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REGULATION OF GENE EXPRESSION IN PULMONARY INFLAMMATION AND DIFFERENTIATION:
A ROLE FOR C/EBP TRANSCRIPTION FACTORS

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ABSTRACT

CCAAT/enhancer-binding protein (C/EBP) transcription factors play essential roles in gene regulation. The lung-enriched isoform C/EBPα is known to inhibit proliferation, promote differentiation and stimulate gene expression characteristic of the mature differentiated pulmonary epithelium. C/EBPβ, also enriched in the lung, plays a role in cell differentiation and the regulation of inflammatory and host defense genes in several organs. The activity of C/EBPβ is decreased in smokers with chronic obstructive pulmonary disease (COPD), indicating a role in COPD pathogenesis. The objective of this thesis was to investigate the unique or overlapping roles of C/EBPα and C/EBPβ in lung epithelial differentiation, and to assess the contribution of C/EBPβ in regulating pulmonary inflammation.

To investigate unique *vs.* overlapping roles of C/EBPα and C/EBPβ in the lung, the pulmonary phenotype of mice lacking C/EBPα (*Cebpa*$^{\Delta LE}$ mice), C/EBPβ (*Cebpb*$^{\Delta LE}$ mice) or both C/EBPα and C/EBPβ (*Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice) specifically in the lung epithelium, all generated by *SFTPC*-Cre mediated excision, was investigated. Cell culture experiments suggested that C/EBPα and C/EBPβ bind the same elements within a lung-specific promoter, and that their functions are partially overlapping. Pre-natal *Cebpa*$^{\Delta LE}$ mice and *Cebpa*$^{\Delta LE}$*;* *Cebpb*$^{\Delta LE}$ mice displayed immature lungs similar to the lungs of premature infants, and *Cebpa*$^{\Delta LE}$*;* *Cebpb*$^{\Delta LE}$ mice exhibited even more impaired airway epithelial cell differentiation than the *Cebpa*$^{\Delta LE}$ mice. The proportion of *Cebpa*$^{\Delta LE}$ mice that survived and reached adulthood spontaneously developed a majority of the histopathological hallmarks of COPD, possibly caused by infiltrating inflammatory cells – similar to what is observed in COPD and what is mechanistically proposed to drive COPD pathogenesis. These findings are indicative of a relationship between immature lungs at birth, C/EBPs and the development of inflammatory lung disease.

Considering the previous documentation of decreased airway epithelial C/EBPβ activity in smokers with COPD, C/EBPβ could have a role in COPD pathogenesis. The role of C/EBPβ in regulating inflammatory and innate immune responses in the lung was on this account investigated by employing a translational approach encompassing clinical samples as well as *in vitro* and *in vivo* experiments. *CEBPB* was significantly down-regulated in the airway epithelium of both current and former smokers compared to never-smokers, and in cigarette smoke extract-treated primary human airway epithelial cells *in vitro*, suggesting that C/EBPβ plays a role in smoking-induced disease. Supporting this, inhibition of *CEBPB* in human airway cells *in vitro* resulted in a compromised inflammatory response to smoke. Moreover, cigarette smoke-exposed *Cebpb*$^{\Delta LE}$ mice displayed reduced respiratory neutrophilia and induction of inflammatory mediators, including the neutrophil chemoattractant *Groa*, compared to smoke-exposed controls. LPS-challenged *Cebpb*$^{\Delta LE}$ mice also exhibited blunted respiratory neutrophilia and lower pulmonary expression of *Groa*, compared to LPS-challenged control littermates. In addition, suppression of LPS-induced neutrophilia and inflammatory gene expression by formoterol, a long acting β_2-adrenoceptor agonist used in treatment of COPD, was impaired in *Cebpb*$^{\Delta LE}$ mice. C/EBP transactivation was increased by treatment with formoterol *in vitro*, possibly through a β_2-adrenoceptor and cAMP-dependent mechanism. This demonstrates that both inflammatory as well as anti-inflammatory stimuli involve regulation of gene transcription by C/EBPβ.

Taken together, these findings demonstrate that C/EBPα and C/EBPβ play pivotal and partly overlapping roles in airway epithelial differentiation, and that C/EBPβ and the lung epithelium orchestrates inflammatory responses as well as anti-inflammatory signaling by β_2-adrenoceptor agonists in the lung. Thus, C/EBPs may influence tissue regeneration in lung homeostasis and disease as well as inflammatory and anti-inflammatory signaling, and are potential contributors to COPD pathogenesis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Transkriptionsfaktorer är proteiner som binder till reglerande element i gener och kontrollerar cellens genuttryck. CCAAT/enhancer-binding proteiner, C/EBP:er, är högt uttryckta transkriptionsfaktorer som är betydelsefulla för en rad vitala cellulära processer. C/EBPα, som är anrikad i lungan, bidrar till att stoppa celldelning och stimulerar samtidigt utmognad av de specialiserade celler som är karaktäristiska för den fullt utvecklade lungan. En annan C/EBP faktor som är högt uttryckt i lungan, C/EBPβ, spelar också en viktig roll för cellers utmognad, och har dessutom föreslagits vara viktig för reglering av inflammatoriska och immunförsvarsgener. Förmågan hos C/EBPβ att binda till DNA och därmed aktivera geners uttryck är lägre hos rökare med kronisk obstruktiv lungsjukdom (KOL), men inte hos rökare utan luftvägssymptom, vilket antyder att C/EBPβ skulle kunna bidra till sjukdomsutvecklingen. KOL är en långsamt förlöpande lungsjukdom orsakad av de inflammatoriska processer som tobaksrökning framkallar, och utmärks av en minskad lungfunktion. Sjukdomsprocessen omfattar inflammation och förträngning av lungans små luftvägar (bronkiolit), samt nedbrytning (emfysem) av de små lungblåsorna som ansvarar för gasutbyte i lungan (alveolerna). Dessutom ses förändringar av luftvägsepitelet, där en ökning av antalet slemproducerade celler är karakteristisk. Målet med denna avhandling var att undersöka funktionerna av C/EBPα och C/EBPβ i utmognaden av luftvägsepitelet, och utreda ifall C/EBPβ bidrar till luftvägsinflammation och hämning av denna inflammation, vilket kan ha implikationer för sjukdomsprocesser och behandlingsstrategier i KOL.

Initiala cellförsök antydde att C/EBPα och C/EBPβ binder till samma genreglerande DNA-element, och att dessa transkriptionsfaktorer har delvis överlappande roller. I likhet med förtidigt födda barn, som ofta drabbas av akut andnöd och behöver andningsstöd, uppvisar möss som saknar C/EBPα specifikt i lungans epitel omogna lungor. Hos möss som saknar både C/EBPα och C/EBPβ i lungans epitel är lungorna än mer underutvecklade och utmärks av bristfällig utmognad av luftvägsepitelet samt ökat antal slemproducerande celler, i likhet med patologin i inflammatoriska luftvägssjukdomar. Dessutom utvecklar vuxna möss som saknar C/EBPα spontant en majoritet av de lungvävnadsförändringar som är karakärisktiska för KOL. Dessa patologiska förändringar skulle kunna förklaras av den inflammatoriska bild mössen uppvisar, liknande den inflammation som orsakar och driver KOL. Detta antyder ett orsaksförhållande mellan omogna lungor vid födeln, C/EBP-faktorer och utvecklingen av inflammatorisk obstruktiv lungsjukdom senare i livet. Stora kliniska studier som undersöker förekomsten av lungfunktionsnedsättning och luftvägsobstruktion hos vuxna individer som fötts för tidigt är därför av stort intresse. Större kunskap inom detta område kan förbättra behandlingsmetoderna av förtidigt födda barn, och därmed minska riskerna för dessa individer att utveckla luftvägsobstuktion senare i livet.

Eftersom C/EBPβ potentiellt skulle kunna bidra till insjuknandet och sjukdomsförloppet i KOL undersöktes den roll C/EBPβ spelar i luftvägsinflammation. Genuttrycket av C/EBPβ är lägre i luftvägsepitelet hos rökare, jämfört med icke-rökare, samt i odlade epitelceller som exponerats för cigarettrök. Dessutom är den inflammatoriska reaktionen mot cigarettrök försvagad i epitelceller med inhiberat uttryck av C/EBPβ, samt i möss som saknar C/EBPβ i lungans epitel. Detta stödjer att C/EBPβ i lungans epitel bidrar till de inflammatoriska processer som vållas av cigarettrökning, vilka betraktas som centrala i sjukdomsbilden i KOL. Bevarade immunreaktioner är emellertid viktiga för att bekämpa luftvägsinfektioner, som är ofta förekommande bland patienter med KOL och anses accelerera sjukdomsförloppet. Möss som saknar C/EBPβ i lungans epitel har mycket riktigt ett dämpat inflammatoriskt svar mot bakteriekomponenten endotoxin, vilket antyder att försvaret mot bakterieinfektioner är nedsatt i dessa möss. β_2-agonister används rutinmässigt i behandlingen av inflammatorisk lungsjukdom, såsom KOL. Dessa läkemedel verkar luftrörsvidgande, men har även inflammationshämmande effekter. Dock är de positiva effekterna av inhalerade β_2-agonister relativt små bland KOL patienter. I likhet med detta var hämningen av luftvägsinflammation med β_2-agonister utebliven i möss som saknar C/EBPβ. Ytterligare försök visade att och β_2-agonister ökar inbindning av

C/EBP-faktorer till DNA, som potentiellt skulle kunna hämma luftvägsinflammation. Den minskade DNA-bindande aktiviteten av C/EBPβ hos rökare med KOL skulle därför kunna förklara den ringa anti-inflammatoriska effekten av inhalerade β_2-agonister, samt den ökade känslighet mot luftvägsinfektioner, som föreligger vid denna sjukdom.

Tillsammans visar resultaten i den här avhandlingen att C/EBP-faktorer är betydelsefulla för utmognaden av luftvägsepitelet och att C/EBPβ bidrar till inflammation såväl som hämning av inflammatoriska processer i lungan. C/EBP:er kan således påverka immunförsvar samt regenerering av luftvägsepitelet i olika inflammatoriska sjukdomstillstånd, och är potentiellt bidragande faktorer i utvecklingen av KOL. Ökade kunskaper om C/EBP-faktorer kan öka förståelsen kring sjukdomen KOL och därmed också bidra till bättre behandlingmöjligheter för denna utsatta patientgrupp.

LIST OF PUBLICATIONS

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CONTENTS

LIST OF ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
ALI	Acute lung injury
BASC	Bronchoalveolar stem cell
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary dysplasia
BUD	Budesonide
C/EBP	CCAAT/enhancer-binding protein
cDNA	Complimentary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CSE	Cigarette smoke extract
DsRNA	Double stranded ribonucleic acid
E	Embryonic day
E-cadherin	Epithelial cadherin
EMSA	Electrophoretic mobility shift assay
FEV_1	Forced expiratory volume in one second
Fl/Floxed	Flanked by lox P sites
FM	Formoterol
FoxJ1	Forkhead box transcription factor J1
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
G-CSF/*Csf3*	Granulocyte-colony stimulating factor
GM-CSF/*Csf2*	Granulocyte macrophage-colony stimulating factor
GRO	Growth regulated oncogene
HPRT	Hypoxanthine phosphoribosyltransferase
ICS	Inhaled corticosteroid
IL	Interleukin
iNOS/*Nos2*	Inducible nitric oxide synthase
LABA	Long-acting β2-agonist
LPS	Lipopolysaccharide
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid

MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor κ B
NHBE	Normal human bronchial epithelial
NKX2.1	NK2 homeobox 1
Nod1	Nucleotide-binding oligomerization domain-containing protein 1
P	Postnatal day
PCLS	Precision cut lung slices
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
RDS	Respiratory distress syndrome
SAA	Serum amyloid A
SCGB1A1	Secretoglobin, family 1A, member 1 (Clara cell secretory protein)
siRNA	Small interfering RNA
SP-A	Surfactant protein A
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α

1 INTRODUCTION

1.1 THE LUNG AND AIRWAYS

The mammalian lung and airways consist of a series of morphologically distinct cell types with unique functions, and include cells of epithelial, lymphoid, vascular, muscular as well as nervous origin. The main function of the respiratory system is to facilitate gas exchange and supply the circulatory system with oxygen, at the same time as carbon dioxide is removed from the bloodstream. The lung epithelium plays a central role in maintaining the conduit for air to and from the lung parenchyma, as well as in facilitating gas exchange in the alveoli. The surface epithelium lining the airways also serves as a first line of defense against invading pathogens, as it is the site of initial contact for both environmental and inflammatory stimuli [1, 2]. More specifically, the airway epithelium attracts and activates inflammatory cells, clears inhaled agents but also regulates lung fluid balance and smooth muscle cell activity.

The proximal part of the airways consists of the trachea, bronchi and bronchioles, while respiratory bronchioles constitute the distal airways (Figure 1). In the human lung, the respiratory bronchioles represent an extensive zone of transition ranging between the most distal part of the conducting airways (i.e. the terminal bronchioles) and the alveoli. In contrast, this zone is completely absent in the murine airways. The airway wall of the respiratory bronchioles are interrupted by alveolar outpockets, which in similarity to the alveolar sacs of the acinus mediate gas exchange [3].

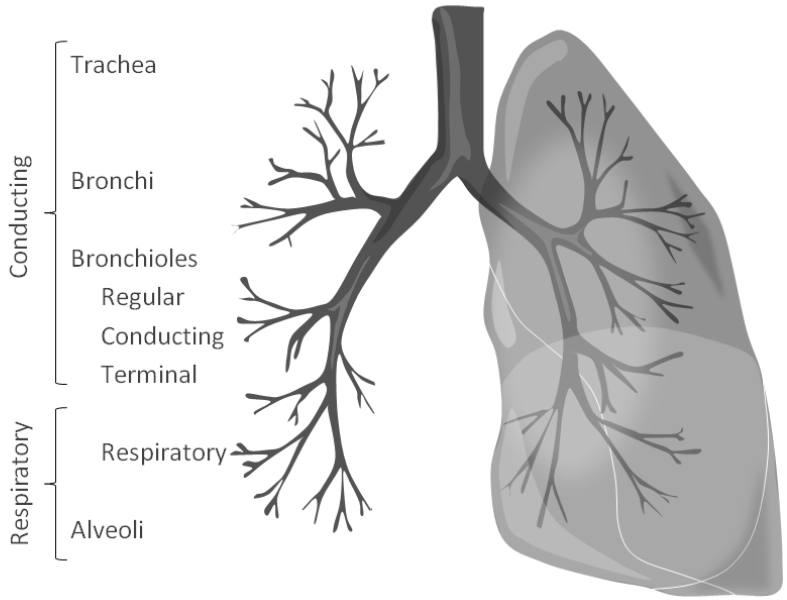

Figure 1. The human respiratory tree. *The trachea divides into the main stem bronchi and subsequently several generations of bronchi. These in turn give rise to regular, conducting, terminal and respiratory bronchioles, the former part of the conducting airways and the latter part of the respiratory airways.*

1.1.1 Lung development

Lung formation is divided into five structural periods: embryonic initiation, and the pseudoglandular, canalicular, saccular and alveolar stages. These periods are all shared between species, although temporal and regional diversities exist [4]. During lung organogenesis, the respiratory epithelium distal to the trachea grows and divides, and gives rise to the bronchi, bronchioles as well as the alveolar ducts [3]. In total, the respiratory tree consists of between 2^{21} and 2^{23} branches in the adult human lung. Our understanding of branching morphogenesis has been significantly advanced by studies in *Drosophila melanogaster,* as well as the assessment of these processes in mice [5, 6]. Also, extensive documentation of different signaling molecules and transcription factors involved in lung developmental processes exist from animal models. Of note, many of these signaling pathways are implicated in pathological conditions in the adult lung [7].

Embryonic stage/Initiation
At approximately day 28 (week 3) in humans and embryonic day (E)9.5 in mice, the establishment of localized NK2 homeobox (NKX)2-1 expression in the foregut endoderm induces the formation of two primary lung buds that evaginate into the visceral mesenchyme [8, 9]. Removal of the visceral mesenchyme blocks lung growth and branching morphogenesis [10], which confirms that paracrine signaling between mesenchymal cells surrounding the endodermally-derived tube is crucial for lung organogenesis, as originally proposed by Alescio and colleagues in 1962 [11]. This intricate signaling includes sonic hedgehog (SHH) in the pulmonary epithelium and fibroblast growth factors (FGFs) in the surrounding mesenchyme, which are crucial for early lung development [6, 7, 12]. As the embryonic stage of lung development continues, the major bronchi are formed and septation of the tracheal-esophageal tube occurs.

Pseudoglandular stage
The subsequent step in lung development, the pseudoglandular period, occurs between week 5-17 in humans and from E11.5 to E16.5 in mice. Stereotypic branching and budding, a repetitive process of endbud invasion into the mesenchyme, peaks in the pseudoglandular period and is a hallmark of this stage [6, 10, 12, 13]. Data obtained from the developing mouse lung has been used to generate a complete map of branching morphogenesis, reveling a highly complex and strictly controlled pattern of branching [5]. The temporal-spatial expression pattern and cooperative function of gene regulating proteins represent the principal mechanisms by which branching morphogenesis is controlled, and are hence of particular importance for lung development. NKX2-1, expressed in epithelial cells of the dividing lung bud in the pseudoglandular period, is together with signaling molecules and gene regulating proteins such as transforming growth factor (TGF)β, FGFs and GATA binding proteins (GATAs) putatively central in lung branching morphogenesis [5, 9, 10, 12-14]. In addition, CCAAT/enhancer-binding proteins (C/EBPs) are first detected at the end of the pseudoglandular period (around E15.5 in mice), and these transcription factors are also implicated in lung development [15].

The general lung structure is well-established by E16.5 in mice. Furthermore, the mesenchyme surrounding the budding epithelium forms blood vessels by vasculogenesis, smooth muscle cells are first detected and innervation of the lung

begins in this stage [6, 10]. Although the structure of the airways is established by the end of the pseudoglandular period, the epithelial cells lining the airways are relatively undifferentiated before the canalicular stage. As discussed in further detail below, autocrine-paracrine and cell-cell interactions are vital in epithelial cell differentiation and lung maturation [10].

The canalicular stage

In the canalicular period, week 16-26 in humans and E16.5-17.5 in mice, peripheral branching morphogenesis continues and the respiratory bronchioles appear in the human airways. In addition, the vascular bed develops, innervation continues and the pulmonary acinus forms [4, 10]. Capillaries are arranged to surround the airspaces, with many sites of close contact with the cuboidal epithelium. Thus, the formation of the blood-air barrier starts in the canalicular period, as differentiation of type I pneumocytes begins [4, 16]. An important role for forkhead box (FOX) proteins and reduced paracrine SHH signaling has been proposed in many of the processes in the canalicular stage of lung development, specifically in lung maturation and cellular differentiation [17].

The saccular and alveolar stage

Differentiation of the epithelium is a key feature of the saccular stage, between week 24-38 in humans and E17.5-postnatal day (P)5 in mice [4, 10]. In this period of lung development, the peripheral airways dilate and the lung saccules are increasingly vascularized. Also, in the beginning of this period, production and secretion of surfactant is initiated, a process that is associated with the differentiated epithelium. Thus, the saccular period is a crucial step in lung development, in which the lung is prepared for the first breath [10]. In line with this, the beginning of the saccular period currently represents the limit of viability for premature births [4]. As in the previous canalicular stage, an important role for C/EBPα has been demonstrated for lung maturation in this stage [15, 18-20].

In the final, alveolar stage of lung development, week 38 to maturity in humans and P5-P28 in mice, the alveoli grow and septate, and the maturation of the pulmonary vasculature continues [4, 10].

Table 1

Histological stages of lung development

Stage	Week (humans)	Day (mice)	Process
Embryonic	w3 - w5	E9.5 - E11.5	Budding
Psudoglandular	w5 - w17	E11.5 - E16.5	Branching
Canalicular	w16 - w26	E16.5 - E17.5	Proximo-distal differentiation
Saccular	w24 - w38	E17.5 - P5	Continued differentiation
Alveolar	w38 -	P5 - P30	Alveologenesis

Table 1. Histological stages of the human and murine lung development. *Indicated are the stages, time spans and processes involved in lung formation and maturation. E: embryonic day; P: post-natal day; W: week.*

1.1.2 Airway epithelial cells

A greater knowledge of the structure and function of the airways is essential to advance our understanding of pulmonary disorders. Pathological airway remodeling, including impaired epithelial differentiation, goblet and basal cell hyperplasia, goblet and squamous cell metaplasia/dysplasia and epithelial to mesenchymal transition are integral parts of many lung diseases [21, 22]. In addition, alterations that do not directly involve the epithelium, including smooth muscle hyperplasia, thickening of the basal lamina as well as fibrosis and inflammatory cell accumulation also occur in lung diseases. Although the fundamental roles of these changes have been documented, the etiology of airway remodeling remains poorly understood [21].

The respiratory epithelium consists of at least eight distinctly different epithelial cell types with unique morphology (Figure 2) [10] . Based on ultrastructure, function and biochemistry, these cells can be grouped as basal, ciliated, secretory or neuroendocrine cells. Airway epithelial cells are polarized, with an apical side facing the airway lumen, and a basal side resting on the basement membrane and lamina propria. The latter rests upon the submucosa and contains bronchial blood vessels, nerve bundles, mononuclear cells as well as fibroblasts. The submucosa of the trachea and large bronchi includes a large amount of mucus producing glands, muscle cells and cartilage, and lies on a thin adventitial coat. The epithelial cells of the airways form a semi-impermeable sheet, held together by anchoring junction proteins, i.e. tight junctions, intermediate junctions and desmosomes. These proteins contribute to homeostasis by selectively regulating the passage of water, ions, small neutral molecules and inflammatory cells. Tight junctions are the most apical junction proteins and consist of intricate belt-like networks of strands and grooves across neighboring airway cells, and are reportedly altered in inflammatory lung diseases [23]. In addition, integrins mediate contact to inflammatory cells and adhesion to components within the extracellular matrix, and thereby contribute to the stability of the airway epithelium as well as immune responses [24].

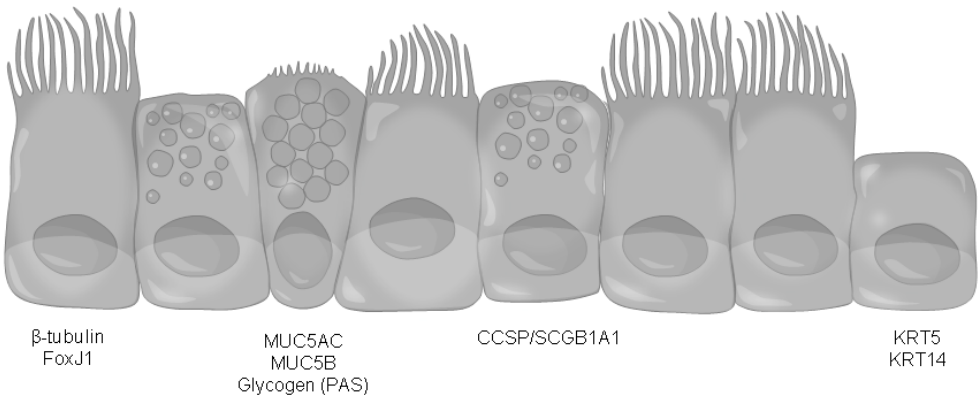

Figure 2. Airway epithelial cells. *Ciliated cells, the most abundant cells in the human airways (depicted on the far left) express β-tubulin and FoxJ1. Mucus cells (seen third from the left) express mucins such as MUC5AC and MUC5B, and the glycogen in these cells stain positive with periodic-acid Schiff staining. A Clara cell, the most abundant cell type in the murine airway, is illustrated with the characteristic granules, (seen as the unciliated cell in the middle). Clara cells are defined by the expression of the Clara cell secretory protein (SCGB1A1). Basal cells (far right) express keratin (KRT)5 and KRT14.*

The epithelium of the larynx is composed of stratified squamous cells, but in the trachea and main bronchi this is replaced by a pseudostratified columnar epithelium, which is predominated by ciliated and basal cells and interspersed with mucus cells. In the more distal conducting airways, there is a simplified columnar epithelium consisting of ciliated, basal, and Clara cells. Finally, in the most distal parts of the airways, the respiratory bronchioles, a simple cuboidal cell layer is found, consisting entirely of ciliated and Clara cells. In the distal parts of the lung, Clara cells replace mucus cells, which are rarely found in the distal airways [25, 26]. The mechanisms that control differentiation of the different cell types constituting the airway epithelium are highly complex and generally involve cell-cell, as well as autocrine-paracrine interactions, with activation of key transcription factors [1, 10, 27-30]. There is still an incomplete understanding of these mechanisms, although the processes are under intense scrutiny.

Basal cells

Basal cells, which have been proposed to be the primary stem cell in the human airway, give rise to both ciliated and secretory cells [2, 31, 32]. Basal cells constitute 6-30% of the epithelial cells in the airways [21] but are sparse in the distal airways, where Clara cells are suggested to act as the primary stem cells [2]. The transcription factor TRP-63 (p63) is together with members of the FOX and sex determining region box (SOX) family, as well as cytokeratin (KRT)5 and KRT14 expressed in basal cells [33, 34]. Evidence also suggests that Notch signaling is essential for the differentiation of this epithelial lineage [35]. Furthermore, C/EBPγ is upregulated in basal cells compared to differentiated epithelial cells [34], indicating a role for this C/EBP family member in processes associated with lung epithelial differentiation.

Ciliated cells

The predominant cell type in the human airway, the columnar ciliated epithelial cell, is critical for the unidirectional transport of mucus from the lung and thus plays an important role in host defenses by removing inhaled particles and microorganisms [2]. It is fairly well-established that ciliated, basal and secretory cells are derived from a common progenitor that expresses SOX-2 and that basal cells can differentiate into both ciliated and secretory cells. The SOX-2 transcription factor, which is negatively regulated by Wnt signaling, is proposed to be central in airway epithelial differentiation [1, 36-38]. In addition, expression of the transcription factor FoxJ1 is necessary for ciliated cell commitment from undifferentiated cells. FoxJ1 is detected specifically in ciliated cells and is used as a marker for this cell lineage [39, 40].

Secretory cells

In the airways, the correct amount of mucus, with the optimal composition and viscoelasticity is, together with cilia activity, central for efficient mucociliary clearance and thus innate immune defenses. Mucus hypersecretion is an intricate part of inflammatory lung diseases, and a hallmark of chronic obstructive pulmonary disease (COPD). Two different cell types secrete mucus; goblet cells and the mucus cells of the submucosal glands, which morphologically resemble goblet cells. The result is a complex mixture of mucus proteins. One of the main constituents of mucus, mucins (i.e. MUC5AC and MUC5B), are large glycoproteins that serve as markers for goblet cells [2]. Although dysfunctional mucus production in diseases like COPD is incompletely understood, studies have suggested important roles of, for instance, the

SAM pointed domain containing ETS transcription factor (SPDEF) [41], as well as Notch signaling in mucus cell hyper- and/or metaplasia [42, 43].

Another secretory cell type, Clara cells (Figure 3), contain electron-dense granules and are found in the bronchial and bronchiolar airways. In humans, their abundance is relatively low compared to the airways of mice, in which this is the most abundant cell type. Clara cells express high levels of secretoglobin (SCGB)1A1 (also known as uteroglobin or Clara cell (secretory) protein [CCSP/CC10/CC16]), a 10-16 kDa secreted protein with suggested immune modulatory properties [44, 45]. The secretion of host defense molecules such as surfactant proteins (SP) A and D and SCGB1A1 suggests that Clara cells are important for innate immune responses, and may participate in the regulation of inflammatory responses in the lung. The expression of SCGB1A1 is reduced in patients with COPD [46], acute lung injury [47, 48] and infants with bronchopulmonary dysplasia (BPD) [49, 50], indicating a role in inflammatory lung diseases. Clara cells also express high levels of cytochrome P450 monooxygenases, which are key in metabolizing xenobiotics.

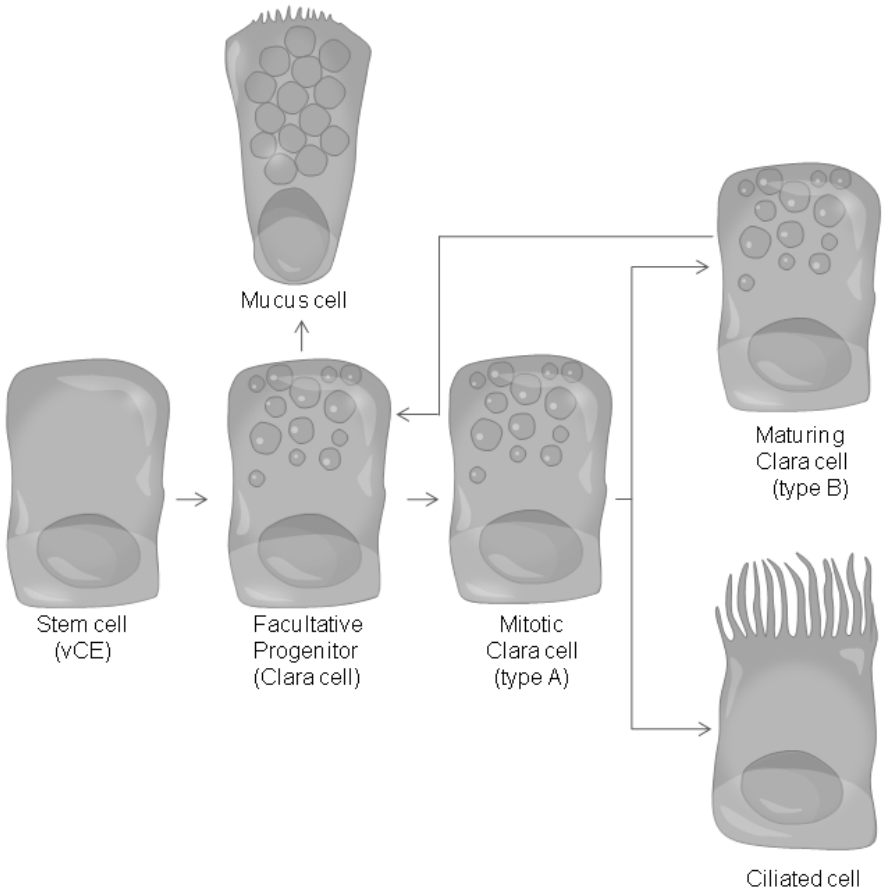

Figure 3. Clara cell differentiation. *Clara cells are non-mitotic cells at homeostasis. Quiescent stem cells (vCE) are activated by epithelial injury and gives rise to facultative progenitor Clara cells, as well as vCE by self-renewal. Facultative progenitors can give rise to dedifferentiated mitotic Clara cells (type A). These in turn differentiate into type B Clara cells, or Ciliated cells. Type B can differentiate into facultative progenitors, and these may differentiate into mucus cells* [51].

Similar to basal cells, Clara cells may also to have stem cell capabilities [2]. More specifically, (variant) Clara cells are suggested to maintain the facultative progenitor pool by proliferation (Figure 3). These cells may also have the capability to alter their phenotype as the surrounding environment changes and to restore terminally differentiated cells of the small conducting airways (i.e. ciliated cells) [31, 51]. An important role for Clara cells has also been proposed in bronchoalveolar duct junctions, where pollutant-resistant Clara cells could account for the proliferative cells within terminal bronchioles (bronchoalveolar stem cell, BASC, depicted in Figure 4), generating Clara cells as well as type I and type II cells [52, 53]. This is, however, challenged by the observation of distinct epithelial progenitor cells in the alveoli [54]. It has also been suggested that at least two distinct Clara cell populations reside in the lung, those originating from the saccular and canalicular period of lung development, and those resulting from differentiation of cells in the mature, post-natal lung [51]. Differentiation into mature Clara cells has been proposed to be promoted by a number of transcription factors [55, 56], as well as Notch and Wnt signaling [57, 58], although contrasting evidence on the importance of Wnt/β-catenin signaling exists [59]. Thus, the precise processes and mechanisms that control Clara cell differentiation remain unsubstantiated.

Alveolar cells

The alveolus consists of two highly specialized epithelial cells, the flat and elongated alveolar type I pneumocyte as well as the cuboidal alveolar type II pneumocyte (Figure 4). Alveolar type I cells facilitate gas exchange and are derived from type II cells. The cuboidal type II cells mediate fluid and ion transport, in similarity with type I cells and produce both the lipid as well as the protein components of surfactant [60]. Pulmonary surfactant plays a crucial role in lowering alveolar surface tension, and additionally has important functions in host defenses [60, 61].

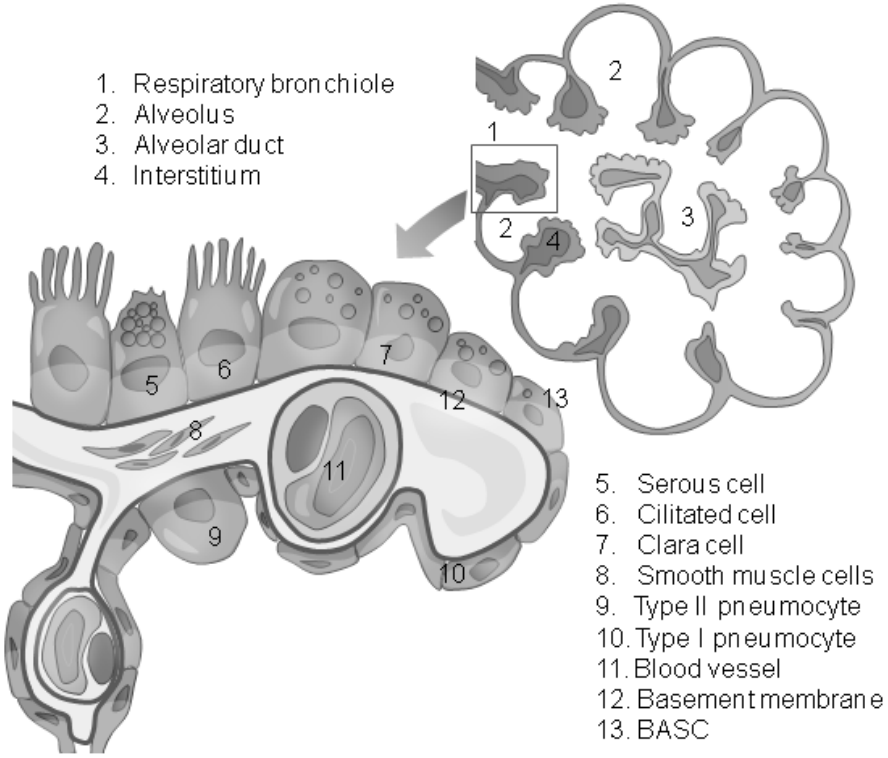

***Figure 4. Structure of the distal lung**. Serous, ciliated and Clara cells line the epithelium in the respiratory bronchioles. In the alveoli, airway epithelial cells are replaced by flat type II pneumocytes and cuboidal type II pneumocytes. The bronchioalveolar stem cell (BASC),which is reportedly SP-C and SCGB1A1 positive, is shown at the proposed location.*

1.1.3 The murine respiratory tree

The adult murine trachea and primary bronchi are, similar to human airways, composed of a pseudostratified epithelium. There are, however, several distinguishing differences between the murine and human respiratory tree. The mouse airway is significantly thinner than the human airway. Additionally, in contrast to the basal, ciliated, serous, and goblet cells of the human proximal airways, the mouse proximal airways are lined with Clara and ciliated cells, with only a few basal cells. In the distal airways, which in mice are devoid of respiratory bronchioles, ciliated cells are scarce and the Clara cells make up >80% of the cells [3, 26]. In addition, the zone of transition, separating the conducting airways and gas exchange area with bronchial epithelial cells mixed with alveoli, is considerably larger in humans than in mice. Mouse airways also lack cartilage in the proximal airways and almost completely lack submucosal glands, except in the most proximal part of the trachea [3]. The differences between the human and murine airways could potentially have an impact on the outcome and interpretation of data obtained from murine models, and are thus important to consider in translational research.

1.2 INNATE IMMUNITY OF THE AIRWAYS

By providing the different constituents of the mucociliary layer and a physical barrier formed by tightly connected epithelial cells, the airway epithelium plays a crucial role in lung defenses. The airway epithelium is in addition a central contributor to innate and adaptive immune responses by the production and secretion of molecules involved in host defenses as well as recruitment and interaction with inflammatory cells.

1.2.1 Pathogen recognition receptors

The immediate innate immune response includes the secretion of antimicrobial peptides and inflammatory mediators that attract and activate phagocytes. This response provides protection against pathogenic invasion and is initiated by the recognition of highly conserved microbial structures (i.e. pathogen-associated molecular patterns) by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) [62].

Toll-like receptors
TLRs are type 1 integral membrane glycoproteins located on the cell membrane or intracellular vesicles. Thirteen TLRs (named TLR1-13) have been documented in mammals. TLR1-10 are expressed in the humans, of which TLR2-6 exhibit the highest expression in lung epithelial cells [63]. Of the TLRs expressed in the airway epithelium, TLR1, 2, 4, 5, 6, and 9 recognize pathogen-associated molecular patterns of bacteria. The first identified pathogen recognition receptor (PRR), TLR4 is activated by lipopolysaccharide (LPS), a complex glycoprotein in the outer cellular membrane of Gram-negative bacteria such as *Pseudomonas aeruginosa*. TLR4 mainly signals via myeloid differentiation primary response gene (MyD)88, although alternative signaling pathways exist [64-67]. TLR4/MyD88 is, in addition to activation by LPS, also involved in inflammatory signaling evoked by for instance cigarette smoke [68-70],

even though this inflammatory response may be in part be due to LPS activation as well.

1.2.2 Host defense molecule production by epithelial cells

In response to inflammatory stimuli, lung epithelial cells produce enzymes, permeabilizing peptides, opsonins, protease inhibitors, toxic small molecules, and pathogen neutralizing macromolecules, all involved in pathogen resistance. The secreted host defense molecules includes collectins (including SP-A and D), defensins, lysozyme, complement, and serum amyloid (SA)A, which together display broad microbicidal activity [62, 71]. SP-A, for instance, secreted by Clara cells and alveolar type II cells, binds to Gram-positive and Gram-negative bacteria, fungi and viruses and thereby enhances engulfment by phagocytosing cells (i.e. acting as an opsonin) [62, 72]. In addition to the anti-microbial activities at high concentrations, low concentration of anti-microbial peptides have been reported to attract inflammatory cells. Some anti-microbial molecules have been shown to increase interleukin (IL)-8 levels and even induce epithelial cell lysis, in a possible attempt to promote inflammation. Antimicrobal peptides can also induce wound repair, proliferation, or differentiation, dependent on cell type [62].

1.2.3 Inflammatory mediator production by epithelial cells

In addition to the production and secretion of anti-microbial molecules, airway epithelial cells also to secrete a number of inflammatory mediators, including cytokines, chemokines, and other cell signaling molecules. Cytokines are soluble proteins or peptides with autocrine, paracrine, or endocrine activity at low concentrations. Chemokines are cytokines classified according to their capacity to induce leukocyte infiltration. Epithelial cells produce the neutrophil chemoattractant IL-8 (chemokine (C-X-C motif) ligand (CXCL)8), as well as the murine counterparts growth related oncogene (GRO)α (CXCL1) and macrophage inflammatory protein (MIP)-2 (CXCL2), the pro-inflammatory IL-1β, IL-6 and tumor necrosis factor (TNF)α, granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), as well as transforming growth factor (TGF)α and β [64]. In addition, epithelial cells express inflammatory genes such as nitric oxide synthases (NOS) [73] as well as cyclooxygenases (COX), lipooxygenases, and prostaglandin synthases, all involved in the generation of lipid mediators involved in inflammatory signaling [74]. Taken together, lung epithelial cells are thus pivotal players in the orchestration as well as the regulation of immune responses in the lung.

1.2.4 Inflammatory cell recruitment by the airway epithelium

The secretion of inflammatory mediators signals the presence of pathogens (and/or microbial products) and attract innate immune cells, such as neutrophils, macrophages, dendritic cells and natural killer (NK) cells, as well as cells within the adaptive immune response. As epithelial cells express cytokine receptors on the cell surface, epithelial-derived cytokines also serve to amplify inflammatory responses. Airway epithelial cells moreover regulate adaptive immune responses through direct interactions with different cell surface receptors on dendritic cells, T helper $(Th)_1$ and Th_2 cells, as well as B cells

[71]. In the work included in this thesis, the focus has been on the role of the lung epithelium in innate immune responses, with particular emphasis on neutrophils.

Neutrophils
The polymorphonuclear neutrophil is a short-lived phagocyte that is produced in the bone marrow and mobilized to the circulation upon inflammatory stimulation. In healthy individuals, neutrophils do not account for more than about 1% of the inflammatory cells in the lungs [75], but are rapidly recruited to sites of inflammation in high numbers. Neutrophils are attracted to inflammatory foci by the chemokines IL-8, GROα, MIP-2, ENA-78 (CXCL5), and lungkine (CXCL15). This recruitment, in which lung epithelial cells play key roles, involves the upregulation of cell adhesion molecules as well as rolling, tethering and passage through capillary walls (i.e. diapedesis) [67]. Neutrophils possess the capability of sensing pathogen-associated molecular patterns and respond by activating and fine-tuning effector functions [76]. Upon phagocytosis, internalized microorganisms are eliminated by the release of reactive oxidative species and proteases into the neutrophil endosome. Furthermore, and in similarity with epithelial cells, neutrophils secrete anti-microbial peptides such as defensins, in addition to proteases and reactive oxidative species. The importance of neutrophil recruitment is emphasized by studies in transgenic mice with enhanced expression of the neutrophil chemoattractant GROα. When infected with *Klebsiella pneumonia*, these transgenic mice display increased neutrophil recruitment and bacterial clearance as well as improved survival [77]. However, molecules secreted by activated neutrophils can cause substantial damage, in particular in chronic inflammatory conditions [67, 78]. In acute respiratory distress syndrome (ARDS), a severe systemic or local microbial infection leads to a large neutrophil recruitment to the lung. The steroid responsiveness of the disease is poor and the mortality is high, in between 40-60% [79].

Mononuclear cells
In similarity with neutrophils, mononuclear phagocytes (i.e. monocytes and macrophages) are bone-marrow derived myeloid leukocytes. Macrophages, which account for approximately 95% of the inflammatory cells in the healthy lung, serve as a first line of defense and are critical for both innate and adaptive immune responses in the lung. Mononuclear phagocytes are recruited to the lung by IL-1β, MIP-1α (CCL3), monocyte chemoattractant protein (MCP)1/CCL2, and TNFα [73, 80]. Alveolar macrophages, located at the interference between lung tissue and air are pivotal in pathogen internalization and phagocytosis [73, 80]. The dysregulated function of these cells, however, putatively contributes to the alveolar destruction and emphysema characteristic of COPD [81].

In summary, lung epithelial cells are essential in pulmonary innate immune responses as well as immune regulation, and control host defenses in close interaction with polymorphonuclear and mononuclear cells. Although the mechanisms controlling the functions of the airway epithelium have been investigated, there are still insufficient data on the regulation and control of airway epithelial cell functions. Growing evidence suggests that members of the C/EBP family of transcription factors play vital roles in controlling differentiation and innate immune defenses in various organs, and possibly also in the airway epithelium.

1.3 CCAAT/ENHANCER BINDING PROTEINS

C/EBPs belong to the basic region-leucine zipper (bZIP) family of transcription factors and are ubiquitously expressed [82, 83]. C/EBPα, the founding member of the family, was identified by McKnight and colleagues as a protein in rat liver nuclei capable of binding the CCAAT box motif present in various gene promoters [84]. C/EBPβ was later recognized as a factor binding to an IL-1 responsive element in the *IL6* gene [85]. During the following decade, four additional members were identified in mammalian species and subsequently named in succeeding order according to the Greek alphabet (C/EBPα, β, γ, δ, ε, ζ) [82, 83] (Table 2).

1.3.1 C/EBP structure and function

All C/EBPs are composed of separate, highly conserved domains (Figure 5). Some regions, however, are unique and distinguish individual C/EBP factors. For instance, C/EBPβ contains characteristic regions that allows for additional post-transcriptional modifications, and thereby further control of the activity [86]. The positively charged basic region binds directly to the negatively charged DNA [86]. The DNA sequences that all lung-enriched C/EBPs (C/EBPα, β, and δ) interact with are virtually identical, although some differences in binding site specificities have been reported, in particular for C/EBPβ [87-91]. Thus, functional replacement with regard to activating gene transcription by different C/EBPs is conceivable. The basic-leucine zipper (bZIP) domain is the most C-terminal region and is crucial for homo- and heterodimerization (Figure 6), which in turn is required for DNA binding [91]. All C/EBPs can form heterodimers in all intrafamiliar combinations. Dimerization is of particular interest for inflammatory and anti-inflammatory signaling, since different combinations may result in pro- or anti-inflammatory responses (reviewed in [92]). In addition, the bZIP region also serves as a nuclear localization signal and extensions of the bZIP region mediate various protein-protein interactions with other cellular proteins [86]. The transactivation domain, harbored in the amino-terminal portion of the protein, is not as conserved as the leucine zipper or basic domains. The transactivation domain interacts with different components of the basal transcription apparatus and thereby stimulates transcription. C/EBPγ, however, lacks the transactivation domain and therefore acts to repress gene transcription, even though the function may diverge in different cell types [86]. In addition to the domains mentioned above, C/EBPβ contains negative regulatory regions in the N-terminus, although the precise role of these regions remains unknown [91].

Table 2

Nomenclature of the C/EBP family

Name	Alternate name
C/EBPα	p42, p30
C/EBPβ	NF-IL6, LAP, LAP1, LAP*, LAP2, LIP, CRP2, IL-6DRP, NF-M, C/EBPβ-1, C/EBPβ-2
C/EBPγ	Ig/EBP, GPE1BP
C/EBPδ	NF-IL6β, CRP3, CLEF
C/EBPε	CRP1
C/EBPζ	CHOP, CHOP10, GADD153, DDIT3

Table 2. Nomenclature of the CCAAT/enhancer-binding protein and isoforms.

Phosphorylation and dephosphorylation of C/EBP family members commonly cause conformational changes or modulate the ability to interact with other transcription factors or co-factors, leading to effects on the transactivation capacity. Phosphorylation of human C/EBPβ at Thr235 (homologous to mouse Thr188) has been shown to be pivotal for transactivation and the induction of immediate-early inflammatory genes in response to inflammatory stimuli. In similarity, phosphorylation of Ser276 (homologous to human Ser325) of the murine C/EBPβ has been attributed with a comparable function. There are numerous reports that collectively describe a complex system where phosphorylation at different sites, possibly simultaneous, directs C/EBPβ and determines the gene expression profile of a cell [86]. In addition, inhibitory SUMOylation and stimulatory or inhibitory acetylation of C/EBPβ have been described [86], endowing the system with additional complexity.

1.3.2 Lung-enriched C/EBPs

Of the C/EBP-factors, C/EBPα, β, and δ are lung-enriched and the ubiquitously expressed C/EBPγ and ζ are also expressed in the lung [93]. Among these, C/EBPβ has been reported to be the dominant DNA binding factor in the adult human airway epithelium [94]. The number of C/EBP isoforms, however, widely exceeds the C/EBP genes expressed in a specific cell type. While C/EBPζ contains four introns, C/EBPα, β, δ, and γ are intronless. Due to leaky ribosomal scanning, the same intronless mRNA molecule can be translated into different polypeptides by alternative use of translation initiation codons. Thus, two polypeptides, 42 kDa and 30 kDa, with different activation potential, can be produced from the C/EBPα mRNA sequence [95]. In similarity, three C/EBPβ isoforms have been identified, the 38 kDa liver-enriched transcriptional activator protein (LAP*/LAP1), the 35 kDa LAP/LAP2 and the 20 kDa liver-enriched transcriptional inhibitory protein (LIP). The LIP protein lacks a transactivation domain and represses gene expression, suggestively by inhibiting the function of other C/EBP isoforms in a dominant negative fashion [91, 96]. This is further complicated by the preference of the negative regulatory C/EBPζ to bind LIP [86]. Collectively, various combinations of isoforms with different transactivation potential could have a profound effect on the regulation of target genes.

Figure 5. Schematic illustration of the CCAAT/enhancer-binding proteins (C/EBPs). *The different domains and their primary functions are indicated. Adapted from [93].*

Figure 6. Protein structure of CCAAT/Enhancer-binding proteins. *Two C/EBPβ monomers dimerize and bind to DNA. Adapted from [91].*

Role of C/EBPα in differentiation and proliferation

The founding C/EBP family member, C/EBPα, plays a pivotal role in inhibiting proliferation in several organs, including the lung [97-99]. For example, a notable role for C/EBPα as a master inhibitor of proliferation, and in promoting the differentiation of progenitor cells into the myeloid linage has been documented. C/EBPα is highly expressed in granulocyte and monocyte progenitor cells and mutations in the gene or DNA methylation of the promoter are frequently detected in acute myeloid leukemia [100]. In addition, glucocorticoid (GC)-induced differentiation of preadipocytes is mediated via C/EBPα dependent transcription of C/EBPβ, which interacts with histone deacetylase (HDAC)1 and facilitates transcription of genes associated with differentiated cells [101]. GC induction of hepatocyte differentiation has similarly been reported to be dependent on C/EBPβ [102].

Deletion and ectopic expression of C/EBPα has revealed a vital role in regulating proliferation in the alveolar epithelium [15, 18, 19, 98, 103]. Deletion of C/EBPα during lung development causes impaired lung maturation and hyperproliferation of alveolar type II cells [18, 19, 91, 98, 104]. It has previously been reported that C/EBPα mediates growth arrest by interacting with cyclin-dependent kinase (cdk) 2 and 4, two critical regulators of cell cycle progression [105]. C/EBPα also represses the activation of E2F, which is necessary for passage through the cell cycle restriction point [106, 107]. In addition, C/EBPα is required for GC-induced transcription of the cdk inhibitor $p21^{WAF/Cip1}$ [108, 109], an anti-proliferative factor exhibiting increased expression in the bronchial epithelium of asthmatics as well as COPD patients [110, 111]. Also, the anti-proliferative effect of GCs in lung mesenchymal cells has been reported to be mediated via the formation of a C/EBPα-GR complex [112]. Thus, C/EBPα mediates the anti-

proliferative effects of GCs by at least two different mechanisms, suggesting that C/EBPα is central for the anti-proliferative effects of GC therapy in asthma.

The role of C/EBPβ in proliferation
In sharp contrast to the anti-proliferative role of C/EBPα, C/EBPβ has previously been suggested to support proliferation in hepatocytes after partial hepatectomy. The initial conclusions were, however, based on the observed decrease in C/EPBα expression and simultaneous upregulation of C/EBPβ after hepatectomy [113]. Further studies have revealed a more complex system, where the C/EBPβ isoform LAP decreases cyclin A, cyclin E as well as E2F and delays transition into S-phase, while the LIP isoform induces cyclin A and E, as well as C/EBPα [114-116]. Following cigarette smoke extract stimulation of lung fibroblasts, cell proliferation is reduced and C/EBPα and C/EBPβ are upregulated, supporting anti-proliferative roles for both these transcription factors [117]. In other cell types, such as adipocytes, C/EBPβ has been suggested to promote proliferation [118]. In addition to these documented roles in controlling proliferation, C/EBPβ has been attributed with anti-apoptotic functions [91, 93]. There is no existing evidence from mouse models that supports a vital role for C/EBPβ in baseline lung function or lung development [119, 120]. There is, however, still insufficient data on the proliferative role of C/EBPβ in the pulmonary epithelium.

Role of C/EBPβ in inflammatory and acute phase responses
Current evidence suggests that C/EBPβ plays a critical role in the hepatic acute phase response induced by inflammatory stimuli. C/EBP binding motifs are present in the promoters of many class I acute phase responsive genes, such as the gene coding for α_1-acid glycoprotein and the different *SAA* genes. In addition, many genes associated with inflammation, for instance the *TNFA, IL1B, IL6, IL8, IL12, CSF3, MIP1A/CCL3,* and *MIP1B/CCL4* genes, as well as genes coding for the receptors binding for instance G-CSF and GM-CSF contain C/EBP responsive elements, and/or have been reported to be regulated by C/EBPβ [86, 121, 122]. Also, the mRNA expression, isoform ratio and activity level of C/EBPβ are all modulated by inflammatory stimuli such as recombinant cytokines (i.e. TNFα, IL-1 and IL-6), as well as LPS, indicating a specific role for C/EBPβ in the acute phase response [90]. In line with this, C/EBPβ-deficient mice display an increased susceptibility to *Listeria monocytogenes* and *Candida albicans* infection, with reported impaired cellular immunity. Following infection with *C. albicans,* humoral and innate immune responses are also affected [90]. Some evidence of the involvement of C/EBPs in inflammatory responses in the lung also exists. Both C/EBPβ and C/EBPδ are elevated in the lung following inflammatory stimuli [85, 90, 91, 123-125]. Also, C/EBPβ has been suggested to transactivate pulmonary expressed host defense proteins, such as SP-A [93, 126], which are induced following inflammatory stimuli [121, 127]. C/EBP transactivation is induced by nucleotide-binding oligomerization domain-containing protein (Nod)1 activation in bronchial epithelial cells, although the transactivation capacity is not immediately increased by LPS stimulation *in vitro* [128]. Notably, the outcome of the inflammatory response is both stimulus- and cell type-specific [90], prompting further investigations into lung-specific responses.

1.3.3 C/EBPs and lung diseases

C/EBPs have been implicated in several lung diseases, including non-small lung cancer, asthma, pulmonary and cystic fibrosis, as well as chronic bronchitis and COPD [94, 104, 129-131]. Smokers with chronic bronchitis or COPD exhibit reduced activity

of C/EBPβ in the lung epithelium compared to asymptomatic smokers [94]. This decreased activity could render the epithelium more sensitive to the damaging effects of cigarette smoke and reduce the efficiency of host defense responses, as the induction of inflammatory and host defense genes would be hampered. Moreover, C/EBPβ has also been suggested to be important for GC signaling [132, 133], a cornerstone of the medical treatment of inflammatory lung diseases. Collectively, these findings stress the need for further studies on the disease-specific role of the C/EBP transcription factors.

1.4 INFLAMMATORY AND SMOKING-RELATED LUNG DISORDERS

Considering the possible contribution of C/EBPs to the pathogenesis of COPD and other detrimental lung diseases [94], there is a need to further investigate the role of C/EBPs in lung disorders. In papers I-IV of this thesis, the role of C/EBPs in lung development (relevant for respiratory distress syndrome (RDS)/BPD, smoking-induced lung disease such as COPD and acute lung injury are investigated and/or discussed.

1.4.1 Bronchopulmonary dysplasia (BPD)

The lungs of premature children (gestational age <28 weeks) with very low birth weight (<1000 g) are severely immature, and respiratory distress syndrome (RDS) is very common in these infants [134]. BPD is the most common chronic respiratory disease associated with treatment of RDS among premature children [135], affecting 20% of all infants with birth weight <1500 g [136]. BPD is a consequence of elevated oxygen and ventilator-induced injury on the immature and surfactant-deficient lungs of premature infants [137]. The most widely accepted definition of BPD is based on gestational age and the requirement for oxygen supplementation. The National Institutes of Health has defined BPD as occurring among infants <32 weeks of post-menstrual age that require supplemental oxygen for at least 28 days after birth. If the need for oxygen persists after week 36, the disease is considered to be more severe [135]. Less aggressive mechanical ventilation along with routinely administered exogenous surfactants and glucocorticoids have led to a change in the disease pattern and the classification of new BPD, to be separated from the old definition of BPD. The latter is characterized by squamous epithelial metaplasia, epithelial and smooth muscle hyperplasia, remodeling of pulmonary arteries, fibroproliferation as well as decreased alveolarization. In contrast, new BPD is not as severe and is dominated by a disruption in distal lung growth due to interrupted gestational growth, with fewer and larger alveoli [138, 139]. The alveolar simplification and enlargement is a result of an impairment, not arrest in postnatal alveolarization [137] and is accompanied by modest airway remodeling and a varying degree of arterial remodeling as well as smooth muscle and fibroproliferation [138, 139]. The pulmonary phenotype of *Cebpa*$^{\Delta LE}$ mice, described in paper I, has greater resemblance to old BPD, although similarities exist with new BPD as well. At present, BPD occurs in preterm infants born at 24 to 26 weeks of gestation, in the late canalicular-early saccular stage. As the alveoli are not uniformly present until week 36, in the alveolar stage, the lungs of preterm newborns at 30 to 32 weeks, during the saccular stage and even later are considered to be immature [4, 137], although these children are not affected with BPD.

There is increasing concern that the lung injury associated with preterm deliveries (i.e. BPD) may lead to chronic conditions and obstructive airways later in life. Abnormal baseline spirometry, as well as impaired exercise capacity and significantly more

respiratory symptoms are noted among childhood survivors of extreme preterm birth. In addition, asthma is twice as common among children born prematurely, compared to children born at full term [140]. Some evidence also suggests that adult survivors of BPD suffer from respiratory symptoms such as shortness of breath and wheeze, as well as airway obstruction [136]. There is still, however, missing data on the consequences of premature birth on the aging lung.

1.4.2 Acute lung injury

Acute lung injury (ALI) and the most severe form of the disorder, ARDS are characterized by lung edema with protein-rich fluid. This is caused by disrupted epithelial barrier functions and microvascular endothelial injury, which lead to increased permeability of the alveolar-capillary barrier. In the later stages, a repair process characterized by fibrosis and remodeling of the alveolar space also occurs [141]. ALI is defined as a disease with acute onset, bilateral pulmonary infiltrates and a PaO_2/FiO_2 ratio < 300 mmHg or 40 kPa (partial pressure of oxygen in arterial blood/fraction of inspired oxygen in a gas mixture), together with the absence of cardiac involvement [142]. A common cause of ALI is pneumonia or sepsis, although non-infectious causes, such as exposure to noxious gases, also are well documented. The mortality of the more severe form, ARDS, is still approximately 40%, despite increasingly effective intensive care routines and thus represents a significant cause of morbidity and mortality in society. The most striking hallmark of the disease is acute inflammation with neutrophil accumulation in the alveolar space. The activated neutrophils subsequently cause significant tissue damage by the release of cytotoxic and immune activating agents such as proteases, cytokines, and reactive oxygen species. In murine models, the most important chemokines for neutrophil recruitment are GROα and MIP-2, which both bind to chemokine (C-X-C motif) receptor (CXCR)2, a receptor with a key role in neutrophil influx to the lung. In addition, multiple cytokines, including TNFα, are upregulated in ALI [79].

1.4.3 Chronic obstructive pulmonary disease

The World Health Organization has estimated that 80 million people suffer from COPD, a disease characterized by progressive airflow limitation in response to noxious particles or gases. COPD is currently the sixth leading cause of death worldwide but the incidence is predicted to increase dramatically in the immediate future, posing a substantial impact on global health and contributing to increasing medical care costs [143]. Cigarette smoking is exclusively the most important risk factor in COPD pathogenesis and smoking cessation is key to preventing disease progression. While continued efforts to reduce smoking prevalence are central to minimizing the burden of COPD [143], the addictive nature of cigarette smoking along with unsuccessful attempts to prevent young adults from developing harmful smoking habits underlines the importance of a greater understanding of the mechanisms that contribute to COPD pathogenesis.

Spirometry is required to make the clinical diagnosis of COPD. The disease is diagnosed when the ratio between forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) is <0.7, or when FEV_1 is <80% of predicted [144, 145]. A series of pathological lesions that cause decreased lung function develop as a consequence of repeated injury and dysfunctional repair processes in response to

cigarette smoke. These lesions include chronic inflammation and structural changes to the proximal and peripheral airways, lung parenchyma and pulmonary vasculature [143, 146]. Small airway disease (obstructive bronchiolitis), inflammation and excessive mucus production (bronchitis), and parenchymal destruction (emphysema) contribute to airflow limitation to varying degrees in different individuals [143]. Corresponding to this, different clinical phenotypes have been described which relate to clinically meaningful outcomes [147]. Increased documentation of the different clinical phenotypes may be used to develop new therapeutic interventions and improve COPD management. Small airway disease and remodeling of the airways is a cardinal feature that is characteristic of COPD and includes airway wall thickening, inflammatory cell infiltrates [146, 148, 149], and pronounced mucus/goblet cell hyperplasia [150]. Extra-pulmonary symptoms, such as heart disease, muscoskeletal depression and underweight may also manifest in individual patients and contribute to disease severity, decreased quality of life, and morbidity [143, 151]. Despite substantial efforts, it is still uncertain exactly how the lesions of COPD develop [148], although it is well-known that the symptoms develop after years of chronic cigarette smoking [143].

The occurrence of COPD exacerbations, defined as an acute and sustained worsening of stable COPD that requires altered medication, accounts for much of the morbidity, mortality and health care costs associated with the disease [152, 153]. Exacerbations are central in driving disease progression, with each individual exacerbation causing further deterioration in lung function [143, 154]. Respiratory pathogens are likely to cause exacerbations [155], and the virulence of the pathogen could together with impaired host defenses theoretically explain the amplified inflammation characteristic of an exacerbation [156]. Altogether, this emphasizes the need for a more comprehensive understanding of the host response to respiratory infections. Additional studies that utilize novel methodological approaches are thus warranted to improve the current knowledge of the mechanisms that contribute to pathogen-induced exacerbations and the detrimental effects of these events.

The risk of developing COPD among continuous smokers has been estimated to be 25% [157], and a small proportion of all COPD patients have never smoked [143], together suggesting that genetic risk factors also contribute to pathogenesis. Hereditary α1-antitrypsin deficiency, which causes reduced inhibition of serine proteases and thereby promotes the development of emphysema, is a well-known genetic factor that influences airflow limitation [158]. Candidate gene and genome-wide association studies, as well as gene expression profiling, have revealed a involvement of a variety of other genes, many of them implicated in inflammatory processes, with possible roles in COPD pathogenesis [159]. Thus, it is well-established that genes with polymorphic expression interact with environmental factors to influence the pathology of COPD. As there currently no medical treatment to prevent the progression of COPD, increased efforts to identify genes associated with COPD pathogenesis that can be used as therapeutic targets are central for improved COPD management.

Inflammation in COPD

The detrimental effects of cigarette smoke are well-established. Cigarette smoke induces inflammatory responses, and these in turn contribute to the airflow limitation observed in COPD (Figure 7) [143]. Cigarette smoking is associated with innate and adaptive immune responses, which are amplified after onset and with the progression of COPD [150, 160]. The chronic inflammation characteristic of COPD is particularly evident in the small airways and involves the accumulation of macrophages, B cells, as

well as T lymphocytes [150]. In addition, the number of neutrophils are increased in bronchoalveolar lavage and sputum of COPD patients [75]. Activated neutrophils release excessive amounts of neutrophil elastase, matrix metalloproteinases, and oxygen free radicals, which are central in host defenses against inhaled pathogens from intracellular granules. Neutrophils are thus likely contributors to the deleterious inflammatory responses in chronic inflammatory diseases such as COPD, due to de-granulation and the release of stored proteases [76]. Is it today generally accepted that cigarette smoke-induced inflammation and inflammatory cell-derived proteases cause the emphysematous changes in COPD [161]. As challenged mice treated with antibodies targeting cytokines such as TNFα display nearly complete protection against experimentally-induced emphysema, inflammatory genes are implicated in tissue destruction and the pathogenesis of emphysema [162].

A wide range of inflammatory mediators, often with overlapping functions such as cytokines interacting in complex networks, have been reported to be involved in COPD [163]. Both interleukins such as IL-1β, IL-6, IL-17 [164, 165], IL-18 [166-168], IL-22 and IL-23 [164], as well as TNFα are implicated in COPD [75]. In addition, several chemokines, including the neutrophil chemoattractants IL-8 and GROα are upregulated in COPD [149, 169], and *in vitro* data suggests that epithelial cells may contribute to these increased levels of IL-8 [170]. Nitric oxide synthases [171] and prostaglandin-generating enzymes (e.g. COX-2) [172, 173] also play important roles disease-specific inflammatory signaling, and are implicated in COPD. Despite substantial efforts in elucidating these mechanisms, it still remains unknown exactly how the dysregulation of inflammatory mediators contributes to disease pathogenesis and progression.

1.5 ANIMAL MODELS OF INFLAMMATORY LUNG DISEASES

Animal models for several inflammatory lung diseases, including asthma [174], ARDS/ALI [141], BPD [137], cystic fibrosis [175], as well as granulatomous diseases such as sarcoidosis [176, 177] and beryllium disease [178] have been relatively well-characterized. In contrast, there is limited documentation of animal models with a considerable resemblance to COPD.

1.5.1 Models of BPD

Lambs and baboons are frequently used to model BPD as both the lung inflammation associated with preterm birth and lung injury induced by mechanical ventilation and hyperoxia can be produced. Rodents have also been used to model BPD, and a pathology resembling both old and new BPD is produced by postnatal hyperoxia in mice and rats. However, an ideal model, which includes all attributes of BPD and allows for studies of both acute and chronic effects, as well as the outcomes of interventions, is yet to be produced [137].

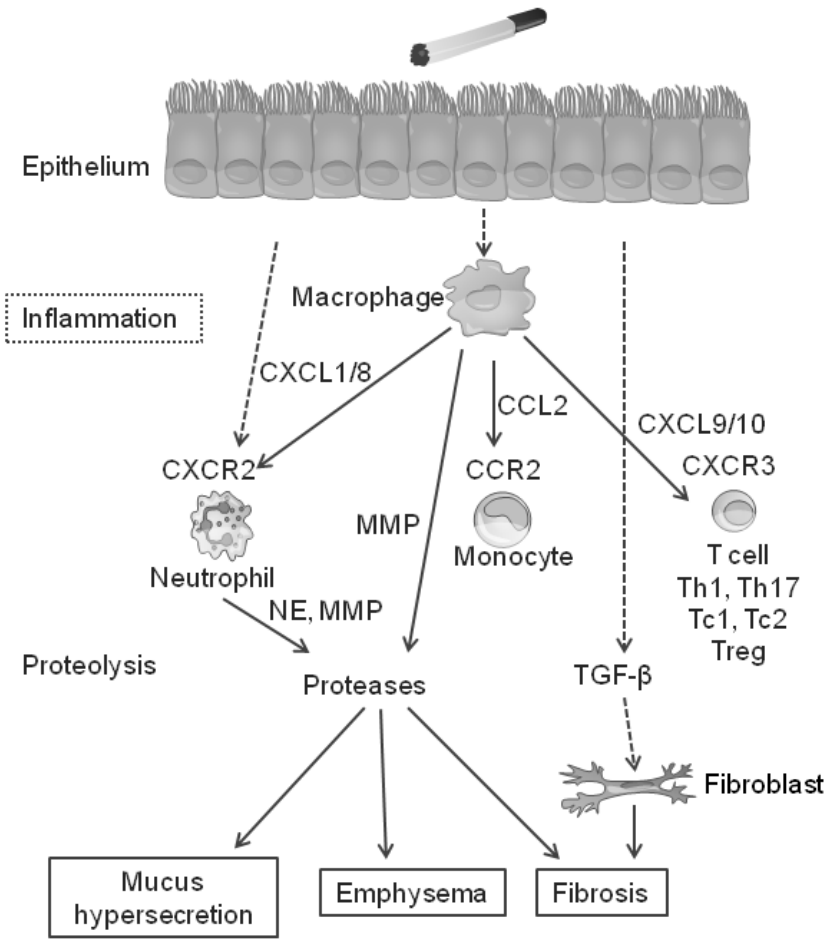

Figure 7. Mechanisms of cigarette smoke induced lung pathogenesis. *Adapted from [179].*

1.5.2 Models of ALI

ALI has been modeled in a wide range of animals, including rodents. There is, however, no animal model mimicking all the characteristics of ALI/ARDS. A variety of insults reproduces the acute neutrophilic alveolitis present in ALI/ARDS, including hyperoxia and LPS administration. LPS administration may be intravenous, intratracheal or intrapulmonary, which affects the outcome of the injury. Intravenous administration primarily causes apoptosis of endothelial cells, which precedes other tissue damage. There are many advantages to LPS-induced lung injury, such as reproducibility and accessible administration, although the epithelial and endothelial damage is not as severe as in clinical ALI/ARDS. In addition, LPS challenge results in an incomplete picture of the tissue damage caused by live bacteria in clinical settings [141].

1.5.3 Models of COPD

Smoke-induced diseases such as COPD are extremely difficult to model, in part since the pathology involves a slow evolution and long chronicity. Animal models recapitulating different pathological lesions of COPD have previously been

documented, although there is currently no animal model that mimics all the pathological hallmarks of COPD, as well as the airway obstruction. Quite a large number of studies have produced emphysematous changes in mice and investigated cellular or molecular components with possible roles in the pathogenesis [180]. Emphysema may, for example, be produced by chronic cigarette smoke exposure, and the observed inflammatory responses associated with cigarette smoke exposure have been suggested to drive the airspace enlargement [162]. There is also evidence of airway remodeling following cigarette smoke exposure [180]; however, only small increases in secretory cells are observed [148]. Nevertheless, mice are not ideal to model COPD. The perfect animal model of COPD should have pulmonary anatomy similar to humans, since the anatomy of the lung is pivotal for the pathophysiology of COPD [148]. As previously discussed, the murine airways differ from the human airways in several aspects. Taken together, this raises the question as to whether all the phenotypes and hallmarks COPD [180] can be modeled in animals, or if efforts should instead be made to identify different suitable models reflecting the diverse phenotypes of COPD. For instance, a hallmark of COPD, pulmonary neutrophilia [149], can be induced by the LPS challenge (used in paper IV) [181]. In addition, cigarette smoke-induced inflammation, as studied in paper III, is an integral part of COPD and the responses to cigarette smoke as well as role in pathogenesis of the disease are important aspects to gain further knowledge on.

Cigarette smoke-induced inflammation
Acute to sub-acute cigarette smoke stimulates the release of inflammatory mediators such as cytokines and chemokines (e.g. IL-8 and IL-1), as well as growth factors (such as G-CSF and TGF-β), leading to inflammatory cell recruitment and increased mucus production [150]. In addition, gene expression studies have revealed increased expression of 175 genes, including xenobiotic enzymes, anti-oxidants, and putative oncogenes, in the bronchial epithelium in cigarette smokers. Expression of genes involved in immune regulation and tumor suppressor genes were in contrast decreased in the bronchial epithelium of smokers [150].

1.6 LONG-ACTING β_2-AGOINST AND GLUCOCORTICOID THERAPY

1.6.1 β_2-adrenoceptor agonists

Short acting β_2-adreonceptor agonists (SABAs) are rapid and effective first choice bronchodilators in asthma therapy [182]. Administration of β_2-adreonceptor agonists may moreover be beneficial in the treatment of BPD, although the subject is controversial, as it is debated whether the airways of preterm neonates respond to β_2-agonists [135]. Documentation of a potential benefit of β_2-agonist therapy for the treatment of ALI in pre-clinical settings exist [183], as β_2-agonists have been proposed to accelerate the resolution of pulmonary edema [142], as well as wound repair [184], although the results from a recent clinical trial was disappointing [185]. The development and clinical application of long acting β_2-adreonceptor agonists (LABAs) and phosphodiesterase (PDE)4 inhibitors have led to the discovery that cAMP elevating agents may come with other beneficial effects, in addition to bronchodilation. For instance, pulmonary neutrophilia and IL-8 levels among patients with severe asthma or asthma exacerbations are reduced by the LABA formoterol [186].

β_2-adrenoceptor signaling

β_2-adrenoceptor agonists exert their effects by binding to the adrenergic β_2-receptor, a member of the seven transmembrane G protein-coupled receptor family. The activated receptor induces the activity of multiple variants of adenylyl cyclase (AC), enzymes with the capability to convert adenine triphosphate (ATP) to cyclic adenine monophosphate (cAMP). The second messenger cAMP interacts with protein kinase (PK)A, as well as different PKA variants, including phosphodiesterases, and PKA anchoring proteins [182]. PKA, in its turn induces smooth muscle cell relaxation through effects on ion channels. Bronchodilation mediated by β_2-adrenoceptor agonist smooth muscle cell relaxation is central to the pharmacological treatment of inflammatory lung diseases. It is also well-established that PKA initiates a phosphorylation cascade, which influences gene transcription. There may also be β_2-adreonceptor signaling pathways that are independent of cAMP and PKA [187].

Anti-inflammatory effects of β_2-adrenoceptor agonists

Although the most important role of β_2-adrenoceptor agonists in medical therapy of inflammatory lung disorders are the bronchodilating properties along with potentiation of glucocorticoid effects, β_2-adrenoceptor agonists display anti-inflammatory effects even when used alone [188]. A suppressive effect on inflammatory mediator expression has been observed in bronchial epithelial cells [189], even though only a few studies have addressed this. Contrasting reports also exist, documenting a stimulatory effect on rhinovirus-induced IL-6 expression in bronchial epithelial cells [190]. These *in vitro* studies are complemented by findings of reduced LPS-induced pulmonary neutrophilia as well as TNFα, IL-6, and MIP-2 expression by adrenoceptor stimulation *in vivo* [191-193].

1.6.2 Glucocorticoids

The inflammation of many respiratory disorders is effectively suppressed by glucocorticoids (GCs). For instance, preterm neonates with RDS/BPD are routinely treated with exogenous steroids [135]. The clinical benefit of GC treatment in ALI/ARDS is limited [142], although the outcome of reported studies are not in agreement. The inflammation in COPD is relatively resistant to the anti-inflammatory effects of GCs [194]. Similarly, the inflammation in asthma is in a minority of cases also less responsive to steroids [195]. Several possible mechanisms of steroid resistance have been postulated including GR modification, increased pro-inflammatory transcription factor expression and defective histone acetylation (for a recent review see [195]). A lack of efficient medical therapy poses a major obstacle in COPD management, and increasing effort is being paid to investigate the relative GC resistance in COPD inflammation. In paper IV, the role of C/EBPβ in mediating the effects of GCs and LABAs was therefore investigated.

GCs are steroid hormones that freely diffuse through the cellular membrane and bind to the glucocorticoid receptor (GR) located in the cytoplasm. The GR is kept inactive by a multi-protein complex and is present in almost all cell types, including bronchial epithelial cells [196-198]. Upon binding of GCs, a conformational change that releases the receptor from the chaperone complex occurs, with subsequent translocation to the nucleus [199, 200].

Gene regulation by the glucocorticoid receptor
GCs play important roles in various cellular functions such as differentiation, proliferation, and apoptosis. In addition, GCs regulate many different inflammatory and host defense genes in the lung through a number of mechanisms [108, 109, 201-205]. For instance, through transactivation, the active GR stimulates the transcription of anti-inflammatory genes by binding to GREs [196, 206]. Transactivation does not, however, solely explain all the anti-inflammatory effects of GCs. The GR also binds to negative glucocorticoid response elements (nGRE) and decreases the transcription of cytokines, chemokines, and inflammatory mediators, such as COX-2 (i.e. transrepression) [207]. The GR also interacts with other transcription factors, such as nuclear factor (NF)κB, activator protein (AP)-1, and C/EBPs, and can thereby mask domains required for activation, dimerization, nuclear translocation signals, or DNA binding sites. Through these mechanisms, the activity of pro-inflammatory transcription factors is inhibited [208]. In addition, GR also has the ability to form dimers with other transcription factors when acting by transactivation or transrepression, and may compete for other transcription factor activating signals or inhibit interactions with the transcriptional machinery as reviewed elsewhere [206]. GC signaling also induces histone modifications as well as chromatin remodeling, and thereby decreases the transcription of inflammatory genes (for a review on histone modifications in lung diseases, see [209]). Finally, sparing or even an induction of host defense molecules by GCs have been reported [210] (for a review, see [211]).

1.6.3 Long-acting β_2-agonists and glucocorticoids in COPD therapy

Both short-acting and long-acting β_2-agonists, as well as glucocorticoids, are cornerstones in COPD management, although there is limited efficiency of these drugs, either when used alone or in combination. The lack of a truly effective medical therapy for COPD thus represents a challenge for the scientific community as well as the pharmaceutical industry.

Long-acting β_2-agonists in COPD management
SABAs are the first choice of medication in COPD patients experiencing an exacerbation, as recommended by the GOLD guidelines [155]. In addition, long-acting anticholinergics (tiotropium) alone [212] or in combination with a LABA (salmeterol) reduce the number and severity of exacerbations, while salmeterol has no significant effect alone [213]. An up-to-date multi-center trial reported increased time to first exacerbation in COPD patients on daily tiotropium treatment as compared to salmeterol [214], suggesting that long-acting anticholinergics rather than β_2-adreonceptor agonists are the preferred choice of bronchodilators in COPD therapy. Previous evidence, however, indicates that there are some beneficial effects with β_2-adreonceptor agonists as maintenance monotherapy for COPD [215].

Inhaled glucocorticoids in COPD management
Inhaled glucocorticosteroids (ICS) are routinely used in COPD management, while systemic corticosteroids are recommended in severe COPD (baseline FEV_1 <50% of predicted) [155]. There is, however, no consensus on whether ICS influence the natural course of COPD, or attenuate the long-term decline in lung function [143]. Two recent studies evaluating large cohorts have challenged previous conceptions and concluded that an ICS (fluticasone) with or without a LABA (salmeterol) significantly reduces the deterioration in lung function [216, 217]. ICS also improve health status by reducing the frequency of COPD exacerbations [218], lower all-cause

mortality [219], and slow down the decline in quality of life [220]. Thus, there are some beneficial effects of ICS in patients with COPD, although the inflammation of COPD is relatively steroid unresponsive.

Combination therapy with inhaled corticosteroids and long-acting β_2-agonists
Compelling evidence from the clinic suggests that there are beneficial effects of combining ICS with LABAs. The dose of the ICS can be maintained at a lower level, since ICS with a LABA demonstrate improved effectiveness compared to ICS alone. It has furthermore been demonstrated that the rate of asthma exacerbations and hospital admissions are reduced with combination treatment [221]. In addition, as mentioned earlier, reduced decline in lung function is observed in COPD patients on ICS treatment with or without an added LABA [216, 217]. Moreover, the frequency and severity of COPD exacerbations are decreased by maintenance treatment with combination therapy of GCs and LABAs [222, 223]. Synergistic inhibition of inflammatory mediator expression in lung cells *in vitro* has also been reported [224], providing a possible mechanistic explanation to how the synergy of ICSs and LABAs. In addition, recent evidence suggests that glucocorticoid insensitivity is reversed by FM in peripheral blood monocytes obtained from COPD patients and that this may be mechanistically explained by an inhibition of phosphoinositide 3 kinase δ [225], which plays a central role in inflammatory responses [226].

1.6.4 C/EBPβ as a mediator of glucocorticoid and β_2-adrenoceptor signaling

Evidence suggests that GCs regulate the transcription of some genes through C/EBPs [112, 132, 133, 210], and that the GR interacts with, or signals to C/EBPβ via an unknown intermediate factor [133, 227]. In the lung, GCs contribute to the maturation of the developing lung and antenatal GC administration increases lung maturation and surfactant production [228]. In line with this, GR-deficient mice display lung immaturity with increased proliferation as well as reduced expression of pulmonary surfactant, and die shortly after birth due to respiratory failure [204, 205]. In spite of this, functional binding sites for the GR has not been found in the promoter of any genes coding for surfactant proteins [229]. Moreover, mice with a mutation in the GR causing an inability to bind to DNA are viable and exhibit normal lungs, demonstrating that impaired lung maturation caused by the ablation of GR is not dependent on DNA binding [230]. This suggests that protein-protein interactions between the GR and other transcription factors are more important during lung development than GR transactivation. The absence of functional binding sites for the GR in the promoters of lung-specific genes associated with the differentiated lung epithelium, such as the surfactant proteins and *CCSP/SCGB1A1*, also support the notion that the GR does not transactivate these genes directly but rather interacts with other proteins with transactivation capability, which mediate the effects of the GR. Since the activity of C/EBPβ is suppressed in the airway epithelium of COPD patients [94], it is tempting to speculate that the suppressed C/EBPβ activity may contribute to the reduced GC responsiveness in COPD. Furthermore, C/EBPβ is a known downstream target of β_2-adrenoceptor signaling in several cell types [231, 232], suggesting that C/EBPs could also be involved in the effects of LABAs in the lung epithelium.

2 SCOPE OF THE CURRENT INVESTIGATIONS

The overall aim of this thesis was to investigate the functional roles of the lung-enriched transcription factors C/EBPα and C/EBPβ. To this end, cell culture experiments were performed, and the pulmonary phenotype of mice lacking C/EBPα and/or C/EBPβ specifically in the lung epithelium was assessed. The pulmonary phenotype of mice lacking C/EBPβ specifically in the lung epithelium was studied with regard to response to both immunological stimuli and pharmaceutical agents used in the therapy of inflammatory lung disease, to assess in detail the consequences of the reduced activity of C/EBPβ in the airway epithelium of smokers with COPD. Since C/EBP transcription factors bind to virtually identical DNA sequences and functional replacement therefore possible, overlapping roles of C/EBPα and C/EBPβ were also investigated. The following three separate aims have been explored:

To investigate the unique and overlapping roles of lung epithelial C/EBPα and C/EBPβ in pulmonary organogenesis with regard to cellular differentiation, as well as the consequences of C/EBPα deletion on adult lung morphology and pathology.

To investigate the role of lung epithelial C/EBPβ in the inflammatory response to acute cigarette smoke exposure and LPS challenge, as both of these inflammatory stimuli are associated with COPD.

To assess C/EBPβ in the lung epithelium as a possible mediator of the effects of LABAs and/or GCs, the mainstay medical therapy of inflammatory lung diseases, in a model of pulmonary neutrophilia.

3 COMMENTS ON METHODOLOGY

3.1 EPITHELIAL CELLS

Normal human bronchial epithelial (NHBE) cells i.e. primary cells collected by bronchial brushings are the preferred cells to study epithelial cells *in vitro,* as these cells are differentiated and resembles the airway epithelium (used in paper II and paper III). It is however difficult to control the differentiation state in culture, and there is a limited proliferative capability of primary cells. Another limitation with primary cells is of course the cost and difficulty in recruiting healthy volunteers; if not commercially available cells are used. For the transfection studies described in paper II and IV, transformed human normal bronchial epithelial cells (BEAS-2B), immortalized with adenovirus [233] were used. There are many different lung epithelial cell lines available, including adenocarcinoma derived A549, originating from alveolar type II cells, and mucoepidermoid carcinoma cells (NCI-H292), as used in paper III. The latter are representative of bronchial epithelial cells and, in similarity to BEAS-2B cells, express C/EBPβ [128, 234-237]. Previous studies have demonstrated a similar inflammatory response to cigarette smoke extract in NCI-H292 cells and NHBE cells [238, 239]. Thus, NCI-H292 cells represent a suitable cell line to study the inflammatory response to cigarette smoke extract and the role of C/EBPβ in mediating *in vitro* induction of inflammatory mediators.

3.2 IN VITRO TRANSFECTIONS

Small interfering (si)RNA or RNA interference (RNAi) was used to inhibit the expression of C/EBPβ in NHBE and NCI-H292 cells. siRNA was first described by Fire and Mello in 1998 [240], a discovery that awarded them the Nobel prize in 2006. siRNA has since been used for more than a decade to knock down or suppress gene expression. Growing concerns that off target effects may influence the outcome of RNAi experiments have led to the development of commercially available pools of several siRNAs targeting different sequences of a gene. With pooled siRNAs (used in papers II and III), the concentration of each individual siRNA is lower, leading to mimimized off target effects.

In paper II, plasmids containing the rat *Cebpa* and *Cebpb* genes controlled by the human cytomegalovirus (CMV) promoters (pCMV-*Cebpa* and pCMV-*Cebpb*) were used together with a *Scgb1a1-luciferase* reporter plasmid (containing a 170 bp segment of the *Scgb1a1* promoter, Figure 8) to study transactivation of the mouse *Scgb1a1* gene [241]. The CMV promoter exhibits a constitutive, high level expression in mammalian cells. A *Cebp-luciferase* reporter plasmid with the reporter gene under the control of a consensus C/EBP binding site, as described elsewhere [242] was used in study IV. When the expressed C/EBPs are activated, the factors bind to the consensus sequence and activate transcription of the luciferase gene, which is easily detected by a luminometer. The luciferase enzyme converts the substrate D-luciferin to oxyluciferin in a light emitting reaction. A great advantage of this system is the linearity between the emitted light and transcription as well as translation of the luciferase gene, which enables gene promoter studies. This methodology allows for a more accessible and functional detection of C/EBP transactivation than electrophoretic mobility shift assay

(EMSA). In contrast to the EMSA technique, the *Cebp-lucferase* reporter plasmid does not allow for determination of which C/EBP activates transcription.

3.3 TRANSGENIC MICE

As compared to other organisms, mice as a model organism offer a number of advantages, however, also a few drawbacks. First and foremost, the mouse genome has been fully sequenced [243], and the murine and human genome corresponds well eachother, although some discrepancies exist, including differences in the immune system. Importantly, the pulmonary expression of C/EBPs is identical between mice and humans [93]. Lung development is furthermore relatively similar between the two species.

C/EBPα knockout mice die shortly after birth due to hypoglycemia due to defetcts in liver metabolism. Mice lacking C/EBPβ display a complex phenotype with affected glucose homeostasis and compromised immune system, and half of the mice die within 24 hours of birth. Together, this emphasizes the need for conditional knockout strains to study the role of these transcription factors in lung development, as well as in the adult lung [91]. Thus, in order to investigate the role of C/EBPs in lung development, pathology, inflammatory response and pharmaceutical therapy, mice with lung epithelial-specific deletion of C/EBPα and C/EBPβ were generated using the Cre-*loxP* system, with Cre expressed under control of the 3.7 kb human *SFTPC* (SP-C) promoter. The *SFTPC* promoter is active in all lung epithelial cells from at least E10 [244, 245]. With the Cre-*loxP* system, an allele with the gene of interest is flanked by short base pair sequences (*LoxP sites*) (Figure 9). The so called floxed allele is next introduced to the genome of embryonic stem (ES) cells from mice of the 129 strain by homologous recombination. By using a selection marker, such as a neomycin resistance gene, ES clones with the floxed gene stably inserted into the genome can be selected for. Selected ES clones are subsequently injected into a C57/BL6 mouse embryo at the blastocyst stage. Offspring are mated with C57/BL6 mice to select for mice in which the ES cells have contributed to the germline, to generate mice that carry the recombinant floxed allele. The Cre recombinase is expressed under control of a cell-specific promoter (*SFTPC*) in mice with an outbread ICR background. The recombinase will excise the floxed element in the cells to be targeted, and their progeny, in which the Cre-recominase has been expressed. These knockout strains allow for studies of the role of C/EBP transcription factors in the epithelium specifically, throughout lung development, as well as in different experimental settings in adult mice. Other lung specific promoters, such as the *Scgb1a1* promoter are also frequently used. The first observed activity of the *Scgb1a1* promoter is, however, observed at a later point in development (i.e. E14), in close temporal proximity to the first detected expression of C/EBPs in the lung, which is at E15.5 [15]. Other promoters with high activity early in lung development and activity in relatively few other organs, such as the *Nkx2-1* promoter may also utilized in studies with conditional deletion of genes, although the activity in other organs is problematic when investigating gene products that are ubiquitously expressed, such as C/EBPs.

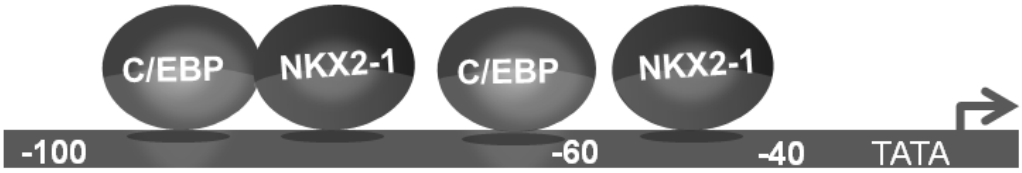

***Figure 8. Schematic representation of the proximal Clara cell secretory protein (Scgb1a1) promoter**. The illustration indicates the location of the C/EBP and NKX2-1 binding within the promoter, which was used in paper II. Numbers indicate distance from origin of transcription. Adapted from [93].*

Mice with a genetically mixed background, with a combination of the inbread 129 and C57BL/6 and the outbred ICR mouse strains were used in the studies included in this thesis, since generation of transgenic and knockout mice involves several mouse strains. This can be avoided by repeated back crossing to one strain, producing isogenic mice. This is nevertheless a time consuming and expensive procedure that may represent a problem, since different mouse strains are more or less sensitive to different challenges, and the choice of background strain would influence possible future investigations. In addition, investigating outbread mice, which in similarity with humans display a variable genetic background, includes the contribution of other genes on the effects of a specific gene deletion. A mixed genetic background, however, influences the variance and normal distribution within groups, and additionally requires that all the control groups have relevant genetic background. Accordingly, all wild-type controls used in papers I-IV were littermate controls, lacking the *SFTPC*-Cre allele. As normal distribution was not assumed among the samples, due to the mixed genetic background and relatively small group sizes, non-parametric statistical analysis were performed. The risk of small sample groups with mixed genetic background is that differences between groups may be undetected, due to lack of statistical power. An absence of a significant difference does not necessarily indicate that there is not a difference between the groups, simply that none could be detected. This is particularly true when the difference between groups approaches significance. Therefore, p-values below 0.1 are reported when appropriate.

The basis of COPD diagnosis is important to consider when studies of pulmonary inflammation in animal models are conducted. Lung function tests are difficult to perform in rodents, and do not directly correlate to lung function tests in human subjects. In the studies included in this thesis, pulmonary inflammation as well as other pathologic lesions of COPD such as airway remodeling, but not lung function, is investigated. In light of that, it is essential to remember that even though the animal models used in the studies in this thesis may have similarities with the pathologic changes associated with COPD; these are by definition not models of COPD.

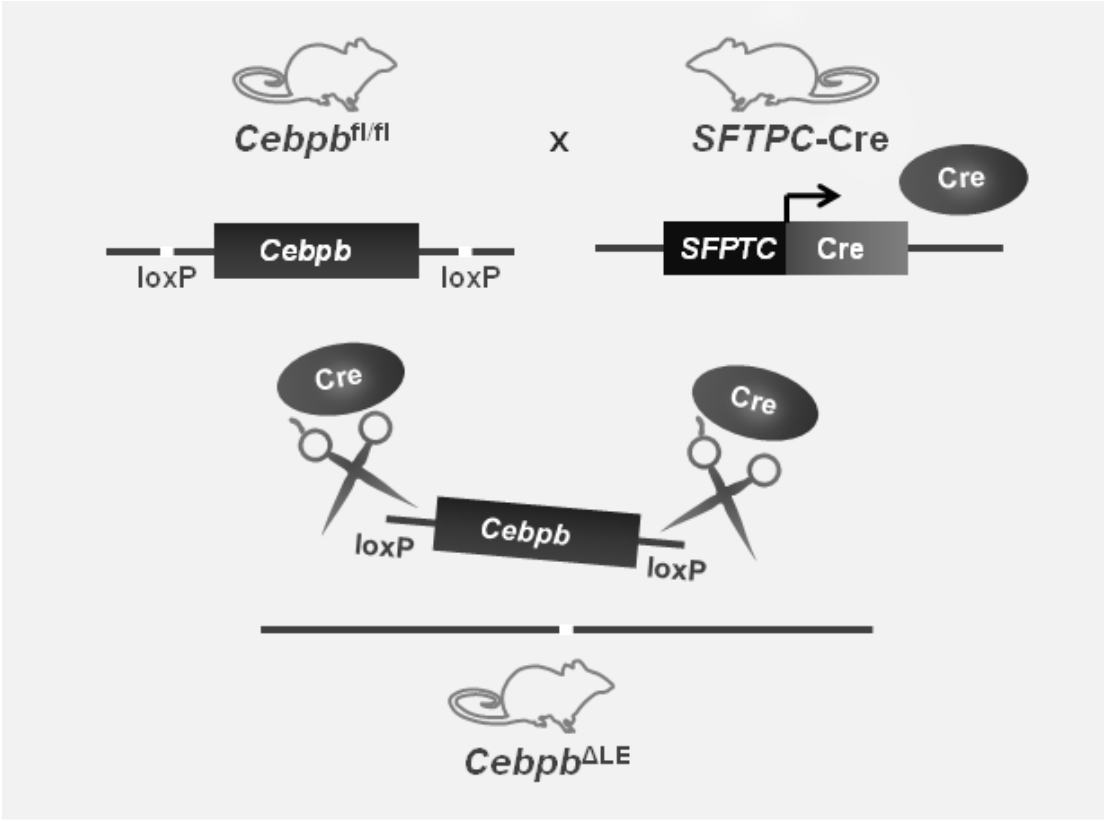

Figure 9. The Cre-loxP system. *In one mouse strain (Cebpb$^{fl/fl}$ mice), the Cebpb gene is flanked by loxP sites (floxed, fl). In the other strain (SFTPC-Cre), the Cre enzyme is expressed under the control of the SFTPC promoter. In the offspring (Cebpb$^{\Delta LE}$ mice), the Cre enzyme recognizes the loxP sites and excises the fragment in between the two sites (e.g. the Cebpb gene).*

3.4 CIGARETTE SMOKE EXPOSURE

Several different procedures for cigarette smoke exposure have been described. For instance, both main stream and a combination of main stream and side stream smoke can be used and the animals can be restrained, or allowed to move around freely. Of course, restraint is disadvantageous, however, this allows for nose only inhalation, while whole body exposure will result in particles being deposited on the coat, with ingestion upon grooming. The passive nose inhalation by mice, which differs from the active oral inhalation by humans who smoke, is a particularly intricate problem with murine smoke models. In addition, although the time of exposure can be increased, due to practical limitations, mice are often exposed for longer periods, followed by more extensive periods of recovery, which also differs from the smoking pattern of humans. The smoke exposure systems can differ by a series of variables, such as the number of puffs/minute, the duration of the exposure, as well as the frequency of the exposures, differences that influence the outcome and can make individual studies difficult to compare. On the other hand, the smoking patterns of humans vary significantly between individuals, and it is therefore difficult to establish what constitutes a general smoking habit [246]. The cigarette smoke exposure system used in paper III, utilizing main and side stream smoke and a whole body exposure for 1 hour, two times a day (Figure 10A), has been extensively documented and generates a reproducible pulmonary inflammation [247, 248].

Cigarette smoke extract (CSE) exposure represents an accessible experimental procedure to stimulate cultured cells to the water soluble fraction of cigarette smoke. Thus, this extract excludes the non-soluble (hydrophobic) fraction of cigarette smoke. Several studies have, however, demonstrated that CSE has immunostimulatory capabilities, although conflicting documentation exists, especially for epithelial cells [249]. Cultured cells may also be exposed to cigarette smoke in air liquid interface. In this model, cells are grown on a membrane, with the basolateral side submerged in media and the apical side exposed to ambient air. This allows for direct exposure with cigarette smoke with a smoking chamber. Some differences in inflammatory responses have been documented between the smoking chamber and CSE model [250], suggesting that the outcome of *in vitro* cigarette smoke models should be interpreted with caution.

3.5 LPS CHALLENGE

LPS challenge is well documented model for ALI/ARDS [251] but also mimics a hallmark of COPD, pulmonary neutrophilia [181]. Intravenous injection of LPS, which primarily damages the endothelium, and subsequently causes destruction of the pulmonary epithelium, is the most commonly used administration in ALI models [79]. Nebulization of dissolved LPS produces an aerosol of fine particles that penetrates the conducting and lower airways, making this administration ideal to assess pulmonary inflammation. Recruited neutrophils causes significant alveolar tissue damage, which leads to edema characterized by a protein rich fluid in the alveolar region [79]. In the LPS-challenged mice described in paper IV (Figure 10B), with LPS dose and timing tuned to get neutrophil recruitment without much alveolar injury and edema, minimal or little edema was detected. In addition, the majority of neutrophils were observed surrounding the blood vessels and airways, not the alveoli. This suggests that, as intended, the level of alveolar tissue destruction observed in ALI was not produced by LPS challenge after 5 hours in this model.

3.6 DRUG ADMINISTRATION

Inhalation therapy is the most effective administration of both formoterol (FM) and budesonide (BUD) in patients with chronic inflammatory lung disorders. In paper IV, FM and BUD were, however, administered via intra-peritoneal injection, as effective inhalation therapy is difficult to achieve in mice. In addition, the largest portion of inhaled BUD is deposited in mouse gut [252]. Extensive hepatic metabolism of BUD motivated use of a relatively high dose [253, 254]. The hepatic metabolism of FM in mice is still unknown, however, the proportion of β_2-adrenocoptors is lower in mouse lungs than human lungs [255, 256], suggesting that mice may be less sensitive to β_2-adrenoceptor agonists and require a relatively high dose of this drug as well. The effects of FM and BUD on baseline expression of inflammatory mediators were studied 3 hours post administration, to detect the effects of both the more rapidly acting FM and the slower acting BUD. To study the drug suppression of LPS-induced inflammation, mice were pre-treated with the drugs 1 hour before LPS challenge, and sacrificed 5 hours after this challenge, to allow for analysis of both early-induced and late-induced inflammatory mediators (Figure 10B). Pre-treatment with FM and BUD was performed to effectively address the contribution of C/EBPβ in preventing LPS-induced neutrophil recruitment in a setting relevant to COPD maintenance therapy with GCs and LABAs [222, 223].

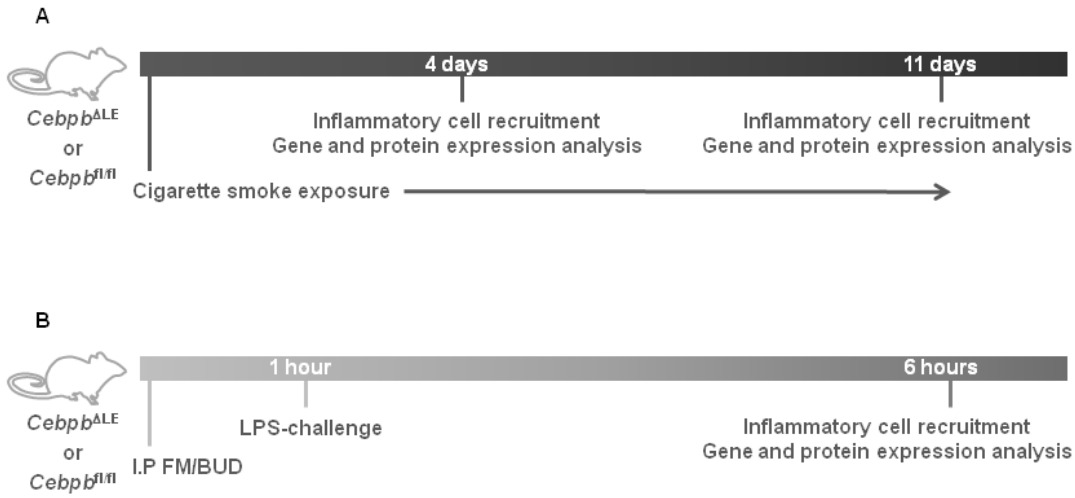

Figure 10. Experimental design of the cigarette smoke and LPS-induced lung inflammation models. *$Cebpb^{\Delta LE}$ or $Cebpb^{fl/fl}$ mice were exposed to (A) cigarette smoke for 4 or 11 days in paper III. In paper IV (B), mice were pre-treated to formoterol (FM), budesonide (BUD) or FM with BUD, challenged with aerosolized Pseudomonas aeruginosa lippolysacharide (LPS) and sacrificed 5 hours post-challenge. At the respective end-points, bronchoalveolar lavage (BAL) was performed and the number of inflammatory cells in BAL was assessed, together with the concentration of key inflammatory mediators. The gene expression of inflammatory mediators that have been proposed to be regulated by C/EBPs previously, was assessed in preserved lung tissue.*

3.7 SEMI-QUANTITATIVE REAL TIME PCR

In paper III and IV, the main objective was to investigate and present gene expression data, as this directly reflects the effect of C/EBPβ deletion on gene regulation. Protein levels of for instance cytokines and chemokines are, in addition to being affected by transcriptional regulation, influenced regulatory mechanisms influencing mRNA stability, protein translation and stability [257]. This suggests that analysis of mRNA expression rather that protein levels may be more accurate to assess gene regulation. The gene expression data was validated on a protein level as far as possible, however, due to the limited material available, only the protein concentration of a selected number of gene products could be measured.

Hypoxanthine phosphoribosyltransferase (*Hprt*)*1* and the human homologue *HPRT1*, as well as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used to normalize gene expression. Both *Hprt1/HPRT1* and *GAPDH* are widely and commonly used housekeeping genes. Previous documentation of their stability in cultured lung cells has been reported [258], although conflicting evidence of the stability in malignant lung tissue exist [258, 259]. The expression of *Hprt1*, *HPRT1*, or *GAPDH* did not differ between the groups with any treatment, in any of the studies. The data included in this thesis are presented as expression relative to the control gene (ΔCt normalization) or relative to the control gene and control condition (ΔΔCt normalization). In paper III and IV, the individual values of $Cebpb^{\Delta LE}$ and $Cebpb^{fl/fl}$ mice of each treatment group were normalized to the corresponding control group, to allow for efficient evaluation of treatment effects.

3.8 STATISTICAL ANALYSIS

All statistical analysis on the experimental studies performed in murine models presented in this thesis were performed with non-parametric tests, namely Kruskal-Wallis one way ANOVA to test for treatment effects, and Mann-Whitney unpaired U-test to test for individual differences between groups. The statistical analysis on experiments performed in cultured or freshly isolated cells were performed with parametric, unpaired t-tests. Alternatively, statistical analysis with multiple comparisons, such as described in paper IV, could be performed with Kruskal-Wallis tests combined with post-hoc tests, such as a Dunn's test. While performing multiple unpaired t-tests increases the risk of discovering false positive differences (type I errors), the risk of correcting for multiple comparison by performing one way ANOVA together with a post-hoc test increases the chance of not detecting differences (type II errors) due to lack of statistical power. This is particularly true for data sets with small sample sizes, such as the data presented in paper IV. In light of this, unpaired t-tests were performed.

4 RESULTS AND DISCUSSION

4.1 FUNCTIONAL ROLE OF C/EBPα IN THE LUNG EPITHELIUM

C/EBPα plays a key role in cell differentiation and the induction of genes associated with the mature differentiated organ in several tissues [91]. In addition, C/EBPα inhibits proliferation by interacting and repressing cdk2, cdk4, and E2F [106, 107], as well as by inducing $p21^{WAF/Cip1}$ [108, 109]. In paper I, the significance of C/EBPα lung epithelial deletion on lung organogenesis was investigated. Furthermore, the embryonic lung immaturity in mice lacking lung epithelial-C/EBPα presented an opportunity to assess the long term consequences of pulmonary immaturity at birth. Hence, the pulmonary phenotype was assessed in adult mice, with the hypothesis that developmental defects may induce pathological changes in the adult lung.

4.1.1 Impaired lung development in *Cebpa*$^{\Delta LE}$ mice

To address the outcome of C/EBPα deletion *in vivo*, the pulmonary phenotype of mice with a lung-epithelial specific deletion of C/EBPα (*Cebpa*$^{\Delta LE}$ mice) was assessed at the saccular (E18.5) and alveolar (P9) stages of lung development, as well as in adult mice (3 months old). *Cebpa*$^{\Delta LE}$ mice displayed prominent interstitial tissue in the alveolar septa along with enlarged airspaces, which appeared to be lined with cuboidal cells in place of slender type I pneumocytes. These changes were present throughout both the saccular as well as the alveolar stages of lung development (paper I and II). Additional assessment revealed relatively fewer Clara cells in *Cebpa*$^{\Delta LE}$ mice, as well as the presence of PAS-positive cells in the conducting airways of *Cebpa*$^{\Delta LE}$ mice at E18.5, suggesting that some of the Clara cells may have differentiated into a mucus cell lineage (presented in paper II). Preceding publications support the findings presented in paper I [18, 103]; however, these studies failed to assess the consequences of lung immaturity caused by embryonic C/EBPα deletion on the adult lung phenotype.

The pulmonary phenotype of embryonic/newborn *Cebpa*$^{\Delta LE}$ mice is consistent with the pathological lesions seen in preterm infants with pulmonary immaturity [138, 139]. Although surfactant deficiency contributes to the disease, inflammatory responses caused by invasive oxygen treatment also drive the pathological changes in BPD. In support of this, inflammatory responses during postnatal development contribute to the impaired alveolarization observed in both the old and new definitions of BPD [260]. Others have described a central role for C/EBPα in hyperoxia. Xu and colleagues used inducible Cre expression to conditionally delete C/EBPα in the lung epithelium after birth. While these mice display normal lung structure when unprovoked, the lungs of C/EBPα deficient mice are severely damaged by oxygen treatment, suggesting that C/EBPα is required for cytoprotection during hyperoxia [261] and that *Cebpa*$^{\Delta LE}$ mice may exhibit amplified responses to hyperoxia. Based on this, it would be of great interest to incorporate oxygen treatment into the model of pulmonary immaturity in *Cebpa*$^{\Delta LE}$ mice.

4.1.2 Lung immaturity in *Cebpa*$^{\Delta LE}$ mice causes histopathological features similar to COPD in the adult lung

As the lungs of *Cebpa*$^{\Delta LE}$ mice mature, bronchiolar metaplasia and goblet cell hyperplasia persisted and the airspaces continued to enlarge. Moreover, adult *Cebpa*$^{\Delta LE}$ mice exhibited an increased number of proliferating cells compared to littermate controls, confirming that C/EBPα plays a key role in inhibiting proliferation of the pulmonary epithelium. In support of this, C/EBPα expression is associated with proliferative arrest [15] and hyperoxia-challenged mice pre-treated with a siRNA targeting *Cebpa* display increased proliferation but decreased type II pneumocyte differentiation two weeks post challenge [262]. In addition to increased proliferation, a majority of the histopathological lesions observed in COPD was detected in adult *Cebpa*$^{\Delta LE}$ mice, including free non-anchored arterioles with an irregular pattern, i.e. emphysema, as well as centrilobular interstitial fibrosis, bronchiolar ectasia, and mucus plugging. Morphological analysis, presented as mean linear intercept, confirmed that the emphysematous changes, i.e. the enlargement and/or destruction of alveoli occurred over time. This may be mechanistically explained by the inflammatory cell infiltrates observed in the bronchioles, alveoli, and interstitial space, along with expression profile of inflammatory mediators and acute phase reactants, similar to what is observed in COPD [149, 263]. The normal lungs of mice where C/EBPα is deleted after birth, as reported by others [261], suggests that the lung immaturity of *Cebpa*$^{\Delta LE}$ mice observed here, not the C/EBPα deficiency in itself, instigated impaired lung homeostasis later in life. It is thus possible that the inflammatory changes in the lungs of *Cebpa*$^{\Delta LE}$ mice drive the progression of emphysema as well as the other observed lesions.

Pulmonary immaturity at birth contributes to adult airway obstruction – a link between BPD and COPD

While the diagnosis of COPD is exclusively based on lung function, the disease is strongly associated with infiltration of inflammatory cells and thickening of the small airway wall, together with mucus plugging and peribronchiolar fibrosis. In addition, destruction of the lung parenchyma causes enlarged airspaces and loss of lung elasticity [149, 264, 265]. Although tobacco smoke is the etiological agent in a vast majority of cases of COPD, far from all smokers develop airway obstruction [143]. *Cebpa*$^{\Delta LE}$ mice spontaneously developed the pathological lesions associated with COPD, without tobacco smoke exposure. This highlights the importance of genetic factors, as well as early life events such as pulmonary immaturity at birth, which may increase the susceptibility of developing chronic lung disorders [136, 140, 266-269]. Investigations into the components that contribute to airway obstruction are, however, complicated by the lack of a definite mechanistic explanation for the processes that drive emphysema. Furthermore, COPD has been proposed to be not a single disease but several different clinical conditions with non-reversible airway obstruction as the common feature. It is therefore likely that preterm infants with BPD develop airway obstruction that is phenotypically separate from cigarette smoke-induced disease. Careful investigations of the clinical phenotypes, including inflammatory profiles and airway remodeling, are thus imperative. Additionally, with increased survival rates of prematurely born infants, there is a growing need to understand the processes that drive this airway obstruction caused by lung immaturity at birth. Such knowledge may be used to improve future clinical practices and treatments for preterm infants with BPD.

4.2 FUNCTIONAL REPLACEMENT OF C/EBPs

More than 50% of *Cebpa*$^{\Delta LE}$ mice die at birth due to respiratory distress (paper I). The reason as to why far from all *Cebpa*$^{\Delta LE}$ mice survive until adulthood is unclear, although the virtually identical binding preferences of the C/EBPs [89] suggests that individual C/EBPs may be functionally replaced by other C/EBP family members under some circumstances. In support of this, C/EBPα, C/EBPβ, and also C/EBPδ have the capability of binding the promoter of the gene coding for the mouse α1-acid glycoprotein [86], indicating that these factors may functionally replace each other. Functional replacement could also explain the lack of pulmonary phenotype in mice lacking C/EBPβ [119], which is somewhat surprising in light of the suggested importance of C/EBPβ in controlling differentiation and proliferation in several other organs [91, 120, 270-272]. Thus, deletion of both *Cebpa* and *Cebpb* may cause a more severe pulmonary phenotype than deletion of either gene alone. In paper II, transgenic mice as well as cultured cells were used to address the hypothesis that lung epithelial C/EBPα and C/EBPβ have overlapping roles in lung organogenesis and lung epithelial cell differentiation. Furthermore, the function of these transcription factors in the lung epithelium was assessed, as functional replacement could mask their definite roles.

4.2.1 C/EBPα compensates for C/EBPβ and both factors activate the *Scgb1a1* promoter

The findings in paper II demonstrate that both *Cebpa* and *Cebpb* increase transactivation of the *Scgb1a1* promoter in BEAS-2B cells. Transactivation was, however, not increased with transfection of both *Cebpa* and *Cebpb* together. This implies that C/EBPα and C/EBPβ bind the same elements within the *Scgb1a1* promoter and may not act synergistically, as has been demonstrated for C/EBPα and NKX2-1 or C/EBPδ [241, 273]. Moreover, in NBEC, suppression of *CEBPB* led to an increase in both *CEBPA* as well as *SCGB1A1* expression. Although this does not provide definite evidence, it is suggestive of a compensatory mechanism where *CEBPA* is upregulated following *CEBPB* suppression, with subsequently more efficient activation of *SCGB1A1* transcription.

4.2.2 *Cebpa*$^{\Delta LE}$*; Cebpb*$^{\Delta LE}$ mice exhibit histopathology similar to *Cebpa*$^{\Delta LE}$ mice

To further investigate the suggested compensatory roles of C/EBPα and C/EBPβ in the lung epithelium, as suggested by the *in vitro* findings in paper II, the functional replacement of individual C/EBPs was assessed *in vivo*. The pulmonary phenotype of mice lacking both C/EBPα and C/EBPβ in the lung epithelium (*Cebpa*$^{\Delta LE}$*; Cebpb*$^{\Delta LE}$ mice) was consequently compared to single *Cebpa*$^{\Delta LE}$ or *Cebpb*$^{\Delta LE}$ transgenic mice, or to wild-type littermate controls (*SFTPC*-Cre^{-} mice). *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice exhibited immature lungs with interstitial tissue in the alveolar septa and enlarged airspaces lined with cuboidal cells during the saccular period of lung organogenesis, similar to what was observed in *Cebpa*$^{\Delta LE}$ mice (paper I and II). The proliferation ratio was comparable between all mice during saccular development, suggesting that C/EBPα and C/EBPβ do not influence proliferation at this stage.

4.2.3 Impaired airway epithelial cell differentiation in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice

Based on the *in vitro* data, which suggested a partially overlapping role for C/EBPα and C/EBPβ in regulating *SCGB1A1*, the expression of SCGB1A1 was assessed in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice (paper II). SCGB1A1 is secreted by Clara cells and serous cells in the proximal conducting airways, and putatively defines the Clara cell population [274]. In both *SFTPC*-Cre^{-} mice or *Cebpb*$^{\Delta LE}$ mice, SCGB1A1-expressing Clara cells represented the majority of the cells lining the large and small airways. The number of Clara cells surrounding the airway lumen was reduced in *Cebpa*$^{\Delta LE}$ mice (paper II), in agreement with the observation in adult *Cebpa*$^{\Delta LE}$ mice (paper I). This suggests that differentiation of the Clara cell linage is affected in the saccular stage of lung development in these mice. In contrast, Clara cells were almost completely absent in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice, suggesting even more impaired Clara cell differentiation in the bronchioles of *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice, compared to *Cebpa*$^{\Delta LE}$ mice. Thus, the functions of C/EBPα and C/EBPβ are central to Clara cell differentiation, with possible implications for lung homeostasis and host defenses. Providing additional support for a role for C/EBPα and C/EBPβ in regulating SCGB1A1 and Clara cell differentiation, the mRNA expression of *Scgb1a1* was reduced by 90% in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice compared to *SFTPC*-Cre^{-} mice, while no significant decrease was detected in *Cebpa*$^{\Delta LE}$ mice. The expression of both C/EBPδ and NKX2-1 was, in contrast, preserved in all mice. Hence, in contrast to the previous suggestion [241], these transcription factors do not appear to be vital for SCGB1A1 regulation in the absence of C/EBPα and C/EBPβ *in vivo*. Based on these findings, it is tempting to speculate that activation of the *Scgb1a1* promoter involves NKX2-1 together with either C/EBPα or C/EBPβ, but that the latter are interchangeable. It is thus likely that C/EBPs may compensate for one another and have partially overlapping functions regarding differentiation of the airway epithelium. The results presented herein suggest that either C/EBPα or C/EBPβ is required for Clara cell commitment. In support of this, functional redundancy of C/EBPs has been documented in other tissues [275, 276]. A clue to how differences in gene regulation may occur between different C/EBPs, even though they bind to virtually the same DNA sequences, is provided by the observation that C/EBPα together with NKX2-1 synergistically activates *Scgb1a1* by binding to adjacent response elements in the *Scgb1a1* promoter [273]. In other words, by their ability to differentially interact with other transcription factors, C/EBPs can achieve specificity, even though they bind to the same or very similar DNA segments.

4.2.4 Ectopic mucus producing cells in the conducting airways of *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice

Clara cells play important roles in the regulation of inflammatory responses [277] and are in addition considered to be progenitor cells for the respiratory epithelium in mice [278, 279]. Evidence supports a plasticity between epithelial cell populations, and Clara cells possess the capability to trans-differentiate into both ciliated as well as mucus producing cells in the adult murine lung [32, 41, 54, 280]. Hence, a defect in Clara cell maturation in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice might affect other cell populations in the airway epithelium, such as ciliated and mucus producing cells. In agreement with this,

ectopic mucus producing cells were observed in the conducting airways of both *Cebpa*$^{\Delta LE}$ mice and *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice, suggesting that some Clara cell progenitors had committed to the mucus producing cell linage. The majority of SCGB1A1 negative cells were, however, most probably undifferentiated progenitor cells, supported by increased expression of *Sox2*, a marker of progenitor cells [281], in the lungs of *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice. Mucus hyperplasia is a key pathological lesion of several chronic lung diseases such as asthma, cystic fibrosis and COPD [282-284]. Therefore, improved knowledge of mucus producing cell commitment and plasticity is of great clinical interest, and key to understand pathological remodeling in lung diseases.

The evidence presented here suggests that the outcome of *Cebpb* deletion may differ when C/EBPα or other C/EBP family members have the ability to functionally replace C/EBPβ. This may, however, not be the case during acute phase responses to inflammatory stimuli, such as cigarette smoke.

4.3 C/EBPβ MEDIATES INFLAMMATORY RESPONSES IN THE LUNG EPITHELIUM

The increased activity of C/EBPβ in the airway epithelium of asymptomatic smokers and decreased C/EBPβ activity in smokers with COPD or chronic bronchitis [94] suggests a possible role for C/EBPβ in COPD pathogenesis. While C/EBPβ has been attributed with a pro-inflammatory role in several tissues [86, 90], the precise role of C/EBPβ in the airway epithelium is unknown. In light of this, the objective of paper III and IV was to investigate the role of C/EBPβ in inflammatory responses in the airways, with the hypothesis that C/EBPβ contributes to the inflammatory responses to cigarette smoke and LPS.

Smoking is the by far most important risk factor for early-onset COPD [285]. An inflammatory response similar to what is observed in COPD, namely pulmonary neutrophilia and increased expression of inflammatory mediators, is detected in mice exposed to cigarette smoke [75, 76, 163-168, 171-173]. In paper III, a translational approach was adopted to evaluate the role of C/EBPβ in cigarette smoke-induced inflammation, investigating clinical samples, transgenic mice as well as human epithelial cells.

4.3.1 *CEBPB* is downregulated in current and former smokers

As a first step, the mRNA expression of *CEBPB* was analyzed in human airway epithelial biopsies from the large airways of current and former smokers, as well as never-smokers [286]. The expression of *CEBPB* was significantly lower in the airway epithelium of both current and former smokers, compared to never-smokers. In agreement with this, CSE stimulation immediately downregulated the expression of *CEBPB* mRNA as well as the C/EBPβ protein isoforms LIP and LAP in NCI-H292 cells and/or NHBE cells. Based on the decreased expression of *CEBPB* in former and current smokers as well as in cultured cells exposed to smoke, it is tempting to speculate that downregulation of C/EBPβ is associated with both the short and long-term consequences of cigarette smoking. This has possible clinical consequences in smoking-associated diseases that develop over the long term, and sometimes long after

smoking cessation, such as COPD, and warrants further studies on the role of C/EBPβ in mediating the airway epithelial response to cigarette smoke.

In support of a role for C/EBPβ in mediating the inflammatory response to cigarette smoke in bronchial epithelial cells, NHBE cells treated with siRNA against *CEBPB* exhibited blunted induction of several inflammatory mediators, including *IL8*, *IL6*, *IL1B* and *TNFA* after CSE stimulation, compared to control cells. Similarly, a blunted inflammatory mediator response was also observed in CSE-stimulated NCI-H292 cells with suppressed expression of *CEBPB*.

4.3.2 Impaired inflammatory cell recruitment to the lungs of cigarette smoke-exposed *Cebpb*$^{\Delta LE}$ mice

To further elucidate the functional role of lung epithelial-C/EBPβ in smoke-induced inflammation, *Cebpb*$^{\Delta LE}$ and *Cebpb*$^{fl/fl}$ mice were next exposed to cigarette smoke for 4 or 11 days, or exposed to unfiltered smoke-free room air for the same time periods. Reduced neutrophil recruitment was observed in *Cebpb*$^{\Delta LE}$ mice after both 4 and 11 days of smoke-exposure, compared to smoke-exposed *Cebpb*$^{fl/fl}$ mice. The induction of inflammatory mediators was also affected in smoke-exposed *Cebpb*$^{\Delta LE}$ mice. Blunted induction of *Groa* and *Saa3* was observed in *Cebpb*$^{\Delta LE}$ mice after both 4 and 11 days of smoke exposure. In addition, impaired induction of *Il1b*, *Tnfa*, *Mip1g* and *Csf3* (coding for G-CSF) was detected with cigarette smoke exposure for 4 days in these mice. These inflammatory mediators attract neutrophils, promote neutrophil degranulation and are associated with COPD [75, 81, 149, 169, 287]. Evidence also suggests a role for several of these cytokines in promoting cigarette smoke-induced inflammation as well as emphysema, suggestively through neutrophil infiltration, although other inflammatory cells such as macrophages also contribute to the pathogenesis of emphysema [288, 289]. Taken together, these findings indicate that C/EBPβ is central to the inflammatory responses to cigarette smoke, possibly by promoting neutrophil recruitment by increasing the expression of inflammatory mediators with neutrophil chemotactic activity such as *Groa*. In light of this, activation of C/EBPβ among asymptomatic smokers likely amplifies inflammatory responses, while the functional outcome of the decreased activity of C/EBPβ in smokers with COPD is uncertain. It is, however, possible that decreased C/EBPβ signaling in the airway epithelium of smokers with COPD contributes to Clara cell hypoplasia and mucus cell hyperplasia, as indicated by the findings in paper II, although further studies are needed to clarify this. In addition, it is also conceivable that impaired C/EBPβ signaling compromises the host defense mechanisms to respiratory pathogens, which are associated with both stable and exacerbated COPD [290-293]. Evidence suggests that the permanent presence of bacteria, as seen with low-grade chronic infection in the lungs of COPD patients, could induce an inflammatory response directly or alter the host defenses to cigarette smoke, as findings from experimental models suggest [248, 294-296]. Acquisition of a new bacterial strain, or a virus infection, with the virulence of the new pathogen, together with impaired host defenses as drivers, may theoretically explain the amplified inflammation characteristic of an exacerbation [156]. Further assessment of C/EBPβ in inflammatory signaling, in particular with respect to inflammation associated with respiratory infections, is hence pivotal for our understanding of the consequences of the activity of lung epithelial C/EBPβ in smokers with COPD.

4.3.3 Reduced respiratory neutrophilia in LPS-challenged *Cebpb*$^{\Delta LE}$ mice

In paper IV, aerosolized *Ps. aeruginosa* LPS was used to induce pulmonary neutrophilia [181], a hallmark of COPD [149] in *Cebpb*$^{\Delta LE}$ and control *Cebpb*$^{fl/fl}$ mice. LPS is a structural component of the Gram-negative bacterial cell wall and is thus associated with respiratory infections. LPS challenge is furthermore used as a model of acute lung injury, a syndrome with acute inflammation and edema characterized by neutrophil accumulation in the alveolar space [79]. The LPS-induced neutrophilia described in paper IV was significantly blunted in *Cebpb*$^{\Delta LE}$ mice, possibly explained by a reduced expression of *Groa*, a neutrophil chemoattractant and murine homologue of IL-8 [297, 298]. In support of the chemoattractant role of GROα, the number of neutrophils correlated positively with the expression of *Groa*. In addition, blunted induction of *Cox2*, *Il6* and *Il1b* was also observed in *Cebpb*$^{\Delta LE}$ mice, although the difference compared to *Cebpb*$^{fl/fl}$ mice failed to reach statistical significance for the two latter parameters (p=0.064 and p=0.11, respectively). These findings support the findings in paper III and demonstrate that C/EBPβ has a pro-inflammatory role in the lung epithelium. The relatively small difference in *Il6* induction observed between *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice, in contrast to earlier suggestions of C/EBPβ regulation of IL6 [85], suggests that the regulatory networks controlling inflammatory genes in the lung epithelium may vary from those in other tissues, possibly because C/EBPs regulate lung-specific genes with immunomodulatory functions, such as *Sftpa1* (coding for SP-A) and *Scgb1a1* [93]. This furthermore implies that the regulation of target genes by C/EBPs is cell and tissue specific, as previously proposed [91].

Possible mechanisms of LPS stimulation in bronchial epithelial cells
The activity level of C/EBPβ is increased by LPS stimulation in extra-pulmonary cells [299]. In addition, other inflammatory stimuli, such as TNFα induce C/EBPβ transactivation in bronchial epithelial cells [236]. Current evidence, however, suggests that the activity of C/EBP in bronchial epithelial cells is unaffected by LPS stimulation *in vitro* [128]. The results presented in paper IV further support the idea that LPS stimulation fails to significantly induce C/EBP transactivation immediately, although a trend towards increased activity was observed (p=0.097). The limited induction of C/EBP transactivation following LPS stimulation may be related to the low expression of the major LPS receptor TLR4 in BEAS-2B cells [300]. With this in mind, it is advisable that further studies address this in primary cells, such as NBEC. It is, however, also conceivable that other mechanisms than increased transcriptional activation, such as the total level of C/EBP proteins or the C/EBPβ LIP/LAP ratio, and may be important following LPS stimulation of airway epithelial cells. These mechanisms are probably slower than induction of transactivation and could take longer than the 1 hour time point studied *in vitro*. Immune responses to respiratory pathogens in the lung are mediated by a complex interaction between epithelial and inflammatory cells, that may be difficult to mimic *in vitro* [301]. While epithelial cells sense the presence of microorganisms and actively recruit inflammatory cells, the secondary response is dependent on the activation of epithelial cells by infiltrating inflammatory cells [76], which is difficult to model *in vitro*. An example of this is the LPS-induced expression of GROα that, in Clara cells, is enhanced by macrophage derived TNFα [302]. Thus, *in vitro* studies investigating the individual response of a single cell population to an inflammatory stimulus may reveal intriguing functions of cells within the lung, although the picture is incomplete.

Impaired C/EBPβ signaling may weaken pulmonary host defenses
The involvement of C/EBPβ in LPS-induced respiratory neutrophilia suggests that this transcription factor may play an essential role in the innate immune responses to bacterial infections. Reduced C/EBPβ activity in smokers with COPD [94] may attenuate innate immune responses and predispose the lungs to reoccurring bacterial infections or permanent microbial colonization, as observed among COPD patients [303], since neutrophils are central in bacterial elimination [304]. The consequence of reduced neutrophilia could, however, also be beneficial, as persistent neutrophil influx has been suggested to contribute to the pathogenesis of COPD [304]. Although LPS induces a strong inflammatory response in the airways, its similarity to actual infections is limited since bacteria are recognized by several PRRs. To definitively conclude that lung epithelial C/EBPβ plays a role in bacterial infection, it is therefore necessary to infect mice with live bacteria and assess the immune responses in the lung (further discussed in the Future Perspectives section).

4.4 C/EBPβ CONTRIBUTES TO THE EFFECTS OF LONG-ACTING β_2-AGONISTS AND GLUCOCORTICOIDS

The LPS model of pulmonary neutrophilia also allows for investigations of the involvement of C/EBPβ in pharmacological suppression of inflammatory responses. Thus, in paper IV, the role of C/EBPβ in simultaneous LPS challenge and LABA and/or GC treatment was investigated in paper IV.

β_2-adrenergic agonists have been suggested to activate C/EBPα *in vitro* [231, 232], and C/EBPα is important for the effects of GCs on proliferation and differentiation in several cell types [92], including bronchial smooth muscle cells [129, 305]. The binding activity of C/EBPβ is increased by GCs in bronchial epithelial cells [133], possibly explaining the ability of GCs to enhance expression of host defense molecules [40], since functional GREs are absent in the promoters of some lung-specific, GC-induced genes [132, 133]. The role of C/EBPβ in the suppression of inflammatory genes by LABAs and GCs has not been addressed, and it is presently not known whether LABAs activate C/EBPs in the airway epithelium. This is particularly intriguing considering that C/EBPβ contributes to the regulation of many genes coding for cytokines and chemokines following inflammatory stimuli [90, 91, 93, 306, 307], implying that C/EBPβ may be influenced by both inflammatory and anti-inflammatory signaling.

4.4.1 C/EBPβ mediates the suppressive action of formoterol on inflammatory signaling

To assess the role of C/EBPβ in mediating the effects of LABAs and GCs in the lung epithelium, *Cebpb*$^{\Delta LE}$ and *Cebpb*$^{fl/fl}$ mice were pre-treated with a LABA, formoterol (FM), a GC, budesonide (BUD), or FM together with BUD, and subsequently challenged with aerosolized *Ps. aeruginosa* LPS. As demonstrated in paper IV, the suppressive effects of FM on neutrophil infiltration along with inflammatory mediator expression were impaired in *Cebpb*$^{\Delta LE}$ mice. Furthermore, FM significantly increased expression of LPS-induced *Groa* and *Il6* in *Cebpb*$^{\Delta LE}$ mice but not in *Cebpb*$^{fl/fl}$ mice. The blunted suppression of *Groa* and *Il6* by combination treatment with FM and BUD, observed in *Cebpb*$^{\Delta LE}$ mice, may consequently be related to the stimulatory effect of FM alone in these mice. As mentioned previously, GROα is an important neutrophil

attractant [297, 298] and is implicated in COPD [169]. IL-6 has been suggested as a target for the medical therapy of COPD, on account of the amplifying function of this cytokine, which acts upstream of other inflammatory mediators [163]. The stimulation of these inflammatory mediators in *Cebpb*$^{\Delta LE}$ mice indicates an inhibitory role of C/EBPβ in LABA signaling.

In order to investigate the possible mechanisms by which LABAs such as FM affect C/EBP signaling, human bronchial epithelial cells (BEAS-2B) transfected with a C/EBP-luciferase reporter construct were stimulated with FM and LPS, FM with or without propranolol, or to forskolin. C/EBP transactivation was significantly induced by FM treatment with or without LPS, and the effect was reversed by pre-treatment with the β-adrenoceptor agonist propranolol. This demonstrates that the effect of FM on C/EBP activity is mediated by β-adrenoceptors, most probably the β_2-adrenoceptor given that FM specifically activates this receptor. The similar stimulation of C/EBP transactivation by FM with LPS and by FM alone suggests that the mechanism by which FM stimulates C/EBP activity is the same at baseline and in the presence of an inflammatory stimulus. The cAMP elevating agent forskolin also significantly increased C/EBP dependent transactivation, further indicating that the β_2-adrenoceptor dependent activation of C/EBPs by FM may involve cAMP signaling. In summary, FM putatively stimulates C/EBP transactivation via the β2-adrenoceptor and cAMP in bronchial epithelial cells *in vitro*, suggesting that the inflammatory suppression by FM observed *in vivo* is mediated by increased C/EBPβ activity. Overall, these findings demonstrate a key role for lung epithelial C/EBPβ in the suppression of inflammatory mediators implicated in COPD by LABAs, which are frequently used in COPD therapy.

4.4.2 A role for C/EBPβ in mediating glucocorticoid suppression of inflammatory mediators

The results presented in paper IV also support a role for C/EBPβ in mediating the suppressive effects of GCs in the airway epithelium. Significantly blunted suppression of LPS-induced *Il6* and *Cox2* expression by BUD was observed in LPS-challenged *Cebpb*$^{\Delta LE}$ mice. COX-2 is involved in the synthesis of prostanoids from arachidonic acid, with a key role in inflammatory signaling in lung disease [308]. Upon GC treatment, C/EBPβ has been demonstrated to physically interact with the GR and bind to the *Cox2* promoter [309]. In addition to the differences in *Il6* and *Cox2* expression observed in LPS-challenged *Cebpb*$^{\Delta LE}$ mice, the suppression by BUD with FM of baseline *Tnfa* expression in naïve, unchallenged *Cebpb*$^{\Delta LE}$ mice was significantly blunted, and, similarly, a trend towards blunted suppression was observed with BUD alone ($p=0.095$), compared to *Cebpb*$^{fl/fl}$ mice. As mentioned earlier, TNFα is implicated in COPD pathogenesis and effective suppression of this cytokine is suggestively central in COPD management. Also, impaired suppression of baseline *Nos2* expression (coding for inducible nitric oxide synthase, iNOS) was observed in *Cebpb*$^{\Delta LE}$ mice. NOS enzymes expressed in the airway epithelium generate endogenous nitric oxide (NO) by converting L-arginine to L-citrulline, and both exhaled NO and iNOS are elevated in COPD [171, 310]. Taken together, these findings complement the previously observed involvement of C/EBPβ in the sparing or enhancement of host defense molecules by GCs [40] with additional evidence of a role for C/EBPβ in the suppression of inflammatory mediators by GCs, possibly by the previously described mechanism of increased C/EBPβ activity by GC stimulation. In agreement with the previous *in vitro* documentation by Zhang and colleagues [40], the acute phase reactant *Saa3* appeared

to be stimulated by GCs only in LPS-challenged *Cebpb*$^{fl/fl}$ mice, with no effect observed in *Cebpb*$^{\Delta LE}$ mice. As demonstrated by others, C/EBPβ and GR synergistically enhance the transcription of the host defense gene *Orm1* (coding for α1-glycoprotein) by binding to overlapping sites in the promoter [311, 312], providing a possible mechanism that could serve to explain the GC stimulation of *Saa3*. Taken together with the blunted suppression of inflammatory mediators, these findings suggest that impaired C/EBPβ signaling in smokers with or without COPD [94, 313] and reduced C/EBPδ expression in the bronchial smooth muscle cells of COPD patients [314] may represent an additional and novel mechanism that may partly explain the relative GC resistance in COPD.

4.4.3 Possible mechanisms of C/EBPβ as a mediator of simultaneous inflammatory and anti-inflammatory signaling

The potential involvement of C/EBPβ in mediating both LPS-induced inflammation as well as the anti-inflammatory effects of LABAs and GCs are individually supported by previous *in vitro* studies [40, 90, 132, 133, 232], although the mechanism underlying this divergent action is still unknown. A possible explanation is provided by different post-transcriptional modifications such as phosphorylation, SUMOylation and acetylation, which have been demonstrated to affect the activity of C/EBPβ [91, 315-317]. C/EBPβ is the only C/EBP family member that contains additional and unique regions that are targets for post-transcriptional modifications [86]. It has previously been shown that SUMOylation of C/EBPβ represses *Cox2* transcription [318], as opposed to the other documented role of C/EBPβ in promoting transcription of *Cox2* [319]. This represents an interesting mechanism by which the function of C/EBPβ could be altered from inflammatory to anti-inflammatory by affecting DNA-binding activity and specificity dependent on post-transcriptional modification. Phosphorylation of amino acid residues on C/EBPβ is an important determinant of transactivation and is required for the induction of pro-inflammatory genes. Multiple phosphorylation sites have also been documented [86], implying that different signaling pathways converge and influence the activity of C/EBPβ. Some of these post-transcriptional modifications are likely to direct C/EBPβ to bind negative regulatory DNA sequences, thereby inhibiting gene transcription of inflammatory genes [320, 321], as opposed to stimulating transcription of pro-inflammatory genes. It is also plausible that GCs and LABAs stimulate the translation of LIP, which could inhibit the expression of pro-inflammatory genes. In addition, C/EBPβ contains negative regulatory regions in the N-terminus, which could be involved in the shift from inflammatory to anti-inflammatory signaling, although the precise role of these regions is unknown [91].

The ability of C/EBPs to physically interact with different proteins [227, 322, 323] and consequently bind to different DNA sequences is well-documented [323, 324]. It is theoretically possible that inflammatory and anti-inflammatory stimuli differentially influence this capacity by inducing different post-transcriptional modifications on C/EBPs or other proteins (Figure 11 A and B). In support of a role for this mechanism, LPS has been reported to increase *IL1B* transcription via binding of C/EBPβ and activating transcription factor (ATF)4 to an enhancer segment of the gene. On the other hand, when cAMP response element-binding (CREB) is phosphorylated as a consequence of cAMP stimulation, CREB dimerizes with C/EBPβ and compete for binding to the enhancer sequence, with suppression of the LPS-induced response [86]. C/EBPs could accordingly be targeted to certain gene promoters, depending on protein-

protein interactions with other transcription factors. Another mechanism that could explain the involvement of C/EBPβ in GC signaling involves the mitogen-activated protein kinase (MAPK)-phosphatase (MKP)1/dual specificity phosphatase (DUSP)1, which reduces pro-inflammatory signaling by inhibiting MAPKs such as extracellular signaling-regulated kinase (ERK) and p38 [325-330]. Although DUSP1 is induced by GCs, C/EBP binding sites, not GREs are necessary for transcription of the gene [331] and transactivation has been proposed to involve a tethering mechanism with GR and C/EBPβ bound to the promoter [331]. This implies that C/EBPβ may mediate the anti-inflammatory effects of GC by inducing DUSP1, possibly effecting GC suppression of inflammatory mediators such as COX-2 and IL-6 through inhibition of MAPKs. In summary, C/EBPβ could mediate both the pro-inflammatory induction as well as the immune suppressive effects of GCs and LABAs, although the mechanism explaining this dual action is still unknown.

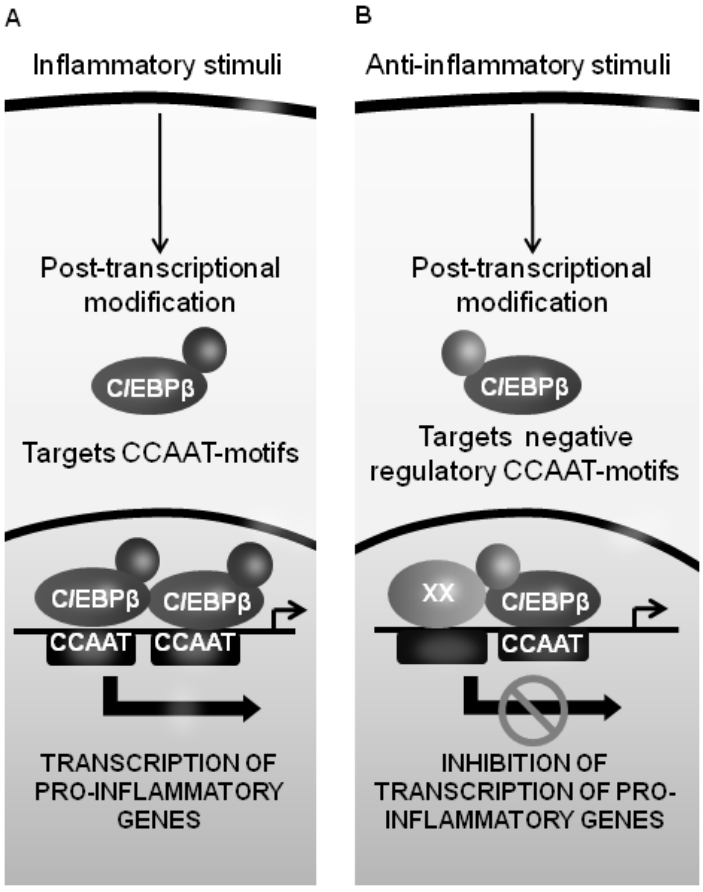

Figure 11. A proposed model for the dual role of C/EBPβ in contributing to the inhibition and induction of inflammatory responses. *(A) Inflammatory signaling induces post-transcriptional modifications that activate C/EBPβ, which homodimerizes, binds to C/EBP-responsive elements and stimulates transcription of pro-inflammatory genes. (B) Anti-inflammatory stimuli induce other post-transcriptional modifications that direct C/EBPβ dimerization with other proteins and binding to negative regulatory DNA sequences, which inhibits gene transcription.*

4.5 PRELIMINARY RESULTS

4.5.1 The role of lung epithelial-C/EBPβ in influenza infection

Influenza A is a common respiratory virus that causes significant morbidity and mortality world-wide, and influenza infection is associated with COPD exacerbations [290, 291, 332]. Influenza infection induces C/EBP activity in the murine lung [333]; however, the role of C/EBPβ in respiratory viral infections remains unknown. With the objective of elucidating whether C/EBPβ promotes inflammatory responses to influenza infection, the inflammatory mediator response to dsRNA stimulation was analyzed in human NCI-H292 airway epithelial cells with siRNA-inhibited expression of C/EBPβ, and in precision cut lung slices (PCLS) from *Cebpb*$^{\Delta LE}$ mice. *Cebpb*$^{\Delta LE}$ mice were also used to examine the inflammatory response to H1N1 Influenza A infection *in vivo,* as well as *ex vivo* in PCLS.

The studies performed so far have revealed that dsRNA induces the expression of *CEBPB* in NCI-H292 cells, while inhibition of *CEBPB* in these cells causes disrupted dsRNA-induced expression of cytokines and chemokines. In our study, influenza infection resulted in respiratory neutrophilia, the induction of inflammatory mediators and, in agreement with the previously observed induction of C/EBP activity [333], increased pulmonary expression of *Cebpb*. Also, influenza-infected *Cebpb*$^{\Delta LE}$ mice demonstrated a delayed onset of clinical symptoms and decline in body weight as well as increased expression of *Tnfa*, *Il1b*, and *Mip1g*, compared to infected wild-type controls. The expression of the host defense genes *Scgb1a1* and *Sftpa1* was, however, lower in *Cebpb*$^{\Delta LE}$ mice as compared to *Cebpb*$^{fl/fl}$ mice. In the immediate-early inflammatory response, assessed in PCLS from *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice, blunted cytokine and chemokine induction in response to both dsRNA stimulation and influenza infection was observed in PCLS from *Cebpb*$^{\Delta LE}$ mice, compared to PCLS from wild-type mice. Thus, lung epithelial-C/EBPβ appears to be a mediator of the immediate-early induction of innate immune responses to influenza infection, and may hence play a role in influenza virus-induced COPD exacerbations. Further studies are warranted to investigate the impaired innate immune responses *in vivo*, and to determine the long-term outcome of C/EBPβ deletion for influenza infection, with regard to clinical presentation, viral titers, and survival.

4.6 CONCLUDING REMARKS

The results presented herein collectively demonstrate that C/EBPs play pivotal roles in epithelial cell differentiation during lung organogenesis, and that both C/EBPα and C/EBPβ are involved in pathological processes related to COPD with an important role for C/EBPβ in inflammatory responses. In addition, the findings presented in this thesis indicate a key role for the airway epithelium in inflammatory responses in the lung.

The development of histopathology similar to COPD in adult *Cebpa*$^{\Delta LE}$ mice suggests that lung immaturity at birth contributes to lung pathology in the adult. Although several studies support an increase in airway symptoms and obstruction among children and adolescents who were delivered prematurely [136, 140, 266-269], the outcome for the development of respiratory disorders later in life is still unknown. This warrants large epidemiological studies of the prevalence of airway obstruction among adults that were prematurely delivered, as these individuals grow older. This is particularly

important since improved critical care has led to increased survival of extremely premature infants with severely immature lungs.

The findings in paper II suggest that C/EBPα and C/EBPβ are required for airway epithelial differentiation. The ectopic mucus producing cells observed in mice lacking C/EBPβ and/or C/EBPα furthermore suggest that C/EBPs may play a part in airway remodeling, with implications in both COPD and asthma. Moreover, evidence suggests that functional replacement of C/EBPβ by other lung-enriched C/EBPs, for instance C/EBPα, as well as additional compensatory mechanisms involving other pro-inflammatory transcription factors, may mask the actual role of C/EBPβ in the lung epithelium. However, these mechanisms may be ineffective following inflammatory stimuli, such as cigarette smoke exposure or LPS challenge, when other C/EBPs appear to have a limited ability to compensate for C/EBPβ. These results are of particular interest considering the reduced activity of C/EBPβ in the airway epithelium of smokers with COPD [94], and implies that other lung-enriched C/EBPs with preserved expression may not compensate for impaired C/EBPβ activity when lung sterility is compromised, such as during respiratory infection. Further studies in infection models are thus needed to investigate these proposed mechanisms.

The results in paper III and IV demonstrate a blunted inflammatory response to LPS as well as cigarette smoke in *Cebpb*$^{\Delta LE}$ mice, and suggest that C/EBPβ is a pro-inflammatory transcription factor in the lung epithelium. *In vivo* as well as *in vitro* evidence also points towards a role for C/EBPβ in mediating the suppressive effects on inflammation of LABAs and GCs in the pulmonary epithelium, suggesting that C/EBPβ may be involved in suppressing inflammatory signaling under some circumstances. Inflammatory responses to cigarette smoke are observed both in COPD patients and asymptomatic smokers [163, 334]. As asymptomatic smokers exhibit markedly increased activity of C/EBPβ, while smokers with COPD display decreased C/EBPβ activity [94], C/EBPβ may contribute to the inflammatory response among asymptomatic smokers, while other inflammatory transcription factors may be more central in driving exaggerated inflammatory signaling in COPD [335, 336]. The role of C/EBPβ in chronic inflammation has, however, not been investigated. It is also possible that the impaired C/EBPβ signaling among smokers with COPD impairs host defenses to respiratory pathogens and increased apoptosis [91, 93], although this remains to be addressed. C/EBPβ signaling may thus also be relevant in other inflammatory lung disorders associated with, or exacerbated by, pathogens, such as asthma. In addition, C/EBPs have been demonstrated to activate anti-oxidative defenses [337, 338], and decreased C/EBPβ activity could result in increased epithelial damage by oxidative stress. In light of this, additional studies investigating the contribution of C/EBPβ to host defenses in a model using both cigarette smoke exposure and a respiratory pathogen is crucial to increase our understanding of C/EBP transcription factors in the lung epithelium further.

5 FUTURE PERSPECTIVES

Since COPD is a complex and multifactorial disease with both environmental and genetic contributions, further studies more closely investigating the role of C/EBPs in disease pathogenesis is warranted. While the studies in this thesis demonstrate that lung epithelial C/EBPβ plays a role in smoke-induced lung inflammation as well as pathogen-related signaling associated with COPD, the relevance of this in COPD pathogenesis needs to be addressed further. From the results presented in this thesis, future investigations should target three central components of COPD; 1) cigarette smoke and pathogen-induced lung inflammation, 2) airway remodeling and 3) exacerbated responses to microbial infection. These pre-clinical studies could translate to translational and clinical research and have the potential to improve the quality of life of millions of patients who suffer from COPD.

The involvement of C/EBPβ in inflammatory responses to long-term cigarette smoke exposure has thus far not been addressed. The findings of impaired acute and sub-acute inflammatory responses to cigarette smoke in mice lacking C/EBPβ presented in this thesis suggest that impaired C/EBPβ signaling may protect against emphysema. *In vivo* investigations into this are required, as the effect of C/EBPβ deletion on chronic cigarette smoke exposure may differ from the observed effect of acute or sub-acute smoke exposure. Such studies would enable the assessment of the degree of airway and pulmonary inflammation as well as emphysematous changes in the lung parenchyma of mice lacking C/EBPβ specifically in the lung epithelium. Moreover, the mechanism by which C/EBPβ contributes to cigarette smoke-induced airway inflammation has not been addressed in detail, and further investigations may reveal mechanisms with the potential to enhance our understanding of the detrimental effects of cigarette smoke. In addition, the involvement of C/EBPs in host defenses to respiratory pathogens is important to investigate. COPD exacerbations drive disease progression, account for a large portion of the morbidity and mortality associated with the disease, and place a significant burden on the health care system. Efforts must therefore be made to minimize the damaging effects of respiratory infections in pulmonary disorders.

As C/EBPβ is implicated in COPD and respiratory infections cause COPD exacerbations, the role of C/EBPβ in innate immune responses and epithelial regeneration following respiratory infection may be central to understanding COPD pathogenesis. To address this, we have so far focused on the inflammatory responses to Influenza A and *Haemophilus influenzae*. Preliminary data shows an impaired activation of innate immune responses observed in precision cut lung slices in the initial phase of influenza infection in *Cebpb*$^{\Delta LE}$ mice, while the responses to influenza A and *H. influenzae* are preserved or elevated 5 days and 12 hours after infection, respectively, in these mice. There is also evidence of airway remodeling in influenza-infected *Cebpb*$^{\Delta LE}$ mice. Additional studies will focus on the immediate-early inflammatory responses as well as infection-induced epithelial differentiation. Since cigarette smoke compromises antimicrobal host defenses [162], a model encompassing cigarette smoke exposure with concomitant bacterial or viral infection, which recapitulates many of the features of COPD exacerbations [294, 339], is also warranted. This is of particular interest as exacerbations are central to driving COPD progression, with each individual exacerbation causing deterioration in lung function that does not fully recover [143, 154]. Models with both cigarette smoke challenge and viral or bacterial infection better mimics the pulmonary insult of chronic lung disease [248,

294, 295, 339, 340] and provides a suitable model to investigate the role of C/EBPs in exacerbations of inflammatory lung diseases. Following completion of a research program as outlined above, we will have a better understanding of the processes that drive the progression of airway disorders. The outcome of future studies has possible implications for future therapies of infection-induced COPD and asthma exacerbations, and may help to prevent disease progression.

The inflammatory response to respiratory infections can be effectively studied in cigarette smoke-exposed mice using precision cut lung slices. This *ex vivo* model allows for assessment of the responses of the structural cells of the lung, but fails to completely replace *in vivo* studies, as cells of the circulatory system (i.e. immune cells) have a large impact on the outcome. In order to investigate the role of C/EBPβ in an exacerbation model, mice will be exposed to cigarette smoke and precision cut lung slices from these mice will be infected with influenza A to determine the time course of the inflammatory response in cells with or without C/EBPβ. *In vivo* studies will subsequently investigate the inflammatory response in detail, at selected time points. It has furthermore been suggested that a failure to repair damaged tissue plays a role in the pathogenesis of emphysema [148]. Considering that C/EBPβ may be involved in regulating proliferation in the lung epithelium (unpublished observations), and could amplify inflammatory responses, suppression of C/EBPβ may be beneficial and protect against emphysema. In further support of this, many of the genes that exhibited blunted induction in challenged *Cebpb*$^{\Delta LE}$ mice (i.e. *Groa*, *Il1b*, *Tnfa* and *Il6*) contribute to or are associated with the development of emphysema. It is thus possible that *Cebpb*$^{\Delta LE}$ mice show attenuated emphysema. Assessment of the role of C/EBPβ in GC and LABA signaling would also be of interest in a model with both cigarette smoke exposure and respiratory infection, since such a challenge has been suggested as a model for COPD exacerbation [339], and maintenance treatment with GCs together with LABAs are used to reduce the frequency and severity of COPD exacerbations.

The ultimate goal of all these studies is, of course, to improve the quality of life of patients suffering from inflammatory lung disorders. Further advancements in our understanding of transcriptional regulation of inflammatory signaling and differentiation programs may lead to the discovery of new potential targets for pharmacological interventions. The signaling pathways that involve C/EBPβ are of great interest in this context and future investigations have the potential to provide important clues to inflammatory signaling pathways and epithelial differentiation. Repression of C/EBPβ may suppress the chronic inflammation observed in pulmonary disorders; however, this could also potentially impair host defenses to respiratory infections. Thus, the most optimal way to improve the clinical outcome of COPD by manipulating C/EBPβ signaling is possibly through precise mapping of post-transcriptional modifications as well as interactions with other proteins, and accurate targeting of the signaling pathways that suppress amplifying cytokines such as IL-6 (i.e. chronic inflammation) while stimulating host defense molecules such as SCGB1A1 and surfactant proteins. Stimulation of such a pathway would most probably be achieved by activating kinases that phosphorylate C/EBPβ. It is, however, necessary to considerably advance the understanding of the precise role of C/EBPs in lung homeostasis and inflammatory responses before such ambitious studies are initiated.

The adult phenotype of *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice still remains unknown. Compromised epithelial cell differentiation in these mice supports further investigations to address the outcome of *Cebpa* and *Cebpb* deletion in the adult lung. These studies would be possible to do using inducible Cre (i.e. the Tet-ON system)

with *Cebpa* and *Cebpb* deleted in adult lungs to circumvent lethality at birth due to respiratory failure. Utilizing inducible Cre, airway remodeling as well as the inflammatory response to long-term cigarette smoke exposure (i.e. emphysema) may also be addressed in *Cebpa*$^{\Delta LE}$ mice and *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice. This may reveal interesting clues to the pathology of the airways, as this thesis has provided evidence that epithelial integrity as well as the differentiation of facultative progenitor cells is impaired in *Cebpa*$^{\Delta LE}$; *Cebpb*$^{\Delta LE}$ mice.

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