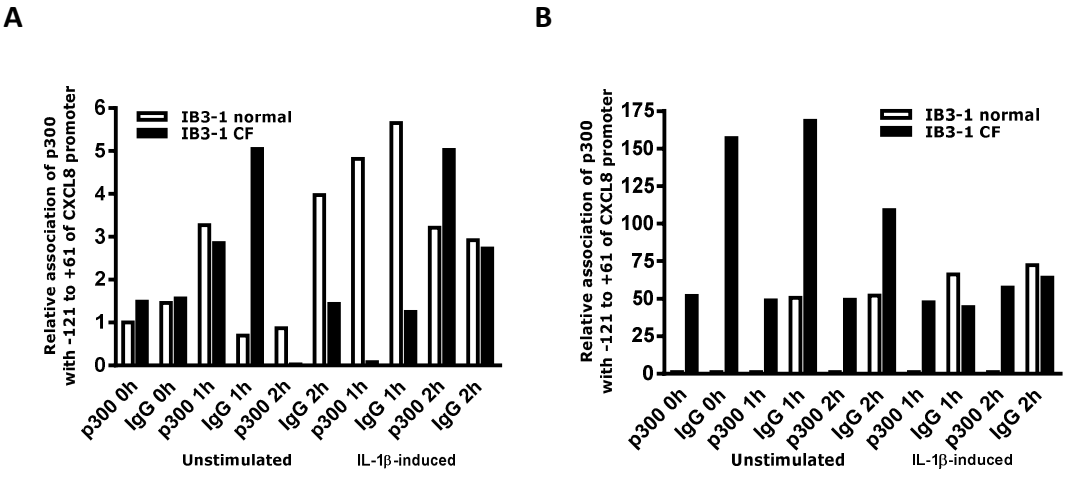


Figure 9-5. p300 binding to the human CXCL8 promoter. Association of acetylated p300 with the CXCL8 promoter was analysed in confluent and growth arrested IB3-1 normal and IB3-1 CF cells. Cells were incubated with/without 1ng/mL IL-1ß for 0, 1 and 2 hours. Extracted chromatin was sheared and immunoprecipitated with 1µg of antibody against p300 or rabbit IgG used as a negative control. Isolated DNA underwent qPCR analysis with CXCL8 promoter-specific primers. Data are expressed as fold change relative to the 0h mean of IB3-1 normal cells first normalised to the Input DNA.

9.3 List of reagents

9.3.1 Antibodies

Anti-acetyl-Histone H3 antibody, 100µg/mL	Millipore, UK
Anti-acetyl-Histone H4 antibody, 100µg/mL	Millipore, UK
Anti-NF-κB p65 (acetyl k310) antibody, ChIP Grade, 100µg/mL	Abcam Biochemicals, UK
Anti-NF-κB p65 antibody ChIP-Grade	Abcam Biochemicals, UK
Bromodomain (BRD) 2 antibody, 200µg/mL	Santa Cruz Biotechnology, UK
Bromodomain (BRD) 3 antibody, 200µg/mL	Santa Cruz Biotechnology, UK
Bromodomain (BRD) 4 antibody, 200µg/mL	Santa Cruz Biotechnology, UK
C/EBPβ	Santa Cruz Biotechnology, UK
C-Jun antibody, 200µg/mL	Active Motif, USA
Histone H3K4me3 (pAb), 100µl	Active Motif, USA
Normal rabbit IgG antibody, 200mg/0.5mL	Santa Cruz Biotechnology, UK
P300 antibody	Santa Cruz Biotechnology, UK

9.3.2 Kits

Bicinchoninic acid (BCA) assay kit	Pierce, UK
ChIP-IT® Express kit	Active Motif, USA
Dual-Luciferase® Reporter Assay System kit	Promega, UK
ECL™ Western blotting detection kit	GE Healthcare Life Sciences, UK
EpiTect® Bisulfite kit	Qiagen, UK
Human CXCL8/IL-8 DuoSet ELISA kit	R&D Systems, UK
Nuclear Complex Co-IP kit	Active Motif, USA
Nuclear Extract kit	Active Motif, USA
NucleoSpin RNA II kit	Macherey-Nagel, Germany
Plasmid purification midi kit	Qiagen, UK
QIAamp DNA blood mini Kit (50)	Qiagen, UK
Re-ChIP-IT® kit	Active Motif, USA
Nuclear Magnetic Co-IP kit	Active Motif, USA

9.3.3 Materials

Hyper film ECL™	GE Healthcare Life Sciences, UK
Siliconised tubes	Sigma-Aldrich, UK
PVDF Immunblott™ membrane	Bio-Rad, UK

9.3.4 Reagents

Agarose beads	Qiagen, UK
Ammonium persulphate	Sigma-Aldrich, UK
Amphotericin B, 2.5µg/mL	Sigma-Aldrich, UK
Annealing buffer	Qiagen, UK
Bisacrilamide, 30%	Sigma Aldrich, UK
ß-mercaptoethanol	Santa Cruz Biotechnology, UK
Binding buffer	Qiagen, UK
Bovine serum albumin, powder	Sigma Aldrich, UK
Bromophenol blue	Sigma-Aldrich, UK
3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide	Sigma-Aldrich, UK
Denaturation solution	Qiagen, UK
Deoxynucleoside triphosphates (dNTPs)	Promega, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Ethanol, 100%	Sigma-Aldrich, UK
Ethidium bromide	Sigma-Aldrich, UK
Fetal calf serum (FCS), heat-inactivated solution	Sigma-Aldrich, PAA laboratories, UK
Formaldehyde	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
Glycogen, 20mg/mL	Roche Diagnostics Ltd, UK
HotStarTaq plus DNA polymerase	Qiagen, UK
Interleukine (IL)-1 β	Peprotech, UK
Lipofectamine™ 2000 transfection reagent	Invitrogen, UK
LHC-8 without gentamicin (1x) medium	Gibco, Life technologies, UK
Methanol	Sigma-Aldrich, UK
Minimum Essential Medium Eagle	Sigma-Aldrich, UK
M-MLV RT	Promega, UK
M-MLV RT buffer, 1x solution	Promega, UK

N,N,N,N-Tetramethylethylenediamine (Temed)	Sigma-Aldrich, UK
Non-fat dry milk	Insight Biotechnology, UK
Normal goat IgG serum	Santa Cruz Biotechnology, UK
Normal rabbit serum	Santa Cruz Biotechnology, UK
Nuclease-free water	Ambion, UK
OligoDT primer	Roche Diagnostics Ltd, UK
PBS, 10X solution	Active Motif, USA
Penicillin/streptomycin, 100U/100µg/mL	Sigma-Aldrich, UK
PFI-1	Cayman Chemical, USA
Phenol/Chloroform	Sigma Aldrich, UK
Phosphate buffered saline (PBS), tablets	Sigma-Aldrich, UK
Protein G magnetic beads	Active Motif, USA
Protease	Qiagen, UK
PyroMark Gold Q24 reagents	Qiagen, UK
Puromycin, 10mg/mL	InvivoGen, UK
(+)/-JQ1	Cayman Chemical, USA
Rainbow™ colored protein marker	GE healthcare, UK
Ribonuclease (RNAase) inhibitor	Promega, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, UK
Sulphuric acid (H_2SO_4)	Sigma-Aldrich, UK
SYBR® Premix Ex Taq™	Clontech Laboratories, Inc, USA
SYBR® Premix Ex Taq™ II (Tli Rnase H Plus)	Clontech Laboratories, Inc, USA
Triton™ X-100	Sigma-Aldrich, UK
Trypsin/EDTA	Sigma-Aldrich, UK
Trizma® base (Tris-base)	Sigma-Aldrich, UK
Tween-20, 0.05%	Sigma-Aldrich, UK
Wash buffer, 5x	Qiagen, UK

9.4 Buffers and recipes

Reducing loading buffer, 2x

Reagents	Volume/mass
130mM Tris pH 6.8	15.74g
SDS, 4%	4g
Bromophenol blue, 0.02%	20mg
20% glycerol	20g
100mM DTT	1.525g
ddH_2O	100mL

Resolving gel, 10%

Reagents	Volume/mass
Bisacrilamide, 30%	6.66mL
Buffer 1	5.2mL
dH_2O	7.92mL
Ammonium persulphate, 10%	200µl
TEMED	20µl

Stacking gel

Reagents	Volume/mass
Bisacrilamide, 30%	1.3mL
Buffer 2	2.5mL
dH_20	6.1mL
Ammonium persulphate, 10%	50µl
TEMED	10µl

Buffer 1, pH=8.8

Reagents	Volume/mass
Tris base	18.5g
ddH_2O	100mL
SDS, 10%	4mL

Buffer 2, pH=6.8

Reagents	Volume/mass
Tris base	6g
ddH_2O	100mL
SDS, 10%	4mL

TBST, 10x, pH=7.4-7.6

Reagents	Volume/mass
Tris base	24.2g
ddH_2O	1000mL
NaCl	87.6g
Tween 20, 0.5%	10mL

Running buffer, 10x

Reagents	Volume/mass
Tris base	24.2g
ddH_2O	1000mL
Glycine	144g
SDS	10g

Transfer buffer, 10x

Reagents	Volume/mass
Tris base	24.2g
ddH_2O	1400mL
Methanol	400mL

Transfer buffer, 1x

Reagents	Volume/mass
10x Transfer buffer	200mL
ddH_2O	1000mL
Glycine	144g

Sodium acetate, 3M, pH=5.2

Reagents	Volume/mass
Sodium acetate	40.8g
ddH_2O	100mL

Tris buffered saline, pH=7.2-7.4

Reagents	Volume/mass
Trizma base	0.242g
Sodium chloride	8.76 g
ddH_2O	1000mL

9.5 PCR primers and cycling conditions

Table 9-1. Primer sequences and cycling conditions for qPCR analysis of CXCL8 mRNA expression and CXCL8 promoter (ChIP).

Gene	Primer sequence	qPCR and RT-PCR cycling conditions
CXCL8 (cDNA)	Sense: 5'-ATGACTTCCAAGCTGGCCGTGGCT–3' Antisense: 5'–TCTCAGCCCTCTTCAAAAACTTCTC–3'	95°C for 30 seconds 40 cycles of: 95°C for 5 seconds 60°C for 30 seconds 72°C for 15 seconds
β_2-microglobulin (β_2-M)	Forward: 5'–GAG TAT GCC TGC CGT GTG–3' Reverse: 5'– AAT CCA AAT GCG GCA TCT–3'	
CXCL8 (ChIP)	Forward: 5'–GGGCCATCAGTTGCAAATC–3' Reverse: 5'–TTCCTTCCGGTGGTTTCTTC–3'	95°C for 10 seconds 45 cycles of: 95°C for 5 seconds 60°C for 30 seconds 72°C for 15 seconds 95°C for 60 seconds

Table 9-2. Cycling conditions for bisulphite conversion of DNA

95°C for 5 minutes
60°C for 25 minutes
95°C for 5 minutes
60°C for 85 minutes
95°C for 5 minutes
60°C for 175 minutes

Table 9-3. Primers sequences and cycling conditions for PCR of bisulphite converted DNA.

CpG site		Primer sequence	RT- PCR cycling conditions
CpG1	CpG1.1	F: TGTTTATAGTGTGGGTAAATTTATTGT R: ATCCTAAAAAAAAAAATCCAAAACCT	95°C for 5 minutes 45 cycles of: 94°C for 30 seconds 56°C for 30 seconds 72°C for 60 seconds 72°C for 10 minutes
	CpG1.2	F: TGTTTATAGTGTGGGTAAATTTATTGT R: ATCCTAAAAAAAAAAATCCAAAACCT	
CpG2	CpG2.1 and CpG2.2	F: GTGGAGTTTTAGTATTTTAAATGTATAT R: ATCACACTTCCTATTTATTCCTTATCA	
CpG3	CpG2.3	F: TTGAGGGGATGGGTTATTAGTT R: ACTTATACACCCTCATCTTTTCAT	
CpG4	CpG3.1	F: GTGTATAAGTTTTTTAGTAGGGTGATG R: AATCAAAAAAACTACCAAAAAAACC	95°C for 5 minutes 45 cycles of: 94°C for 30 seconds 52.6°C for 30 seconds 72°C for 60 seconds 72°C for 10 minutes
	CpG3.3	F: GTGTATAAGTTTTTTAGTAGGGTGATG R: AATCAAAAAAACTACCAAAAAAACC	
LINE-1		Primers sequence is proprietary and not provided by QIAGEN	95°C for 5 minutes 45 cycles of: 94°C for 30

			seconds 50°C for 30 seconds 72°C for 30 seconds 72°C for 10 minutes

Table 9-4. Primer sequences used for bisulphite pyrosequencing.

CpG site		**Primer sequence**
CpG1	CpG1.1	TGGGTAAATTTATTGTTTTGT
	CpG1.2	ATAAATTATGTATTTGTTTAGAAG
CpG2	CpG2.1 and CpG2.2	ACTTCCTATTTATTCCTTATCAA
CpG3	CpG2.3	GGATGGGTTATTAGTTGTA
CpG4	CpG3.1	AGGGTGATGATATAAAAAGT
	CpG3.2	AGGATAAGAGTTAGGAAGA
	CpG3.3	ATTGTGTGTAAATATGATTTTTAA

10 **BIBLIOGRAPHY**

ABDEL-AZIM, A. 2011. Acute & Chronic Inflammation

ADCOCK, I. & CARAMORI, G. 2001. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. *Immunol Cell Biol,* 79(4)**,** 376-84.

ADCOCK, I., FORD, P., ITO, K. & BARNES, P. 2006. Epigenetics and airways disease. *Respiratory Research,* 7**,** 21.

AGGARWAL, N. R., KING, L. S. & D'ALESSIO, F. R. 2014. *Diverse macrophage populations mediate acute lung inflammation and resolution*.

ANDIA, D. C., DE OLIVEIRA, N. F. P., CASARIN, R. C. V., CASATI, M. Z., LINE, S. R. P. & DE SOUZA, A. P. 2010. DNA Methylation Status of the IL8 Gene Promoter in Aggressive Periodontitis. *Journal of Periodontology,* 81**,** 1336-1341.

ARENZANA-SEISDEDOS, F., TURPIN, P., RODRIGUEZ, M., THOMAS, D., HAY, R. T., VIRELIZIER, J. L. & DARGEMONT, C. 1997. Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. *Journal of Cell Science,* 110**,** 369-378.

ARMSTRONG, D., GRIMWOOD, K., CARLIN, J., CARZINO, R., GUTIÈRREZ, J., HULL, J., OLINSKY, A., PHELAN, E., ROBERTSON, C. & PHELAN, P. 1997. Lower Airway Inflammation in Infants and Young Children with Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine,* 156**,** 1197-1204.

ARMSTRONG, D. S., HOOK, S. M., JAMSEN, K. M., NIXON, G. M., CARZINO, R., CARLIN, J. B., ROBERTSON, C. F. & GRIMWOOD, K. 2005. Lower Airway Inflammation in Infants with Cystic Fibrosis Detected by Newborn Screening. *Pediatric Pulmonology,* 40**,** 500-510.

ARROWSMITH, C. H., BOUNTRA, C., FISH, P. V., LEE, K. & SCHAPIRA, M. 2012. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov,* 11**,** 384-400.

ASADULLAH, K., STERRY, W. & VOLK, H. D. 2003. Interleukin-10 Therapy—Review of a New Approach. *Pharmacological Reviews,* 55**,** 241-269.

ASHCROFT, G. S. 1999. Bidirectional regulation of macrophage function by TGF-β. *Microbes and Infection,* 1**,** 1275-1282.

AVVAKUMOV, N. & COTE, J. 2007. The MYST family of histone acetyltransferases and their intimate links to cancer. *Oncogene,* 26**,** 5395-5407.

BALAKATHIRESAN, N. S., BHATTACHARYYA, S., GUTTI, U., LONG, R. P., JOZWIK, C., HUANG, W., SRIVASTAVA, M., POLLARD, H. B. & BISWAS, R. 2009. Tristetraprolin regulates IL-8 mRNA stability in cystic fibrosis lung epithelial cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 296**,** L1012-L1018.

BALASUBRAMANIAN, D., AKHTAR-ZAIDI, B., SONG, L., BARTELS, C., VEIGL, M., BEARD, L., MYEROFF, L., GUDA, K., LUTTERBAUGH, J., WILLIS, J., CRAWFORD, G., MARKOWITZ, S. & SCACHERI, P. 2012. H3K4me3 inversely correlates with DNA methylation at a large class of non-CpG-island-containing start sites. *Genome Medicine,* 4**,** 47.

BALDWIN, A. S. 1996. THE NF-κB AND IκB PROTEINS: New Discoveries and Insights. *Annual Review of Immunology,* 14**,** 649-681.

BALDWIN, A. S., JR. 2001. Series Introduction: The transcription factor NF-κB and human disease. *The Journal of Clinical Investigation,* 107**,** 3-6.

BALKWILL, F. 2004. Cancer and the chemokine network. *Nat Rev Cancer,* 4**,** 540-550.

BANNISTER, A. J. & KOUZARIDES, T. 2005. Reversing histone methylation. *Nature,* 436**,** 1103-1106.

BANNISTER, A. J., MISKA, E. A., GÖRLICH, D. & KOUZARIDES, T. 2000. Acetylation of importin-α nuclear import factors by CBP/p300. *Current Biology,* 10**,** 467-470.

BARBIERI I, CANNIZZARO E & MA., D. 2013. Bromodomains as therapeutic targets in cancer. *Brief Funct Genomics* 12**,** 219-30.

BARNES, P. J. 2006. Transcription factors in airway diseases. *Lab Invest,* 86**,** 867-872.

BARNES, P. J., ADCOCK, I. M. & ITO, K. 2005. Histone acetylation and deacetylation: importance in inflammatory lung diseases. *European Respiratory Journal,* 25**,** 552-563.

BARRERA, L. O. & REN, B. 2006. The transcriptional regulatory code of eukaryotic cells – insights from genome-wide analysis of chromatin organization and transcription factor binding. *Current Opinion in Cell Biology,* 18**,** 291-298.

BARTLING, T. R. 2009. *INHERENT ALTERATION OF HISTONE ACETYLATION IN CELL CULTURE MODELS OF CYSTIC FIBROSIS.*

BARTLING, T. R. & DRUMM, M. L. 2009. Oxidative Stress Causes IL8 Promoter Hyperacetylation in Cystic Fibrosis Airway Cell Models. *American Journal of Respiratory Cell and Molecular Biology,* 40**,** 58-65.

BEAR, C. E., LI, C., KARTNER, N., BRIDGES, R. J., JENSEN, T. J., RAMJEESINGH, M. & RIORDAN, J. R. 1992. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell,* 68**,** 809-818.

BELKINA, A. C., NIKOLAJCZYK, B. S. & DENIS, G. V. 2013a. BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses. *J Immunol,* 190**,** 3670-8.

BELKINA, A. C., NIKOLAJCZYK, B. S. & DENIS, G. V. 2013b. BET Protein Function Is Required for Inflammation: Brd2 Genetic Disruption and BET Inhibitor JQ1 Impair Mouse Macrophage Inflammatory Responses. *The Journal of Immunology,* 190**,** 3670-3678.

BERDIEV, B. K., QADRI, Y. J. & BENOS, D. J. 2009. Assessment of the CFTR and ENaC association. *Molecular BioSystems,* 5**,** 123-127.

BERGER, S. L. 2007. The complex language of chromatin regulation during transcription. *Nature,* 447**,** 407-412.

BERGQVIST, S., GHOSH, G. & KOMIVES, E. A. 2008. The IκBα/NF-κB complex has two hot spots, one at either end of the interface. *Protein Science,* 17**,** 2051-2058.

BERNSTEIN, B. E., KAMAL, M., LINDBLAD-TOH, K., BEKIRANOV, S., BAILEY, D. K., HUEBERT, D. J., MCMAHON, S., KARLSSON, E. K., KULBOKAS III, E. J., GINGERAS, T. R., SCHREIBER, S. L. & LANDER, E. S. 2005. Genomic Maps and Comparative Analysis of Histone Modifications in Human and Mouse. *Cell,* 120**,** 169-181.

BHATTACHARYYA, S., GUTTI, U., MERCADO, J., MOORE, C., POLLARD, H. B. & BISWAS, R. 2010. MAPK signaling pathways regulate IL-8 mRNA stability and IL-8 protein expression in cystic fibrosis lung epithelial cell lines. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 300**,** L81-L87.

BHAUMIK, S. R., SMITH, E. & SHILATIFARD, A. 2007. Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol,* 14**,** 1008-1016.

BIRD, A. 2002. DNA methylation patterns and epigenetic memory. *Genes & Development,* 16**,** 6-21.

BIRD, A. 2007. Perceptions of epigenetics. *Nature,* 447**,** 396-398.

BLAECKE, A., DELNESTE, Y., HERBAULT, N., JEANNIN, P., BONNEFOY, J.-Y., BECK, A. & AUBRY, J.-P. 2002. Measurement of nuclear factor-kappa B translocation on lipopolysaccharide-activated human dendritic cells by confocal microscopy and flow cytometry. *Cytometry,* 48**,** 71-79.

BLAU, H., KLEIN, K., SHALIT, I., HALPERIN, D. & FABIAN, I. 2007. Moxifloxacin but not ciprofloxacin or azithromycin selectively inhibits IL-8, IL-6, ERK1/2, JNK, and NF-κB activation in a cystic fibrosis epithelial cell line. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 292**,** L343-L352.

BOBADILLA, J. L., MACEK, M., FINE, J. P. & FARRELL, P. M. 2002. Cystic fibrosis: A worldwide analysis of CFTR mutations—correlation with incidence data and application to screening. *Human Mutation,* 19**,** 575-606.

BONFIELD, T. L., PANUSKA, J. R., KONSTAN, M. W., HILLIARD, K. A., HILLIARD, J. B., GHNAIM, H. & BERGER, M. 1995. Inflammatory cytokines in cystic fibrosis lungs. *American Journal of Respiratory and Critical Care Medicine,* 152**,** 2111-2118.

BOUCHER, R. C. 2007. Evidence for airway surface dehydration as the initiating event in CF airway disease. *Journal of Internal Medicine,* 261**,** 5-16.

BRADBURY, E. M., INGLIS, R. J. & MATTHEWS, H. R. 1974. Control of cell division by very lysine rich histone (F1) phosphorylation. *Nature,* 247**,** 257-61.

BRAGONZI, A., WORLITZSCH, D., PIER, G. B., TIMPERT, P., ULRICH, M., HENTZER, M., ANDERSEN, J. B., GIVSKOV, M., CONESE, M. & DÖRING, G. 2005. Nonmucoid Pseudomonas aeruginosa Expresses Alginate in the Lungs of Patients with Cystic Fibrosis and in a Mouse Model. *Journal of Infectious Diseases,* 192**,** 410-419.

BRAT, D. J., BELLAIL, A. C. & VAN MEIR, E. G. 2005. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-Oncology,* 7**,** 122-133.

BRÈS, V., YOH, S. M. & JONES, K. A. 2008. The multi-tasking P-TEFb complex. *Current Opinion in Cell Biology,* 20**,** 334-340.

CALLAGHAN, M. & MCCLEAN, S. 2011. Bacterial host interactions in cystic fibrosis. . *Current opinion in microbiology. ,* 15**,** 71-7.

CAMPBELL, L., MAXWELL, P. & WAUGH, D. 2013. Rationale and Means to Target Pro-Inflammatory Interleukin-8 (CXCL8) Signaling in Cancer. *Pharmaceuticals,* 6**,** 929-959.

CAO, H., WANG, A., MARTIN, B., KOEHLER, D. R., ZEITLIN, P. L., TANAWELL, A. K. & HU, J. 2005. Down-regulation of IL-8 expression in human airway epithelial cells through helper-dependent adenoviral-mediated RNA interference. *Cell Res,* 15**,** 111-119.

CARRABINO, S., CARPANI, D., LIVRAGHI, A., DI CICCO, M., COSTANTINI, D., COPRENI, E., COLOMBO, C. & CONESE, M. 2006. Dysregulated interleukin-8 secretion and NF-κB activity in human cystic fibrosis nasal epithelial cells. *Journal of Cystic Fibrosis,* 5**,** 113-119.

CARROLL, T. P., GREENE, C. M., TAGGART, C. C., MCELVANEY, N. G. & O'NEILL, S. J. 2005. Interleukin-1, Neutrophil Elastase, and Lipopolysaccharide: Key Pro-

Inflammatory Stimuli Regulating Inflammation in Cystic Fibrosis. *Current Respiratory Medicine Reviews,* 1**,** 43-67.

CARROZZA, M. J., UTLEY, R. T., WORKMAN, J. L. & CÔTÉ, J. 2003. The diverse functions of histone acetyltransferase complexes. *Trends in Genetics,* 19**,** 321-329.

CATALÁN, M. A., NAKAMOTO, T., GONZALEZ-BEGNE, M., CAMDEN, J. M., WALL, S. M., CLARKE, L. L. & MELVIN, J. E. 2010. Cftr and ENaC ion channels mediate NaCl absorption in the mouse submandibular gland. *The Journal of Physiology,* 588**,** 713-724.

CELIC, I., MASUMOTO, H., GRIFFITH, W. P., MELUH, P., COTTER, R. J., BOEKE, J. D. & VERREAULT, A. 2006. The Sirtuins Hst3 and Hst4p Preserve Genome Integrity by Controlling Histone H3 Lysine 56 Deacetylation. *Current Biology,* 16**,** 1280-1289.

CFF 2009. ANTIBIOTIC TREATMENT FOR CYSTIC FIBROSIS. *In:* GROUP, U. C. F. T. A. W. (ed.).

CFF 2011. Patient Registry 2011 Annual Data Report.

CFF 2012. Annual Data Report.

CFTR.INFO. 2014.

CHAIDOS, A., CAPUTO V, GOUVEDENOU K, LIU B, MARIGO I, CHAUDHRY MS, ROTOLO A, TOUGH DF, SMITHERS NN, BASSIL AK, CHAPMAN TD, HARKER NR, BARBASH O, TUMMINO P, AL-MAHDI N, HAYNES AC, CUTLER L, LE B, RAHEMTULLA A, ROBERTS I, KLEIJNEN M, WITHERINGTON JJ, PARR NJ, PRINJHA RK & A., K. 2014. Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood,* 123**,** 697-705.

CHAN, M. M., CHMURA, K. & CHAN, E. D. 2006. Increased NaCl-induced interleukin-8 production by human bronchial epithelial cells is enhanced by the ΔF508/W1282X mutation of the cystic fibrosis transmembrane conductance regulator gene. *Cytokine,* 33**,** 309-316.

CHEN, H., LIN, R. J., XIE, W., WILPITZ, D. & EVANS, R. M. 1999. Regulation of Hormone-Induced Histone Hyperacetylation and Gene Activation via Acetylation of an Acetylase. *Cell,* 98**,** 675-686.

CHEN, L.-F., FISCHLE, W., VERDIN, E. & GREENE, W. C. 2001. Duration of Nuclear NF-κB Action Regulated by Reversible Acetylation. *Science,* 293**,** 1653-1657.

CHEN, L. F., MU, Y. & GREENE, W. C. 2002. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-κB. *The EMBO Journal,* 21**,** 6539-6548.

CHENG, X. & BLUMENTHAL, R. M. 2010. Coordinated Chromatin Control: Structural and Functional Linkage of DNA and Histone Methylation. *Biochemistry,* 49**,** 2999-3008.

CHENG, Z., GONG, Y., MA, Y., LU, K., LU, X., PIERCE, L. A., THOMPSON, R. C., MULLER, S., KNAPP, S. & WANG, J. 2013. Inhibition of BET Bromodomain Targets Genetically Diverse Glioblastoma. *Clinical Cancer Research,* 19**,** 1748-1759.

CHEUNG, P., ALLIS, C. D. & SASSONE-CORSI, P. 2000. Signaling to Chromatin through Histone Modifications. *Cell,* 103**,** 263-271.

CHEUNG, P. & LAU, P. 2005. Epigenetic Regulation by Histone Methylation and Histone Variants. *Molecular Endocrinology,* 19**,** 563-573.

CHINENOV, Y. & KERPPOLA, T. K. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene,* 20**,** 2438-2452.

CHMIEL, J. & DAVIS, P. 2003. State of the Art: Why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respiratory Research,* 4**,** 8.

CLARKE, D. L., CLIFFORD, R. L., JINDARAT, S., PROUD, D., PANG, L., BELVISI, M. & KNOX, A. J. 2010. TNFα and IFNγ Synergistically Enhance Transcriptional Activation of CXCL10 in Human Airway Smooth Muscle Cells via STAT-1, NF-κB, and the Transcriptional Coactivator CREB-binding Protein. *Journal of Biological Chemistry,* 285**,** 29101-29110.

CLIFFORD, R. L., SINGER, C. A. & JOHN, A. E. 2013. Epigenetics and miRNA emerge as key regulators of smooth muscle cell phenotype and function. *Pulmonary Pharmacology & Therapeutics,* 26**,** 75-85.

CLOOS, P. A. C., CHRISTENSEN, J., AGGER, K. & HELIN, K. 2008. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes & Development,* 22**,** 1115-1140.

CLOUTIER, A., GUINDI, C., LARIVÉE, P., DUBOIS, C. M., AMRANI, A. & MCDONALD, P. P. 2009. Inflammatory Cytokine Production by Human Neutrophils Involves C/EBP Transcription Factors. *The Journal of Immunology,* 182**,** 563-571.

COHEN-CYMBERKNOH, M., KEREM, E., FERKOL, T. & ELIZUR, A. 2013. Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. *Thorax*.

COHEN, T. S. & PRINCE, A. 2012. Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat Med,* 18**,** 509-519.

COLOBRAN, R., PUJOL-BORRELL, R., ARMENGOL, M. P. & JUAN, M. 2007. The chemokine network. I. How the genomic organization of chemokines contains clues for deciphering their functional complexity. *Clinical & Experimental Immunology,* 148**,** 208-217.

CONDREN, M. E. & BRADSHAW, M. D. 2013. Ivacaftor: a novel gene-based therapeutic approach for cystic fibrosis. *J Pediatr Pharmacol Ther,* 18**,** 8-13.

CONESE, M., ROMANO, M., FURNARI, M., COPRENI, E., DE FINO, I., PARDO, F. & GALIETTA, L. J. 2009. New Genetic and Pharmacological Treatments for Cystic Fibrosis. *Current Pediatric Reviews,* 5**,** 8-27.

CORVOL, H., FITTING, C., CHADELAT, K., JACQUOT, J., TABARY, O., BOULE, M., CAVAILLON, J.-M. & CLEMENT, A. 2003. Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 284**,** L997-L1003.

COTRAN, KUMAR & COLLINS 1998. *Robbins Pathologic Basis of Disease,* Philadelphia, W.B Saunders Company.

COUTINHO, C. P., DOS SANTOS, S. C., MADEIRA, A., MIRA, N. P., MOREIRA, A. S. & SÁ-CORREIA, I. 2011. Long-term colonization of the cystic fibrosis lung by Burkholderia cepacia complex bacteria: epidemiology, clonal variation and genome-wide expression alterations. *Frontiers in Cellular and Infection Microbiology,* 1.

COZENS, A. L., YEZZI, M. J., KUNZELMANN, K., OHRUI, T., CHIN, L., ENG, K., FINKBEINER, W. E., WIDDICOMBE, J. H. & GRUENERT, D. C. 1994. CFTR

expression and chloride secretion in polarized immortal human bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology,* 10**,** 38-47.

DATABASE, C. F. M. 2001.

DAUJAT, S., BAUER, U.-M., SHAH, V., TURNER, B., BERGER, S. & KOUZARIDES, T. 2002. Crosstalk between CARM1 Methylation and CBP Acetylation on Histone H3. *Current Biology,* 12**,** 2090-2097.

DAVIE, J. K. & DENT, S. Y. R. 2002. Transcriptional Control: An Activating Role for Arginine Methylation. *Current Biology,* 12**,** R59-R61.

DAWSON, M. A., PRINJHA, R. K., DITTMANN, A., GIOTOPOULOS, G., BANTSCHEFF M, CHAN, W. I., ROBSON, S. C., CHUNG, C. W., HOPF, C., SAVITSKI, M. M., HUTHMACHER, C., GUDGIN, E., LUGO, D., BEINKE, S., CHAPMAN, T. D., ROBERTS, E. J., SODEN, P. E., AUGER, K. R., MIRGUET, O., DOEHNER, K., DELWEL, R., BURNETT, A. K., JEFFREY, P., DREWES, G., LEE, K., HUNTLY, B. J. & KOUZARIDES, T. 2011. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature,* 478**,** 529-33.

DE LARCO, J. E., WUERTZ, B. R. K., YEE, D., RICKERT, B. L. & FURCHT, L. T. 2003. Atypical methylation of the interleukin-8 gene correlates strongly with the metastatic potential of breast carcinoma cells. *Proceedings of the National Academy of Sciences,* 100**,** 13988-13993.

DE ROSE, V. 2002. Mechanisms and markers of airway inflammation in cystic fibrosis. *European Respiratory Journal,* 19**,** 333-340.

DE RUIJTER, A. J. M., VAN GENNIP, A. H., CARON, H. N., KEMP, S. & VAN KUILENBURG, A. B. P. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.,* 370**,** 737-749.

DELHAES, L., MONCHY, S., FRÉALLE, E., HUBANS, C., SALLERON, J., LEROY, S., PREVOTAT, A., WALLET, F., WALLAERT, B., DEI-CAS, E., SIME-NGANDO, T., CHABÉ, M. & VISCOGLIOSI, E. 2012. The Airway Microbiota in Cystic Fibrosis: A Complex Fungal and Bacterial Community—Implications for Therapeutic Management. *PLoS ONE,* 7**,** e36313.

DELMORE, J. E., ISSA, G. C., LEMIEUX, M. E., RAHL, P. B., SHI, J., JACOBS, H. M., KASTRITIS, E., GILPATRICK, T., PARANAL, R. M., QI, J., CHESI, M., SCHINZEL, A. C., MCKEOWN, M. R., HEFFERNAN, T. P., VAKOC, C. R., BERGSAGEL, P. L., GHOBRIAL, I. M., RICHARDSON, P. G., YOUNG, R. A., HAHN, W. C., ANDERSON, K. C., KUNG, A. L., BRADNER, J. E. & MITSIADES, C. S. 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell,* 146**,** 904-17.

DENG, G., CHEN, A., PONG, E. & KIM, Y. 2001. Methylation in hMLH1 promoter interferes with its binding to transcription factor CBF and inhibits gene expression. *Oncogene,* 20(48)**,** 7120-7.

DEQUEKER, E., STUHRMANN, M., MORRIS, M. A., CASALS, T., CASTELLANI, C., CLAUSTRES, M., CUPPENS, H., DES GEORGES, M., FEREC, C., MACEK, M., PIGNATTI, P.-F., SCHEFFER, H., SCHWARTZ, M., WITT, M., SCHWARZ, M. & GIRODON, E. 2008. Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders - updated European recommendations. *Eur J Hum Genet,* 17**,** 51-65.

DESHMANE, S. L., KREMLEV, S., AMINI, S. & SAWAYA, B. E. 2009. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview *Journal of Interferon & Cytokine Research,* 29**,** 313-326.

DEY, A., CHITSAZ, F., ABBASI, A., MISTELI, T. & OZATO, K. 2003. The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proceedings of the National Academy of Sciences,* 100**,** 8758-8763.

DEY, A., ELLENBERG, J., FARINA, A., COLEMAN, A. E., MARUYAMA, T., SCIORTINO, S., LIPPINCOTT-SCHWARTZ, J. & OZATO, K. 2000. A Bromodomain Protein, MCAP, Associates with Mitotic Chromosomes and Affects G2-to-M Transition. *Molecular and Cellular Biology,* 20**,** 6537-6549.

DIMANGO, E., RATNER, A. J., BRYAN, R., TABIBI, S. & PRINCE, A. 1998. Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *The Journal of Clinical Investigation,* 101**,** 2598-2605.

DONALDSON, S. H., BENNETT, W. D., ZEMAN, K. L., KNOWLES, M. R., TARRAN, R. & BOUCHER, R. C. 2006. Mucus Clearance and Lung Function in Cystic Fibrosis with Hypertonic Saline. *New England Journal of Medicine,* 354**,** 241-250.

DOOHER, J. E., PAZ-PRIEL, I., HOUNG, S., BALDWIN, A. S. & FRIEDMAN, A. D. 2011. C/EBPα, C/EBPα Oncoproteins, or C/EBPβ Preferentially Bind NF-κB p50 Compared with p65, Focusing Therapeutic Targeting on the C/EBP:p50 Interaction. *Molecular Cancer Research,* 9**,** 1395-1405.

DURRIN, L. K., MANN, R. K., KAYNE, P. S. & GRUNSTEIN, M. 1991. Yeast histone H4 N-terminal sequence is required for promoter activation in vivo. *Cell,* 65**,** 1023-31.

EGGER, G., LIANG, G., APARICIO, A. & JONES, P. A. 2004. Epigenetics in human disease and prospects for epigenetic therapy. *Nature,* 429**,** 457-463.

EHRENHOFER-MURRAY, A. E. 2004. Chromatin dynamics at DNA replication, transcription and repair. *European Journal of Biochemistry,* 271**,** 2335-2349.

ELIZUR, A., CANNON, C. L. & FERKOL, T. W. 2008. Airway inflammation in cystic fibrosis*. *Chest,* 133**,** 489-495.

EMING, S. A., KRIEG, T. & DAVIDSON, J. M. 2007. Inflammation in Wound Repair: Molecular and Cellular Mechanisms. *J Invest Dermatol,* 127**,** 514-525.

FARRELL, P. M., ROSENSTEIN, B. J., WHITE, T. B., ACCURSO, F. J., CASTELLANI, C., CUTTING, G. R., DURIE, P. R., LEGRYS, V. A., MASSIE, J., PARAD, R. B., ROCK, M. J. & CAMPBELL III, P. W. 2008. Guidelines for Diagnosis of Cystic Fibrosis in Newborns through Older Adults: Cystic Fibrosis Foundation Consensus Report. *The Journal of Pediatrics,* 153**,** S4-S14.

FERREIRO, D. U. & KOMIVES, E. A. 2010. Molecular Mechanisms of System Control of NF-κB Signaling by IκBα. *Biochemistry,* 49**,** 1560-1567.

FERRERO-MILIANI, L., NIELSEN, O. H., ANDERSEN, P. S. & GIRARDIN, S. E. 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clinical & Experimental Immunology,* 147**,** 227-235.

FILIPPAKOPOULOS, P., PICAUD, S., MANGOS, M., KEATES, T., LAMBERT, J., BARSYTE-LOVEJOY, D., FELLETAR, I., VOLKMER, R., MÜLLER, S., PAWSON, T., GINGRAS, A., ARROWSMITH, C. & S., K. 2012. Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell,* 149**,** 214-31.

FILIPPAKOPOULOS, P., QI, J., PICAUD, S., SHEN, Y., SMITH, W. B., FEDOROV, O., MORSE, E. M., KEATES, T., HICKMAN, T. T., FELLETAR, I., PHILPOTT, M., MUNRO, S., MCKEOWN, M. R., WANG, Y., CHRISTIE, A. L., WEST, N., CAMERON, M. J., SCHWARTZ, B., HEIGHTMAN, T. D., LA THANGUE, N., FRENCH, C. A., WIEST, O., KUNG, A. L., KNAPP, S. & BRADNER, J. E. 2010. Selective inhibition of BET bromodomains. *Nature,* 468**,** 1067-1073.

FIRESTEIN, G. S., BUDD, R. C., GABRIEL, S. E., MCINNES, I. B. & O'DELL, J. R. 2013. *Kelley's Textbook of Rheumatology*.

FITZSIMMONS, S. 1998. Annual Data Report, CFF Patient Registry. *In:* FOUNDATION, C. F. (ed.). Bethesda.

FLOTTE, T. R., AFIONE, S. A., SOLOW, R., DRUMM, M. L., MARKAKIS, D., GUGGINO, W. B., ZEITLIN, P. L. & CARTER, B. J. 1993. Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. *Journal of Biological Chemistry,* 268**,** 3781-3790.

FLUME, P. & VAN DEVANTER, D. 2012. State of progress in treating cystic fibrosis respiratory disease. *BMC Medicine,* 10**,** 88.

FLUME, P. A., LIOU, T. G., BOROWITZ, D. S., LI, H., YEN, K., ORDOÑEZ, C. L. & GELLER, D. E. 2012. IVacaftor in subjects with cystic fibrosis who are homozygous for the f508del-cftr mutation. *CHEST Journal,* 142**,** 718-724.

FLUME, P. A., MOGAYZEL, P. J., ROBINSON, K. A., GOSS, C. H., ROSENBLATT, R. L., KUHN, R. J. & MARSHALL, B. C. 2009. Cystic Fibrosis Pulmonary Guidelines. *American Journal of Respiratory and Critical Care Medicine,* 180**,** 802-808.

FRAGA, M. F., BALLESTAR, E., VILLAR-GAREA, A., BOIX-CHORNET, M., ESPADA, J., SCHOTTA, G., BONALDI, T., HAYDON, C., ROPERO, S., PETRIE, K., IYER, N. G., PEREZ-ROSADO, A., CALVO, E., LOPEZ, J. A., CANO, A., CALASANZ, M. J., COLOMER, D., PIRIS, M. A., AHN, N., IMHOF, A., CALDAS, C., JENUWEIN, T. & ESTELLER, M. 2005. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet,* 37**,** 1061-4036.

FREITAG, M. & SELKER, E. U. 2005. Controlling DNA methylation: many roads to one modification. *Current Opinion in Genetics & Development,* 15**,** 191-199.

FUJIOKA, S., NIU, J., SCHMIDT, C., SCLABAS, G. M., PENG, B., UWAGAWA, T., LI, Z., EVANS, D. B., ABBRUZZESE, J. L. & CHIAO, P. J. 2004. NF-κB and AP-1 Connection: Mechanism of NF-κB-Dependent Regulation of AP-1 Activity. *Molecular and Cellular Biology,* 24**,** 7806-7819.

FUKS, F. 2005. DNA methylation and histone modifications: teaming up to silence genes. *Curr Opin Genet Dev.,* 15**,** 490-5.

GALLAGHER, S., J, , MIJATOV B, GUNATILAKE D, GOWRISHANKAR K, TIFFEN J, JAMES W, JIN L, PUPO G, CULLINANE C, MCARTHUR GA, TUMMINO PJ, RIZOS H & P., H. 2014. Control of NF-kB activity in human melanoma by BET protein inhibitor I-BET151. *Pigment Cell Melanoma Res*.

GAMSJAEGER, R., WEBB SR, LAMONICA JM, BILLIN A, BLOBEL GA & JP., M. 2011. Structural basis and specificity of acetylated transcription factor GATA1 recognition by BET family bromodomain protein Brd3. *Mol Cell Biol,* 31**,** 2632-40.

GENE ENTREZ 2012. CEBPB CCAAT/enhancer binding protein (C/EBP), beta. Entrez Gene.

GILL, G. 2005. Something about SUMO inhibits transcritpion. *Curr Opin Genet Dev,* 15**,** 536-41.

GILMORE, T. D. 2006. NF-kB: from basic research to human disease. *Oncogene (Reviews),* 251**,** 6679-6899.

GOLL, M. G., KIRPEKAR, F., MAGGERT, K. A., YODER, J. A., HSIEH, C. L., ZHANG, X., GOLIC K.G, JACOBSEN, S. E. & BESTOR, T. H. 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science,* 311**,** 395-8.

GREENHALGH, D. G. 1998. The role of apoptosis in wound healing. *The International Journal of Biochemistry & Cell Biology,* 30**,** 1019-1030.

GREER, E. L. & SHI, Y. 2012. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet,* 13**,** 343-357.

GRIMM, S. & BAEUERLE, P. 1993. The inducible transcription factor NF-kappa B: structure-function relationship of its protein subunits. *Biochem J,* 290 (Pt 2)**,** 297-308.

GRUNSTEIN, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature,* 389**,** 349-352.

GUAN, H., HOU, S. & RICCIARDI, R. P. 2005. DNA Binding of Repressor Nuclear Factor-κB p50/p50 Depends on Phosphorylation of Ser337 by the Protein Kinase A Catalytic Subunit. *Journal of Biological Chemistry,* 280**,** 9957-9962.

GUAN, K.-L. & XIONG, Y. 2011. Regulation of intermediary metabolism by protein acetylation. *Trends in Biochemical Sciences,* 36**,** 108-116.

GUO, Y., SU, M., MCNUTT, M. A. & GU, J. 2009. Expression and Distribution of Cystic Fibrosis Transmembrane Conductance Regulator in Neurons of the Human Brain. *Journal of Histochemistry & Cytochemistry,* 57**,** 1113-1120.

GYURIS, A., DONOVAN, D. J., SEYMOUR, K. A., LOVASCO, L. A., SMILOWITZ, N. R., HALPERIN, A. L. P., KLYSIK, J. E. & FREIMAN, R. N. 2009. The chromatin-targeting protein Brd2 is required for neural tube closure and embryogenesis. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms,* 1789**,** 413-421.

HALKIDOU, K., GAUGHAN, L., COOK, S., LEUNG, H. Y., NEAL, D. E. & ROBSON, C. N. 2004. Upregulation and Nuclear Recruitment of HDAC1 in Hormone Refractory Prostate Cancer. *The Prostate,* 59**,** 177-189.

HAPPEL, N. & DOENECKE, D. 2009. Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene* 431**,** 1-12.

HARGREAVES, D., HORNG T & R., M. 2009. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell. 2009 Jul 10;138(1):129-45. doi: 10.1016/j.cell.2009.05.047.,* 138**,** 129-45.

HARRISON, F. 2007. Microbial ecology of the cystic fibrosis lung. *Microbiology,* 153**,** 917–923.

HARRISON, M. J., MURPHY, D. M. & PLANT, B. J. 2013. Ivacaftor in a G551D Homozygote with Cystic Fibrosis. *New England Journal of Medicine,* 369**,** 1280-1282.

HASSELGREN, P.-O. 2007. Ubiquitination, phosphorylation, and acetylation—triple threat in muscle wasting. *Journal of Cellular Physiology,* 213**,** 679-689.

HAYDEN, M. S., WEST, A. P. & GHOSH, S. 2006. NF-[kappa]B and the immune response. *Oncogene,* 25**,** 6758-6780.

HAZZALIN, C. A. & MAHADEVAN, L. C. 2002. MAPK-regulated transcription: a continuously variable gene switch? *Nat Rev Mol Cell Biol,* 3**,** 30-40.

HEECKEREN, A., WALENGA, R., KONSTAN, M. W., BONFIELD, T., DAVIS, P. B. & FERKOL, T. 1997. Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with Pseudomonas aeruginosa. *The Journal of Clinical Investigation,* 100**,** 2810-2815.

HEIJERMAN, H. 2005. Infection and inflammation in cystic fibrosis: A short review. *Journal of Cystic Fibrosis,* 4, Supplement 2**,** 3-5.

HEINTZMAN, N. D., STUART, R. K., HON, G., FU, Y., CHING, C. W., HAWKINS, R. D., BARRERA, L. O., VAN CALCAR, S., QU, C., CHING, K. A., WANG, W., WENG, Z., GREEN, R. D., CRAWFORD, G. E. & REN, B. 2007. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet,* 39**,** 311-318.

HEISSMEYER, V., KRAPPMANN, D., HATADA, E. N. & SCHEIDEREIT, C. 2001. Shared Pathways of IκB Kinase-Induced SCFβTrCP-Mediated Ubiquitination and Degradation for the NF-κB Precursor p105 and IκBα. *Molecular and Cellular Biology,* 21**,** 1024-1035.

HERMAN, J. G. & BAYLIN, S. B. 2003. Gene Silencing in Cancer in Association with Promoter Hypermethylation. *New England Journal of Medicine,* 349**,** 2042-2054.

HESS, J., ANGEL, P. & SCHORPP-KISTNER, M. 2004a. AP-1 subunits: quarrel and harmony among siblings. *Journal of Cell Science,* 117**,** 5965-5973.

HESS, J., ANGEL, P. & SCHORPP-KISTNER, M. 2004b. AP-1 subunits: quarrel and harmony among siblings. *Journal of Cell Science,* 117**,** 5965-5973.

HIRCHE, T. O. 2014. Practical Guidelines: Lung Transplantation in Patients with Cystic Fibrosis. *Pulmonary Medicine*.

HOESEL, B. & SCHMID, J. 2013. The complexity of NF-kappaB signaling in inflammation and cancer. *Molecular Cancer,* 12**,** 86.

HOFFMANN, A. & BALTIMORE, D. 2006. Circuitry of nuclear factor κB signaling. *Immunological Reviews,* 210**,** 171-186.

HOFFMANN, E., DITTRICH-BREIHOLZ, O., HOLTMANN, H. & KRACHT, M. 2002. Multiple control of interleukin-8 gene expression. *Journal of Leukocyte Biology,* 72**,** 847-855.

HOIBY, N. 2011. Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. *BMC Medicine,* 9**,** 32.

HOLLIDAY, R. 1987. The inheritance of epigenetic defects. *Science,* 238(4824)**,** 163-70.

HOLTMANN, H., WINZEN, R., HOLLAND, P., EICKEMEIER, S., HOFFMANN, E., WALLACH, D., MALININ, N. L., COOPER, J. A., RESCH, K. & KRACHT, M. 1999. Induction of Interleukin-8 Synthesis Integrates Effects on Transcription and mRNA Degradation from at Least Three Different Cytokine- or Stress-Activated Signal Transduction Pathways. *Molecular and Cellular Biology,* 19**,** 6742-6753.

HOME, G. & REFERENCE. 2013. Available: http://ghr.nlm.nih.gov/gene/CFTR.

HORTON, J. R., UPADHYAY, A. K., QI, H. H., ZHANG, X., SHI, Y. & CHENG, X. 2010. Enzymatic and structural insights for substrate specificity of a family of jumonji histone lysine demethylases. *Nat Struct Mol Biol,* 17**,** 38-43.

HOUZELSTEIN, D., BULLOCK, S. L., LYNCH, D. E., GRIGORIEVA, E. F., WILSON, V. A. & BEDDINGTON, R. S. P. 2002. Growth and Early Postimplantation Defects in Mice Deficient for the Bromodomain-Containing Protein Brd4†. *Molecular and Cellular Biology,* 22**,** 3794-3802.

HUANG, B., YANG, X.-D., ZHOU, M.-M., OZATO, K. & CHEN, L.-F. 2009. Brd4 Coactivates Transcriptional Activation of NF-κB via Specific Binding to Acetylated RelA. *Molecular and Cellular Biology,* 29**,** 1375-1387.

HUANG, S., M, & MCCANCE, D. 2002. Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. *J Virol.,* 76**,** 8710-21.

HUXFORD, T. & GHOSH, G. 2009. A Structural Guide to Proteins of the NF-κB Signaling Module. *Cold Spring Harbor Perspectives in Biology,* 1.

ILLINGWORTH, R. S., GRUENEWALD-SCHNEIDER, U., WEBB, S., KERR, A. R. W., JAMES, K. D., TURNER, D. J., SMITH, C., HARRISON, D. J., ANDREWS, R. & BIRD, A. P. 2010. Orphan CpG Islands Identify Numerous Conserved Promoters in the Mammalian Genome. *PLoS Genetics,* 6.

IMUNDO, L., BARASCH, J., PRINCE, A. & AL-AWQATI, Q. 1995. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. *Proceedings of the National Academy of Sciences,* 92**,** 3019-3023.

JACOBSON, R. H., LADURNER, A. G., KING, D. S. & TJIAN, R. 2000. Structure and Function of a Human TAFII250 Double Bromodomain Module. *Science,* 288**,** 1422-1425.

JAENISCH, R. & BIRD, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*.

JANG, M. K., MOCHIZUKI, K., ZHOU, M., JEONG, H.-S., BRADY, J. N. & OZATO, K. 2005. The Bromodomain Protein Brd4 Is a Positive Regulatory Component of P-TEFb and Stimulates RNA Polymerase II-Dependent Transcription. *Molecular cell,* 19**,** 523-534.

JENKINS, B. D., PULLEN, C. B. & DARIMONT, B. D. 2001. Novel glucocorticoid receptor coactivator effector mechanisms. *Trends in Endocrinology & Metabolism,* 12**,** 122-126.

JENUWEIN, T. & ALLIS, C. D. 2001. Translating the Histone Code. *Science,* 293**,** 1074-1080.

JIANG, Y.-H., SAHOO, T., MICHAELIS, R. C., BERCOVICH, D., BRESSLER, J., KASHORK, C. D., LIU, Q., SHAFFER, L. G., SCHROER, R. J., STOCKTON, D. W., SPIELMAN, R. S., STEVENSON, R. E. & BEAUDET, A. L. 2004. A mixed epigenetic/genetic model for oligogenic inheritance of autism with a limited role for UBE3A. *American Journal of Medical Genetics Part A,* 131A**,** 1-10.

JOCHUM, W., PASSEGUÉ, E. & EF, W. 2001. AP-1 in mouse development and tumorigenesis. *Oncogene,* 20(19)**,** 2401-12.

JOHN, A. E., CLIFFORD, R. L., BRIGHTLING, C. E. & KNOX, A. J. 2010. Basal Hypersecretion Of CXCL8 In Asthmatic Human Airway Smooth Muscle Cells Is Associated With

Increased Binding Of Histone Acetyltransferases To The CXCL8 Promoter And Acetylation Of Histone H3. *Am J Respir Crit Care Med*

JOHN, A. E., ZHU, Y. M., BRIGHTLING, C. E., PANG, L. & KNOX, A. J. 2009. Human Airway Smooth Muscle Cells from Asthmatic Individuals Have CXCL8 Hypersecretion Due to Increased NF-κB p65, C/EBPβ, and RNA Polymerase II Binding to the CXCL8 Promoter. *The Journal of Immunology,* 183**,** 4682-4692.

JOHNSON, P. F. 2005. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *Journal of Cell Science,* 118**,** 2545-2555.

JONES, P. A. & LIANG, G. 2009. Rethinking how DNA methylation patterns are maintained. *Nat Rev Genet.,* 10**,** 805-11.

JOSEPH, T., LOOK, D. & FERKOL, T. 2005. NF-κB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 288**,** L471-L479.

KATKIN, J. P. 2014. Cystic fibrosis: Clinical manifestations of pulmonary disease.

KEGG 2014.

KEREM, B., ROMMENS JM, BUCHANAN JA, MARKIEWICZ D, COX TK, CHAKRAVARTI A, BUCHWALD M & LC, T. 1989. Identification of the cystic fibrosis gene: genetic analysis. *Science,* 245(4922)**,** 1073-8.

KHALAF, H., JASS, J. & OLSSON, P.-E. 2010. Differential cytokine regulation by NF-κB and AP-1 in Jurkat T-cells. *BMC Immunology,* 11**,** 1-12.

KHAN, T., GILLESPIE, M., PARSONS, P., WAEGENER, J., RICHES, D. & ACCURSO, F. 1995a. Airway inflammation in infants with cystic fibrosis can occur in the absence of detectable endotoxin in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med,* 151:247a.

KHAN, T. Z., WAGENER, J. S., BOST, T., MARTINEZ, J., ACCURSO, F. J. & RICHES, D. W. 1995b. Early pulmonary inflammation in infants with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine,* 151**,** 1075-1082.

KHAN, Y. M., KIRKHAM, P., BARNES, P. J. & ADCOCK, I. M. 2014. Brd4 Is Essential for IL-1β-Induced Inflammation in Human Airway Epithelial Cells. *PLoS ONE,* 9**,** e95051.

KHANJANI, S., TERZIDOU, V., JOHNSON, M. & BENNETT, P. 2012. NFκB and AP-1 drive human myometrial IL8 expression. *Mediators Inflamm*.

KLOSE, R. J. & BIRD, A. P. 2006. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci. ,* 31**,** 89-97.

KLOSE, R. J. & ZHANG, Y. 2007. Regulation of histone methylation by demethylimination and demethylation. *Nat Rev Mol Cell Biol,* 8**,** 307-318.

KNORRE, A., WAGNER, M., SCHAEFER, H. E., COLLEDGE, W. H. & PAHL, H. L. 2002. ΔF508-CFTR Causes Constitutive NF-κB Activation through an ER-Overload Response in Cystic Fibrosis Lungs. *Biological Chemistry.*

KONDO, Y., SHEN, L., AHMED, S., BOUMBER, Y., SEKIDO, Y., HADDAD, B. R. & ISSA, J.-P. J. 2008. Downregulation of Histone H3 Lysine 9 Methyltransferase G9a Induces Centrosome Disruption and Chromosome Instability in Cancer Cells. *PLoS ONE,* 3**,** e2037.

KONDO, Y., SHEN, L., SUZUKI, S., KUROKAWA, T., MASUKO, K., TANAKA, Y., KATO, H., MIZUNO, Y., YOKOE, M., SUGAUCHI, F., HIRASHIMA, N., ORITO, E., OSADA, H., UEDA, R., GUO, Y., CHEN, X., ISSA, J.-P. J. & SEKIDO, Y. 2007. Alterations of DNA methylation and histone modifications contribute to

gene silencing in hepatocellular carcinomas. *Hepatology Research,* 37**,** 974-983.

KONSTAN, M. & DAVIS, P. 2002. Pharmacological approaches for the discovery and development of new anti-inflammatory agents for the treatment of cystic fibrosis. *Adv Drug Deliv Rev,* 54(11)**,** 1409-23.

KOUZARIDES, T. 2000. Acetylation: a regulatory modification to rival phosphorylation? *EMBO J,* 19**,** 1176-1179.

KOUZARIDES, T. 2007. Chromatin Modifications and Their Function. *Cell,* 128**,** 693-705.

KUNZELMANN, K. 2003. *Control of Membrane Transport by the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)*.

KUNZELMANN, K. & MALL, M. 2003. Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. *Am J Respir Med,* 2(4)**,** 299-309.

KUO, M. H. & ALLIS, C. D. 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays,* 20**,** 615-26.

L'HERNAULT, S. W. & ROSENBAUM, J. L. 1985. Chlamydomonas α-tubulin is posttranslationally modified by acetylation on the ε-amino group of a lysine. *Biochemistry,* 24**,** 473-478.

LACHNER, M. & JENUWEIN, T. 2002. The many faces of histone lysine methylation. *Current Opinion in Cell Biology,* 14**,** 286-298.

LAMBIASE, A., RAIA, V., PEZZO, M., SEPE, A., CARNOVALE, V. & ROSSANO, F. 2006. Microbiology of airway disease in a cohort of patients with Cystic Fibrosis. *BMC Infectious Diseases,* 6**,** 4.

LAMOUREUX, F., BAUD'HUIN M, RODRIGUEZ CALLEJA L, JACQUES C, BERREUR M, RÉDINI F, LECANDA F, BRADNER JE, HEYMANN D & B., O. 2014. Selective inhibition of BET bromodomain epigenetic signalling interferes with the bone-associated tumour vicious cycle. *Nat Commun,* 19**,** 3511.

LAUBERTH, SH M., NAKAYAMA, T., WU, X., FERRIS, ANDREA L., TANG, Z., HUGHES, STEPHEN H. & ROEDER, ROBERT G. 2013. H3K4me3 Interactions with TAF3 Regulate Preinitiation Complex Assembly and Selective Gene Activation. *Cell,* 152**,** 1021-1036.

LAURENT, L., WONG, E., LI, G., HUYNH, T., TSIRIGOS, A., ONG, C. T., LOW, H. M., KIN SUNG, K. W., RIGOUTSOS, I., LORING, J. & WEI, C.-L. 2010. Dynamic changes in the human methylome during differentiation. *Genome Research,* 20**,** 320-331.

LEE, D. Y., HAYES, J. J., PRUSS, D. & WOLFFE, A. P. 1993. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell,* 72**,** 73-84.

LEE, K.-Y., ITO, K., HAYASHI, R., JAZRAWI, E. P. I., BARNES, P. J. & ADCOCK, I. M. 2006. NF-κB and Activator Protein 1 Response Elements and the Role of Histone Modifications in IL-1β-Induced TGF-β1 Gene Transcription. *The Journal of Immunology,* 176**,** 603-615.

LEROY, G., RICKARDS B & SJ., F. 2008. The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. *Mol Cel,* 30**,** 51-60.

LI, B., CAREY, M. & WORKMAN, J. L. 2007. The Role of Chromatin during Transcription. *Cell,* 128**,** 707-719.

LI, J., KARTHA, S., IASVOVSKAIA, S., TAN, A., BHAT, R. K., MANALIGOD, J. M., PAGE, K., BRASIER, A. R. & HERSHENSON, M. B. 2002. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 283**,** L690-L699.

LIVRAGHI, A. & RANDELL, S. H. 2007. Cystic Fibrosis and Other Respiratory Diseases of Impaired Mucus Clearance. *Toxicologic Pathology,* 35**,** 116-129.

LONGWORTH, M. S. & LAIMINS, L. A. 2006. Histone deacetylase 3 localizes to the plasma membrane and is a substrate of Src. *Oncogene,* 25**,** 4495-4500.

LYCZAK, J., CANNON, C. & PIER, G. 2002. Lung Infections Associated with Cystic Fibrosis. *CLINICAL MICROBIOLOGY REVIEWS***,** 194-222.

MALIK, S. & BHAUMIK, S. R. 2010. Mixed lineage leukemia: histone H3 lysine 4 methyltransferases from yeast to human. *FEBS Journal,* 277**,** 1805-1821.

MALLORY, G. J. 1996. Lung transplantation for cystic fibrosis: state of the art.1996; (Suppl)13:121). *Pediatr Pulmonol* 13.

MARMORSTEIN, R. 2001. Structure of histone acetyltransferases. *Journal of Molecular Biology,* 311**,** 433-444.

MARUYAMA, T., FARINA, A., DEY, A., CHEONG, J., BERMUDEZ, V. P., TAMURA, T., SCIORTINO, S., SHUMAN, J., HURWITZ, J. & OZATO, K. 2002. A Mammalian Bromodomain Protein, Brd4, Interacts with Replication Factor C and Inhibits Progression to S Phase. *Molecular and Cellular Biology,* 22**,** 6509-6520.

MASSENGALE, A. R. D., QUINN, F., YANKASKAS, J., WEISSMAN, D., THOMAS MCCLELLAN, W., CUFF, C. & ARONOFF, S. C. 1999. Reduced Interleukin-8 Production by Cystic Fibrosis Airway Epithelial Cells. *American Journal of Respiratory Cell and Molecular Biology,* 20**,** 1073-1080.

MATSUI, H., GRUBB, B. R., TARRAN, R., RANDELL, S. H., GATZY, J. T., DAVIS, C. W. & BOUCHER, R. C. 1998. Evidence for Periciliary Liquid Layer Depletion, Not Abnormal Ion Composition, in the Pathogenesis of Cystic Fibrosis Airways Disease. *Cell,* 95**,** 1005-1015.

MCCLEAN, S. & CALLAGHAN, M. 2009. Burkholderia cepacia complex: epithelial cell–pathogen confrontations and potential for therapeutic intervention. *Journal of Medical Microbiology,* 58**,** 1-12.

MCQUIBBAN, G. A., GONG, J.-H., TAM, E. M., MCCULLOCH, C. A. G., CLARK-LEWIS, I. & OVERALL, C. M. 2000. Inflammation Dampened by Gelatinase A Cleavage of Monocyte Chemoattractant Protein-3. *Science,* 289**,** 1202-1206.

MÉLIK-PARSADANIANTZ, S. & ROSTÈNE, W. 2008. Chemokines and neuromodulation. *Journal of Neuroimmunology,* 198**,** 62-68.

MEYERHOLZ, D. K., STOLTZ, D. A., MCCRAY, P. B. J. & WELSH, M. J. 2012. Dissecting the fetal development of cystic fibrosis tracheal abnormalities. *The FASEB Journal,* 26**,** 10.

MILLA, C. E. 2007. Nutrition and Lung Disease in Cystic Fibrosis. *Clinics in Chest Medicine,* 28**,** 319-330.

MISHRA, A., GREAVES, R. & MASSIE, J. 2005. The Relevance of Sweat Testing for the Diagnosis of Cystic Fibrosis in the Genomic Era. *Clin Biochem Rev.,* 26**,** 135–153.

MIZUGUCHI, G., VASSILEV, A., TSUKIYAMA, T., NAKATANI, Y. & WU, C. 2001. ATP-dependent Nucleosome Remodeling and Histone Hyperacetylation

Synergistically Facilitate Transcription of Chromatin. *Journal of Biological Chemistry,* 276**,** 14773-14783.

MOCHIZUKI, K., NISHIYAMA, A., JANG, M. K., DEY, A., GHOSH, A., TAMURA, T., NATSUME, H., YAO, H. & OZATO, K. 2008. The Bromodomain Protein Brd4 Stimulates G1 Gene Transcription and Promotes Progression to S Phase. *Journal of Biological Chemistry,* 283**,** 9040-9048.

MORENO, R., SOBOTZIK, J.-M., SCHULTZ, C. & SCHMITZ, M. L. 2010. Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKKε. *Nucleic Acids Research,* 38**,** 6029-6044.

MOSAMMAPARAST, N. & SHI, Y. 2010. Reversal of Histone Methylation: Biochemical and Molecular Mechanisms of Histone Demethylases. *Annual Review of Biochemistry,* 79**,** 155-179.

MUKAIDA, N. 2003. *Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases*.

MUKAIDA, N., MAHE, Y. & MATSUSHIMA, K. 1990. Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *Journal of Biological Chemistry,* 265**,** 21128-33.

MUKAIDA, N., OKAMOTO, S., ISHIKAWA, Y. & MATSUSHIMA, K. 1994. Molecular mechanism of interleukin-8 gene expression. *Journal of Leukocyte Biology,* 56**,** 554-8.

MULLER, S., FILIPPAKOPOULOS, P. & KNAPP, S. 2011a. Bromodomains as therapeutic targets. *Expert Reviews in Molecular Medicine,* 13**,** null-null.

MULLER, S., FILIPPAKOPOULOS, P. & KNAPP, S. 2011b. Bromodomains as therapeutic targets. *Expert Rev Mol Med,* 13:e29.

MURDOCH, C. & FINN, A. 2000. Chemokine receptors and their role in inflammation and infectious diseases. *Blood,* 95**,** 3032-43.

MURPHY, P. M. 1994. The Molecular Biology of Leukocyte Chemoattractant Receptors. *Annual Review of Immunology,* 12**,** 593-633.

MUSELET-CHARLIER, C., ROQUE, T., BONCOEUR, E., CHADELAT, K., CLEMENT, A., JACQUOT, J. & TABARY, O. 2007. Enhanced IL-1β-induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF-κB signaling. *Biochemical and Biophysical Research Communications,* 357**,** 402-407.

NAGEL, G., SZELLAS, T., RIORDAN, J. R., FRIEDRICH, T. & HARTUNG, K. 2001. Non-specific activation of the epithelial sodium channel by the CFTR chloride channel. *EMBO reports,* 2**,** 249-254.

NAGY, Z. & TORA, L. 2007a. Distinct GCN5//PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene,* 26**,** 5341-5357.

NAGY, Z. & TORA, L. 2007b. Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene,* 26(37)**,** 5341-57.

NAKAMURA, T., OUCHIDA, R., KODAMA, T., KAWASHIMA, T., MAKINO, Y., YOSHIKAWA, N., WATANABE, S., MORIMOTO, C., KITAMURA, T. & TANAKA, H. 2002. Cytokine Receptor Common β Subunit-mediated STAT5 Activation

Confers NF-κB Activation in Murine proB Cell Line Ba/F3 Cells. *Journal of Biological Chemistry,* 277**,** 6254-6265.

NAKANISHI, M. & ROSENBERG, D. 2013. Multifaceted roles of PGE2 in inflammation and cancer. *Seminars in Immunopathology,* 35**,** 123-137.

NARASIMHAN, M. & COHEN, R. 2011. New and Investigational Treatments in Cystic Fibrosis. *Ther Adv Resp Dis.,* 5**,** 275-282.

NASSER, M. W., RAGHUWANSHI, S. K., GRANT, D. J., JALA, V. R., RAJARATHNAM, K. & RICHARDSON, R. M. 2009. Differential Activation and Regulation of CXCR1 and CXCR2 by CXCL8 Monomer and Dimer. *The Journal of Immunology,* 183**,** 3425-3432.

NATOLI, G. 2009. Organization Control of NF-kB-dependent Transcriptional Responses by Chromatin. *Cold Spring Harb Perspect Biol*.

NGUYEN, C. T., WEISENBERGER, D. J., VELICESCU, M., GONZALES, F. A., LIN, J. C. Y., LIANG, G. & JONES, P. A. 2002. Histone H3-Lysine 9 Methylation Is Associated with Aberrant Gene Silencing in Cancer Cells and Is Rapidly Reversed by 5-Aza-2'-deoxycytidine. *Cancer Research,* 62**,** 6456-6461.

NICHOLS, D., CHMIEL, J. & BERGER, M. 2008. Chronic Inflammation in the Cystic Fibrosis Lung: Alterations in Inter- and Intracellular Signaling. *Clinical Reviews in Allergy & Immunology,* 34**,** 146-162.

NICK, J. A., CHACON, C. S., BRAYSHAW, S. J., JONES, M. C., BARBOA, C. M., CLAIR, C. G. S., YOUNG, R. L., NICHOLS, D. P., JANSSEN, J. S., HUITT, G. A., ISEMAN, M. D., DALEY, C. L., TAYLOR-COUSAR, J. L., ACCURSO, F. J., SAAVEDRA, M. T. & SONTAG, M. K. 2010. Effects of Gender and Age at Diagnosis on Disease Progression in Long-term Survivors of Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine,* 182**,** 614-626.

NICODEME, E., JEFFREY, K. L., SCHAEFER, U., BEINKE, S., DEWELL, S., CHUNG, C.-W., CHANDWANI, R., MARAZZI, I., WILSON, P., COSTE, H., WHITE, J., KIRILOVSKY, J., RICE, C. M., LORA, J. M., PRINJHA, R. K., LEE, K. & TARAKHOVSKY, A. 2010. Suppression of inflammation by a synthetic histone mimic. *Nature,* 468**,** 1119-1123.

NIH. *Celebrating America's women physicians* [Online]. Available: http://www.nlm.nih.gov/changingthefaceofmedicine/physicians/biography_8.html.

NIXON, G. M., ARMSTRONG, D. S., CARZINO, R., CARLIN, J. B., OLINSKY, A., ROBERTSON, C. F. & GRIMWOOD, K. 2002. Early airway infection, inflammation, and lung function in cystic fibrosis. *Archives of Disease in Childhood,* 87**,** 306-311.

NOAH, T. L., BLACK, H. R., CHENG, P.-W., WOOD, R. E. & LEIGH, M. W. 1997. Nasal and Bronchoalveolar Lavage Fluid Cytokines in Early Cystic Fibrosis. *Journal of Infectious Diseases,* 175**,** 638-647.

NORZILA, M. Z., FAKES, K., HENRY, R. L., SIMPSON, J. & GIBSON, P. G. 2000. Interleukin-8 Secretion and Neutrophil Recruitment Accompanies Induced Sputum Eosinophil Activation in Children with Acute Asthma. *American Journal of Respiratory and Critical Care Medicine,* 161**,** 769-774.

O'DEA, E. & HOFFMANN, A. 2010. The Regulatory Logic of the NF-κB Signaling System. *Cold Spring Harbor Perspectives in Biology,* 2.

O'SULLIVAN, B. & FREEDMAN, S. 2009. Cystic fibrosis. *Lancet,* 373(9678)**,** 1891-904.

OECKINGHAUS, A. & GHOSH, S. 2009. The NF-κB Family of Transcription Factors and Its Regulation. *Cold Spring Harbor Perspectives in Biology,* 1.

OOI, C. Y., DUPUIS, A., ELLIS, L., JARVI, K., MARTIN, S., GONSKA, T., DORFMAN, R., KORTAN, P., SOLOMON, M., TULLIS, E. & DURIE, P. R. 2012. Comparing the American and European diagnostic guidelines for cystic fibrosis: same disease, different language? *Thorax,* 67**,** 618-624.

OOI, S. K. T., QIU, C., BERNSTEIN, E., LI, K., JIA, D., YANG, Z., ERDJUMENT-BROMAGE, H., TEMPST, P., LIN, S.-P., ALLIS, C. D., CHENG, X. & BESTOR, T. H. 2007. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature,* 448**,** 714-717.

OSPELT, C., REEDQUIST, K. A., GAY, S. & TAK, P. P. 2011. Inflammatory memories: Is epigenetics the missing link to persistent stromal cell activation in rheumatoid arthritis? *Autoimmunity Reviews,* 10**,** 519-524.

OUYANG, W., RUTZ, S., CRELLIN, N. K., VALDEZ, P. A. & HYMOWITZ, S. G. 2011. Regulation and Functions of the IL-10 Family of Cytokines in Inflammation and Disease. *Annual Review of Immunology,* 29**,** 71-109.

PAZ-PRIEL, I., HOUNG, S., DOOHER, J. & FRIEDMAN, A. D. 2011. *C/EBPα and C/EBPα oncoproteins regulate nfkb1 and displace histone deacetylases from NF-κB p50 homodimers to induce NF-κB target genes*.

PETERS, A. H. & SCHÜBELER, D. 2005. Methylation of histones: playing memory with DNA. *Curr Opin Cell Biol. ,* 17**,** 230-8.

PETERSON-CARMICHAEL, S. L., HARRIS, W. T., GOEL, R., NOAH, T. L., JOHNSON, R., LEIGH, M. W. & DAVIS, S. D. 2009. Association of lower airway inflammation with physiologic findings in young children with cystic fibrosis. *Pediatric Pulmonology,* 44**,** 503-511.

PETERSON, C. L. & LANIEL, M.-A. 2004. Histones and histone modifications. *Current Biology,* 14**,** R546-R551.

PIER, G., GROUT, M., ZAIDI, T., OLSEN, J., JOHNSON, L., YANKASKAS, J. & GOLDBERG, J. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science,* 271(5245)**,** 64-7.

PLATH, K., FANG, J., MLYNARCZYK-EVANS, S. K., CAO, R., WORRINGER, K. A., WANG, H., DE LA CRUZ, C. C., OTTE, A. P., PANNING, B. & ZHANG, Y. 2003. Role of Histone H3 Lysine 27 Methylation in X Inactivation. *Science,* 300**,** 131-135.

POKHOLOK, D. K., HARBISON, C. T., LEVINE, S., COLE, M., HANNETT, N. M., LEE, T. I., BELL, G. W., WALKER, K., ROLFE, P. A., HERBOLSHEIMER, E., ZEITLINGER, J., LEWITTER, F., GIFFORD, D. K. & YOUNG, R. A. 2005. Genome-wide Map of Nucleosome Acetylation and Methylation in Yeast. *Cell,* 122**,** 517-527.

POMERANTZ, J. L. & BALTIMORE, D. 2002. Two Pathways to NF-κB. *Molecular cell,* 10**,** 693-695.

POSCHET, J. F., BOUCHER, J. C., TATTERSON, L., SKIDMORE, J., VAN DYKE, R. W. & DERETIC, V. 2001. Molecular basis for defective glycosylation and Pseudomonas pathogenesis in cystic fibrosis lung. *Proceedings of the National Academy of Sciences,* 98**,** 13972-13977.

PRAY-GRANT, M. G., DANIEL, J. A., SCHIELTZ, D., YATES, J. R. & GRANT, P. A. 2005. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature,* 433**,** 434-438.

PRIGENT, C. & DIMITROV, S. 2003. Phosphorylation of serine 10 in histone H3, what for? *J Cell Sci.,* 116**,** 3677-85.

PRZYBILLA, J., BUSKE, P., BINDER, H. & GALLE, J. 2013. Histone modifications control DNA methylation profiles during ageing and tumour expansion. *Frontiers in Life Science,* 7**,** 31-43.

PUKELSHEIM, K., STOEGER, T., KUTSCHKE, D., GANGULY, K. & WJST, M. 2010. Cytokine Profiles in Asthma Families Depend on Age and Phenotype. *PLoS ONE,* 5**,** e14299.

QAZI, B. S., TANG, K. & QAZI, A. 2011. Recent Advances in Underlying Pathologies Provide Insight into Interleukin-8 Expression-Mediated Inflammation and Angiogenesis. *International Journal of Inflammation,* 2011.

RAIA V, MAIURI L, CIACCI C, RICCIARDELLI I, VACCA L, AURICCHIO S, CIMMINO M, CAVALIERE M, NARDONE M, CESARO A, MALCOLM J, QUARATINO S & M., L. 2005. Inhibition of p38 mitogen activated protein kinase controls airway inflammation in cystic fibrosis. *Thorax,* 60**,** 773-80.

RAIA, V., MAIURI, L., CIACCI, C., RICCIARDELLI, I., VACCA, L., AURICCHIO, S., CIMMINO, M., CAVALIERE, M., NARDONE, M., CESARO, A., MALCOLM, J., QUARATINO, S. & LONDEI, M. 2005. Inhibition of p38 mitogen activated protein kinase controls airway inflammation in cystic fibrosis. *Thorax,* 60**,** 773-780.

RAJASEKARAN, S., VAZ, M. & REDDY, S. P. 2012. Fra-1/AP-1 Transcription Factor Negatively Regulates Pulmonary Fibrosis <italic>In Vivo</italic>. *PLoS ONE,* 7**,** e41611.

RAMJI, D. P. & FOKA, P. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J. ,* 365**,** 561-575.

RAMSEY, B. W., DAVIES, J., MCELVANEY, N. G., TULLIS, E., BELL, S. C., DŘEVÍNEK, P., GRIESE, M., MCKONE, E. F., WAINWRIGHT, C. E., KONSTAN, M. W., MOSS, R., RATJEN, F., SERMET-GAUDELUS, I., ROWE, S. M., DONG, Q., RODRIGUEZ, S., YEN, K., ORDOÑEZ, C. & ELBORN, J. S. 2011. A CFTR Potentiator in Patients with Cystic Fibrosis and the G551D Mutation. *New England Journal of Medicine,* 365**,** 1663-1672.

RANGANATHAN, S. C., PARSONS, F., GANGELL, C., BRENNAN, S., STICK, S. M., SLY, P. D. & FIBROSIS, O. B. O. T. A. R. E. S. T. F. C. 2011. Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis. *Thorax,* 66**,** 408-413.

RATJEN, F. & DÖRING, G. 2003. Cystic fibrosis. *The Lancet,* 361**,** 681-689.

REDDY, S. M. & MOSSMAN, B. T. 2002. *Role and regulation of activator protein-1 in toxicant-induced responses of the lung*.

RHEE, K., BRUNORI, M., BESSET, V., TROUSDALE, R. & WOLGEMUTH, D. J. 1998. Expression and potential role of Fsrg1, a murine bromodomain-containing homologue of the Drosophila gene female sterile homeotic. *Journal of Cell Science,* 111**,** 3541-3550.

RICCIOTTI, E. & FITZGERALD, G. A. 2011. Prostaglandins and Inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 31**,** 986-1000.

RICE, J. C., BRIGGS, S. D., UEBERHEIDE, B., BARBER, C. M., SHABANOWITZ, J., HUNT, D. F., SHINKAI, Y. & ALLIS, C. D. 2003. Histone Methyltransferases Direct

Different Degrees of Methylation to Define Distinct Chromatin Domains. *Molecular cell,* 12**,** 1591-1598.

RICHMOND, A. 2002. NF-kB, chemokine gene transcription and tumour growth. *Nat Rev Immunol,* 2(9)**,** 664-74.

ROSSI, D. & ZLOTNIK, A. 2000. The Biology of Chemokines and their Receptors. *Annual Review of Immunology,* 18**,** 217-242.

ROTH, S. Y. & ALLIS, C. D. 1992. Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends Biochem Sci,* 17**,** 93-8.

ROTH, S. Y., DENU, J. M. & ALLIS, C. D. 2001. HISTONE ACETYLTRANSFERASES. *Annual Review of Biochemistry,* 70**,** 81-120.

ROTTNER, M., FREYSSINET, J.-M. & MARTINEZ, M. C. 2009. Mechanisms of the noxious inflammatory cycle in cystic fibrosis. *Respiratory Research,* 10**,** 23.

RUBEN, S. M., NARAYANAN, R., KLEMENT, J. F., CHEN, C. H. & ROSEN, C. A. 1992. Functional characterization of the NF-kappa B p65 transcriptional activator and an alternatively spliced derivative. *Molecular and Cellular Biology,* 12**,** 444-454.

RUBENSTEIN, R. C., LOCKWOOD, S. R., LIDE, E., BAUER, R., SUAUD, L. & GRUMBACH, Y. 2011. *Regulation of endogenous ENaC functional expression by CFTR and ΔF508-CFTR in airway epithelial cells*.

RUFFELL, D., MOURKIOTI, F., GAMBARDELLA, A., KIRSTETTER, P., LOPEZ, R., ROSENTHAL, N. & NERLOV, C. 2009. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A. 2009 Oct 13;:106(41). doi: . ,* 106(41)**,** 17475-80.

RUSSO, R. C., GARCIA, C. C., TEIXEIRA, M. M. & AMARAL, F. A. 2014. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. *Expert Review of Clinical Immunology,* 10**,** 593-619.

RUTHENBURG, A. J., LI, H., PATEL, D. J. & DAVID ALLIS, C. 2007. Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol,* 8**,** 983-994.

SAADANE, A., EASTMAN, J., BERGER, M. & BONFIELD, T. 2011. Parthenolide inhibits ERK and AP-1 which are dysregulated and contribute to excessive IL-8 expression and secretion in cystic fibrosis cells. *Journal of Inflammation,* 8**,** 26.

SAADANE, A., SOLTYS, J. & BERGER, M. 2005. Role of IL-10 deficiency in excessive nuclear factor-κB activation and lung inflammation in cystic fibrosis transmembrane conductance regulator knockout mice. *Journal of Allergy and Clinical Immunology,* 115**,** 405-411.

SACCANI, S., PANTANO, S. & NATOLI, G. 2001. Two Waves of Nuclear Factor κb Recruitment to Target Promoters. *The Journal of Experimental Medicine,* 193**,** 1351-1360.

SAGEL, S., CHMIEL, J. & KONSTAN, M. 2007. Sputum biomarkers of inflammation in cystic fibrosis lung disease. *Proc Am Thorac Soc,* 1**,** 406-17.

SALOZHIN, S. V., PROKHORCHUK, E. B. & GEORGIEV, G. P. 2005. Methylation of DNA--one of the major epigenetic markers. *Biochemistry (Mosc),* 70**,** 525-32.

SANCHEZ, R. & ZHOU, M. M. 2009. The role of human bromodomains in chromatin biology and gene transcription. *Curr Opin Drug Discov Devel.,* 12**,** 659-65.

SATO, Y., OHSHIMA, T. & KONDO, T. 1999. Regulatory Role of Endogenous Interleukin-10 in Cutaneous Inflammatory Response of Murine Wound Healing. *Biochemical and Biophysical Research Communications,* 265**,** 194-199.

SAWAN, C. & HERCEG, Z. 2010. Histone Modifications and Cancer. *In:* ZDENKO, H. & TOSHIKAZU, U. (eds.) *Advances in Genetics.* Academic Press.

SCHMITZ, M. & BAEUERLE, P. 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J,* 10(12)**,** 3805-17.

SCHRAUFSTATTER, I. U., CHUNG, J. & BURGER, M. 2001. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 280**,** L1094-L1103.

SERHAN, C. N. 2008. Controlling the Resolution of Acute Inflammation: A New Genus of Dual Anti-Inflammatory and Proresolving Mediators. *Journal of Periodontology,* 79**,** 1520-1526.

SHANG, E., WANG, X., WEN, D., GREENBERG, D. A. & WOLGEMUTH, D. J. 2009. Double bromodomain-containing gene Brd2 is essential for embryonic development in mouse. *Developmental Dynamics,* 238**,** 908-917.

SHAULIAN, E. & KARIN, M. 2001. AP-1 in cell proliferation and survival. *Oncogene,* 20(19):**,** 2390-400.

SHEPPARD, D. N. & WELSH, M. J. 1999. Structure and Function of the CFTR Chloride Channel. *Physiological Reviews,* 79**,** S23-S45.

SHI, Q., XIONG, Q., LE, X. & XIE, K. 2004. Regulation of Interleukin-8 Expression by Tumor-Associated Stress Factors *Journal of Interferon & Cytokine Research,* 21**,** 553–566.

SHIIO, Y. & EISENMAN, R. N. 2003. Histone sumoylation is associated with transcriptional repression. *Proc Natl Acad Sci USA,* 100**,** 13225-30.

SHILATIFARD, A. 2006. Chromatin Modifications by Methylation and Ubiquitination: Implications in the Regulation of Gene Expression. *Annual Review of Biochemistry,* 75**,** 243-269.

SIGALOTTI, L., FRATTA, E., CORAL, S., CORTINI, E., COVRE, A., NICOLAY, H. J. M., ANZALONE, L., PEZZANI, L., DI GIACOMO, A. M., FONSATTI, E., COLIZZI, F., ALTOMONTE, M., CALABRÒ, L. & MAIO, M. 2007. Epigenetic drugs as pleiotropic agents in cancer treatment: Biomolecular aspects and clinical applications. *Journal of Cellular Physiology,* 212**,** 330-344.

SINHA, A., FALLER, D. V. & DENIS, G. V. 2005. Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. *Biochem. J.,* 387**,** 257-269.

SMITH, J., TRAVIS, S., GREENBERG, E. & WELSH, M. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell,* 85(2)**,** 229-36.

SOEHNLEIN, O. & LINDBOM, L. 2010. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol,* 10**,** 427-439.

SONG, J., NOH, J. H., LEE, J. H., EUN, J. W., AHN, Y. M., KIM, S. Y., LEE, S. H., PARK, W. S., YOO, N. J., LEE, J. Y. & NAM, S. W. 2005. Increased expression of histone deacetylase 2 is found in human gastric cancer. *APMIS,* 113**,** 264-268.

SPEYER, C. L. & WARD, P. A. 2011. Role of Endothelial Chemokines and Their Receptors during Inflammation. *Journal of Investigative Surgery,* 24**,** 18-27.

SPIEGELMAN, B. M. & HEINRICH, R. 2004. Biological Control through Regulated Transcriptional Coactivators. *Cell,* 119**,** 157-167.

STARNER, T. D. & MCCRAY, J. P. B. 2005. Pathogenesis of Early Lung Disease in Cystic Fibrosis: A Window of Opportunity To Eradicate Bacteria. *Annals of Internal Medicine,* 143**,** 816-822.

STECENKO, A., KING, G., TORII, K., BREYER, R., DWORSKI, R., BLACKWELL, T., CHRISTMAN, J. & BRIGHAM, K. 2001. Dysregulated Cytokine Production in Human Cystic Fibrosis Bronchial Epithelial Cells. *Inflammation,* 25**,** 145-155.

STEINKE, J. W. & BORISH, L. 2006. 3. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology,* 117**,** S441-S445.

STERNER, R., VIDALI, G. & ALLFREY, V. G. 1979. Studies of acetylation and deacetylation in high mobility group proteins. Identification of the sites of acetylation in HMG-1. *Journal of Biological Chemistry,* 254**,** 11577-11583.

STRAHL, B. D. & ALLIS, C. D. 2000. The language of covalent histone modifications. *Nature,* 403**,** 41-45.

STRIETER, R. M. 2002. Interleukin-8: a very important chemokine of the human airway epithelium. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 283**,** L688-L689.

TAKECHI, S. & NAKAYAMA, T. 1999. Sas3 Is a Histone Acetyltransferase and Requires a Zinc Finger Motif. *Biochemical and Biophysical Research Communications,* 266**,** 405-410.

TALBERT, P. B. & HENIKOFF, S. 2006. Spreading of silent chromatin: inaction at a distance. *Nat Rev Genet,* 7**,** 793-803.

TERHEGGEN-LAGRO, S. W. J., RIJKERS, G. T. & VAN DER ENT, C. K. 2005. The role of airway epithelium and blood neutrophils in the inflammatory response in cystic fibrosis. *Journal of Cystic Fibrosis,* 4, Supplement 2**,** 15-23.

THELEN, M. 2001. Dancing to the tune of chemokines. *Nat Immunol,* 2**,** 129-134.

TRASK, O. J. J. 2012. *uclear Factor Kappa B (NF-κB) Translocation Assay Development and Validation for High Content Screening,* Eli Lilly & Company and the National Center for Advancing Translational Sciences.

TROTTER, K. & ARCHER, T. 2008. The BRG1 transcriptional coregulator. *Nucl Recept Signal,* 1;6e004.

TSAPROUNI, L., ITO, K., POWELL, J., ADCOCK, I. & PUNCHARD, N. 2011. Differential patterns of histone acetylation in inflammatory bowel diseases. *Journal of Inflammation,* 8**,** 1.

URNOV, F. & WOLFFE, A. 2001. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). *Oncogene,* 20(24)**,** 2991-3006.

VALENZUELA-FERNÁNDEZ, A., CABRERO, J. R., SERRADOR, J. M. & SÁNCHEZ-MADRID, F. 2008. HDAC6: a key regulator of cytoskeleton, cell migration and cell–cell interactions. *Trends in Cell Biology,* 18**,** 291-297.

VALK-LINGBEEK, M. E., BRUGGEMAN, S. W. M. & VAN LOHUIZEN, M. 2004. Stem Cells and Cancer: The Polycomb Connection. *Cell,* 118**,** 409-418.

VAN COILLIE, E., VAN DAMME, J. & OPDENAKKER, G. 1999. The MCP/eotaxin subfamily of CC chemokines. *Cytokine & Growth Factor Reviews,* 10**,** 61-86.

VAN GOOR, F., HADIDA, S., GROOTENHUIS, P. D. J., BURTON, B., CAO, D., NEUBERGER, T., TURNBULL, A., SINGH, A., JOUBRAN, J., HAZLEWOOD, A., ZHOU, J., MCCARTNEY, J., ARUMUGAM, V., DECKER, C., YANG, J., YOUNG, C., OLSON, E. R., WINE, J. J., FRIZZELL, R. A., ASHLOCK, M. & NEGULESCU, P. 2009. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proceedings of the National Academy of Sciences,* 106**,** 18825-18830.

VENTERS, B. J. & PUGH, B. F. 2009. How eukaryotic genes are transcribed. *Critical Reviews in Biochemistry and Molecular Biology,* 44**,** 117-141.

VENZA, I., CUCINOTTA, M., VISALLI, M., DE GRAZIA, G., OLIVA, S. & TETI, D. 2009. Pseudomonas aeruginosa Induces Interleukin-8 (IL-8) Gene Expression in Human Conjunctiva through the Recruitment of Both RelA and CCAAT/Enhancer-binding Protein β to the IL-8 Promoter. *Journal of Biological Chemistry,* 284**,** 4191-4199.

VERHAEGHE, C., DELBECQUE, K., DE LEVAL, L., OURY, C. & BOURS, V. 2007a. Early inflammation in the airways of a cystic fibrosis foetus. *Journal of Cystic Fibrosis,* 6**,** 304-308.

VERHAEGHE, C., REMOUCHAMPS, C., HENNUY, B., VANDERPLASSCHEN, A., CHARIOT, A., TABRUYN, S. P., OURY, C. & BOURS, V. 2007b. Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways. *Biochemical Pharmacology,* 73**,** 1982-1994.

VERMEULEN, M., MULDER, K. W., DENISSOV, S., PIJNAPPEL, W. W. M. P., VAN SCHAIK, F. M. A., VARIER, R. A., BALTISSEN, M. P. A., STUNNENBERG, H. G., MANN, M. & TIMMERS, H. T. M. 2007. Selective Anchoring of TFIID to Nucleosomes by Trimethylation of Histone H3 Lysine 4. *Cell,* 131**,** 58-69.

VIOLA, A. & LUSTER, A. D. 2008. Chemokines and Their Receptors: Drug Targets in Immunity and Inflammation. *Annual Review of Pharmacology and Toxicology,* 48**,** 171-197.

WAGNER, E. F. 2001. AP-1-Introductory remarks. *Oncogene,* 20**,** 2334-5.

WANG, D., PAZ-PRIEL, I. & FRIEDMAN, A. D. 2009a. NF-κB p50 Regulates C/EBPα Expression and Inflammatory Cytokine-Induced Neutrophil Production. *The Journal of Immunology,* 182**,** 5757-5762.

WANG, F., LIU, H., BLANTON, W. P., BELKINA, A., LEBRASSEUR, N. K. & DENIS, G. V. 2009b. Brd2 disruption in mice causes severe obesity without Type. *Biochemical Journal* 425**,** 71-83.

WANG, L. 2009. *Mechanistic and Structural Studies on P300/CBP Transcriptional Coactivator*.

WANG, L. & FREEDMAN, S. 2002. Laboratory tests for the diagnosis of cystic fibrosis. *Am J Clin Pathol,* 117 Suppl**,** 109-15.

WANG, X. & ZHU, W. 2008. Advances in histone methyltransferases and histone demethylases. *Ai Zheng,* 27**,** 1018-25.

WATERBORG, J. H. 2002. Dynamics of histone acetylation in vivo. A function for acetylation turnover? *Biochem Cell Biol,* 80(3)**,** 363-78.

WEAKE, V. M. & WORKMAN, J. L. 2008. Histone ubiquitination: triggering gene activity. *Mol Cell.,* 29**,** 653-63.

WEBER, M., DAVIES, J. J., WITTIG, D., OAKELEY, E. J., HAASE, M., LAM, W. L. & SCHUBELER, D. 2005. Chromosome-wide and promoter-specific analyses

identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet,* 37**,** 1061-4036.

WELSH, M. J., TSUI, L., BOAT. T. F. & BEADET, A. L. 1995. *Cystic fibrosis. The Metabolic and Molecular Basis of Inherited Disease,* New York, McGraw-Hill.

WHO 2010. Genes and human disease.

WILSON, A. G. 2008. Epigenetic Regulation of Gene Expression in the Inflammatory Response and Relevance to Common Diseases. *Journal of Periodontology,* 79**,** 1514-1519.

WINE, J. J. 1999. The genesis of cystic fibrosis lung disease. *The Journal of Clinical Investigation,* 103**,** 309-312.

XIA, C., WATTON, S., NAGL, S., SAMUEL, J., LOVEGROVE, J., CHESHIRE, J. & WOO, P. 2004. Novel sites in the p65 subunit of NF-κB interact with TFIIB to facilitate NF-κB induced transcription. *FEBS Letters,* 561**,** 217-222.

XU, Y., CLARK, J. C., ARONOW, B. J., DEY, C. R., LIU, C., WOOLDRIDGE, J. L. & WHITSETT, J. A. 2003. Transcriptional Adaptation to Cystic Fibrosis Transmembrane Conductance Regulator Deficiency. *Journal of Biological Chemistry,* 278**,** 7674-7682.

YALCIN, A. D., BISGIN, A. & GORCZYNSKI, R. M. 2012. IL-8, IL-10, TGF-, and GCSF Levels Were Increased in Severe Persistent Allergic Asthma Patients with the Anti-IgE Treatment. *Mediators of Inflammation,* 2012**,** 8.

YANG, S.-R., WRIGHT, J., BAUTER, M., SEWERYNIAK, K., KODE, A. & RAHMAN, I. 2007. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-κB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 292**,** L567-L576.

YANG, X. J. 2004. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Research,* 32**,** 959-976.

YANG, X. J. & SETO, E. 2007. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene,* 26**,** 5310-5318.

YANG, Z., YIK, J. H. N., CHEN, R., HE, N., JANG, M. K., OZATO, K. & ZHOU, Q. 2005. Recruitment of P-TEFb for Stimulation of Transcriptional Elongation by the Bromodomain Protein Brd4. *Molecular cell,* 19**,** 535-545.

YASUMOTO, K., OKAMOTO, S., MUKAIDA, N., MURAKAMI, S., MAI, M. & MATSUSHIMA, K. 1992. Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. *Journal of Biological Chemistry,* 267**,** 22506-11.

YEUNG, F., HOBERG, J. E., RAMSEY, C. S., KELLER, M. D., JONES, D. R., FRYE, R. A. & MAYO, M. W. 2004. Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *The EMBO Journal,* 23**,** 2369-2380.

YOSHIDA, M., HORINOUCHI, S. & BEPPU, T. 1995. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *Bioessays,* 17**,** 423-30.

YU, S.-H., CHIANG, W.-C., SHIH, H.-M. & WU, K.-J. 2004. Stimulation of c-Rel transcriptional activity by PKA catalytic subunit β. *Journal of Molecular Medicine,* 82**,** 621-628.

ZABNER, J., SMITH, J. J., KARP, P. H., WIDDICOMBE, J. H. & WELSH, M. J. 1998. Loss of CFTR Chloride Channels Alters Salt Absorption by Cystic Fibrosis Airway Epithelia In Vitro. *Molecular cell,* 2**,** 397-403.

ZEITLIN, P. L., LU, L., RHIM, J., CUTTING, G., STETTEN, G., KIEFFER, K. A., CRAIG, R. & GUGGINO, W. B. 1991. A Cystic Fibrosis Bronchial Epithelial Cell Line: Immortalization by Adeno-12-SV40 Infection. *American Journal of Respiratory Cell and Molecular Biology,* 4**,** 313-319.

ZENZ, R., EFERL, R., SCHEINECKER, C., REDLICH, K., SMOLEN, J., SCHONTHALER, H., KENNER, L., TSCHACHLER, E. & WAGNER, E. 2008. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Research & Therapy,* 10**,** 201.

ZHAO, Y., YANG CY & S., W. 2013. The making of I-BET762, a BET bromodomain inhibitor now in clinical development. *J Med Chem,* 56**,** 7498-500.

ZHENG, Y., JOHN, S., PESAVENTO, J. J., SCHULTZ-NORTON, J. R., SCHILTZ, R. L., BAEK, S., NARDULLI, A. M., HAGER, G. L., KELLEHER, N. L. & MIZZEN, C. A. 2010. Histone H1 phosphorylation is associated with transcription by RNA polymerases I and II. *The Journal of Cell Biology,* 189**,** 407-415.

ZIELENSKI J, T. L. 1995. Cystic fibrosis: genotypic and phenotypic variations. *Annu Rev Genet,* 29**,** 777-807.

ZLOTNIK, A. & YOSHIE, O. 2000. Chemokines: A New Classification System and Their Role in Immunity. *Immunity,* 12**,** 121-127.

ZOU, Z., HUANG B, WU X, ZHANG H, QI J, BRADNER J, NAIR S & LF., C. 2014. Brd4 maintains constitutively active NF-κB in cancer cells by binding to acetylated RelA. *Oncogene* 33**,** 2395-404.

ZUPKOVITZ, G., TISCHLER, J., POSCH, M., SADZAK, I., RAMSAUER, K., EGGER, G., GRAUSENBURGER, R., SCHWEIFER, N., CHIOCCA, S., DECKER, T. & SEISER, C. 2006. Negative and Positive Regulation of Gene Expression by Mouse Histone Deacetylase 1. *Molecular and Cellular Biology,* 26**,** 7913-7928.