REVIEW

The emerging role of C/EBPs in glucocorticoid signaling: lessons from the lung

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Abstract

Glucocorticoids (GCs) have been successfully used in the treatment of inflammatory diseases for decades. However, there is a relative GC resistance in several inflammatory lung disorders, such as chronic obstructive pulmonary disease (COPD), but still the mechanism(s) behind this unresponsiveness remains unknown. Interaction between transcription factors and the GC receptor contribute to GC effects but may also provide mechanisms explaining steroid resistance. CCAAT/enhancer-binding protein (C/EBP) transcription factors are important regulators of pulmonary gene expression and have been implicated in inflammatory

lung diseases such as asthma, pulmonary fibrosis, cystic fibrosis, sarcoidosis, and COPD. In addition, several studies have indicated a role for C/EBPs in mediating GC effects. In this review, we discuss the different mechanisms of GC action as well as the function of the lung-enriched members of the C/EBP transcription factor family. We also summarize the current knowledge of the role of C/EBP transcription factors in mediating the effects of GCs, with emphasis on pulmonary effects, and their potential role in mediating GC resistance.

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Introduction

Glucocorticoids or (gluco)corticosteroids (GCs) are a mainstay in the treatment of chronic inflammatory lung diseases, although their effectiveness is reduced in several diseases. A poor response and a need for higher doses of GCs, defined as a relative GC resistance or insensitivity, are present in disorders such as chronic obstructive pulmonary disease (COPD; for a review, see Barnes & Adcock (2009)). This represents a therapeutic problem affecting millions of patients with chronic inflammatory lung disorders worldwide. As the prevalence of COPD is rising dramatically, GC insensitivity in this patient group may also contribute to increasing medical care costs (Barnes & Adcock 2009). This review summarizes the role of lung-enriched CCAAT/enhancer-binding proteins (C/EBPs) in GC signaling and the significance of C/EBP family in the mechanisms underlying GC resistance.

GR is expressed in almost all cell types, including bronchial epithelial cells, and is retained in the cytoplasm by a multi-protein complex (Howard & Distelhorst 1988, Adcock & Lane 2003, Pujols *et al.* 2009). Steroid binding to the GR triggers a conformational change that releases the receptor from the chaperone complex and allows for nuclear transportation. The translocated GR dimerizes and binds to GC-responsive elements (GREs), i.e. cis-regulatory regions of target genes (Dahlman-Wright *et al.* 1990, Luisi *et al.* 1991).

Gene regulation by the GR

GCs regulate various inflammatory and host defense genes in the lung through different mechanisms. Additionally, GCs influence important cellular functions such as differentiation, proliferation, and apoptosis (Cole *et al.* 1995, 2004, Wen *et al.* 1997, Cha *et al.* 1998, Cram *et al.* 1998, Pelaia *et al.* 2003, Saffar *et al.* 2011). It is estimated that the number of genes that are directly regulated by GCs are between 10 and 100 per cell, and in addition, many genes are indirectly regulated (Adcock & Lane 2003). The GR mode of action is highly complex, involving interactions with both basal and specific transcription factors, co-factors, and adaptor proteins. The activity of

Glucocorticoids

The GC receptor mediates the effects of GCs

GCs freely diffuse across the cellular membrane and exert effects by binding to the cytoplasmic GC receptor (GR). The

GR is modulated by post-transcriptional modifications, similar to C/EBPs, and the GR is involved in complex cross-talk with other signaling pathways, for instance nuclear factor (NF)κB. As a transcription factor, the GR can act by transactivation and increase transcription of anti-inflammatory genes by binding GREs (transactivation; Wikstrom 2003, Liberman *et al.* 2007, Pujols *et al.* 2009). In addition, while GCs suppress pro-inflammatory responses by transrepression, sparing or even induction of host defense molecules by GCs has been reported (Zhang *et al.* (2007); for a review, see Schleimer (2004)). In this review, we will discuss several genes that are regulated by GCs and C/EBPs, either through direct interaction and binding to DNA or via a tethering mechanism.

Transrepression by the GR

Transactivation cannot solely explain all the anti-inflammatory effects of GCs. In transrepression, the GR binds to negative GREs and decreases transcription of cytokines and chemokines such as interleukins (ILs), but also adhesion molecules such as intracellular adhesion molecule (ICAM)-1 and inflammatory enzymes, such as cyclooxygenase 2 (COX2; reviewed in Barnes (2006)). The GR also influences gene regulation by interacting directly with other transcription factors, most noteworthy NFκB, activator protein (AP)-1 and C/EBPs.

Interaction between the GR and other transcription factors

Direct or indirect interaction with pro-inflammatory transcription factors like NFκB (Ray & Prefontaine 1994, Caldenhoven *et al.* 1995) is of great importance and may explain a significant proportion of the anti-inflammatory effects of GCs. Through binding to inflammatory transcription factors, the GR can mask the DNA-binding site, domains required for activation or dimerization, or nuclear translocation signals and thereby inhibit activity of pro-inflammatory transcription factors (Adcock & Barnes 2008). For instance, GC suppression of ICAM1 in bronchial epithelial cells is mediated via direct interaction between the GR and NFκB (Ray & Prefontaine 1994, vanderSaag *et al.* 1996). However, as ICAM1 is C/EBP regulated (Chini *et al.* 1998, Manzel *et al.* 2009), it is possible that GR interaction with C/EBPs reduces C/EBP binding to its responsive element and subsequent gene transcription, thus also contributing to the GC suppression of ICAM1. In support of this concept, reduced C/EBP binding to the CCAAT consensus sequence following GC treatment has been reported in murine macrophages (Das *et al.* 2011). In addition to these mechanisms, the GR can also dimerize with other transcription factors when acting in transactivation or transrepression. GRs have furthermore been shown to inhibit interactions with the transcriptional machinery by steric impairment or displace transcription factors from DNA, as reviewed elsewhere (Liberman *et al.* 2007).

Other mechanisms of GC suppression of inflammatory signaling

In addition to the modes of action mentioned above, GCs decrease inflammatory signaling through repression of mitogen-activated protein kinase activity (MAPKs), such as extracellular regulated kinase (ERK) and Jun N-terminal kinase (JNK) (Rider *et al.* 1996, Caelles *et al.* 1997, Swantek *et al.* 1997, Hirasawa *et al.* 1998). It has also been shown that the activity of GR is modulated by a complex pattern of phosphorylations. The majority of phosphorylation sites on the GR are serine residues in proline-directed consensus sequences, suggesting that MAPKs also play a role in activating GR (Galliher-Beckley & Cidlowski 2009). Thus, interplay between MAPKs and GR occurs at several levels to contribute to the complexity of GC signaling. GCs also decrease transcription of inflammatory genes by inducing histone modifications as well as chromatin remodeling and making the DNA sequences inaccessible for gene transcription (for a review, see Barnes *et al.* (2005)). C/EBP transcription factors have a potential role in several of the mentioned mechanisms that will be discussed in further detail.

GC therapy and resistance in inflammatory lung diseases

While GCs represent the most effective anti-inflammatory treatment for many diseases, several lung diseases with an inflammatory component, such as COPD, acute respiratory distress syndrome, interstitial pulmonary fibrosis, and cystic fibrosis, appear to be largely GC resistant. In addition, some patients with asthma also display steroid unresponsiveness (Barnes & Adcock 2009).

Inhaled GCs in COPD

There is currently no consensus whether inhaled GCs attenuate the long-term decline in lung function or influence the natural course of COPD (Rabe *et al.* 2007). Recent evidence suggests that patients with moderate or even mild COPD, not only patients with severe COPD, may benefit from GC therapy (Telenga *et al.* 2010). Inhaled GCs (fluticasone) with or without an added long-acting β-adrenoceptor agonist (salmeterol) have been demonstrated to reduce the rate of decline in lung function significantly (Celli *et al.* 2008, Lapperre *et al.* 2009). Additionally, inhaled GCs reduce the frequency of COPD exacerbations, thereby improving health status (Spencer *et al.* 2004) and all-cause mortality (Sin *et al.* 2005), and lead to a slower decline in quality of life (Yang *et al.* 2007a). Taken together, this suggests that there are beneficial effects of inhaled GC in patients with COPD, although the inflammation of the disease is considered to be largely steroid unresponsive.

Mechanisms of GC resistance

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 Barnes & Adcock (2009)). Smokers with COPD display a lower activity of C/EBPβ compared with asymptomatic smokers (Didon *et al.* 2005), and lower transcriptional levels of C/EBPβ have been observed in both current and former smokers compared to never smokers (Didon *et al.* 2011). This may represent an additional and novel mechanism explaining relative GC resistance in COPD, since C/EBPs are implicated in GC signaling, as will be discussed.

CCAAT/enhancer-binding proteins

C/EBPs, constituting a family of six members (C/EBPα, β, γ, δ, ε, and ζ), belong to the basic region-leucine zipper (bZIP) class of basic domain transcription factors (Wingender *et al.* 1997, 2001). The founding member of the group, C/EBPα, was identified in rat liver nuclei by Johnson & McKnight (1989) as a protein capable of binding the CCAAT box motif present in various gene promoters (Johnson & McKnight 1989).

The positively charged and highly conserved basic region of C/EBPs (i.e. the C-terminal) directly interacts with DNA. The DNA sequences that C/EBPα, β, and δ bind have been identified as virtually identical, although some differences in binding site specificities exist, in particular for C/EBPβ (Cao *et al.* 1991, Williams *et al.* 1991, Osada *et al.* 1996, Poli 1998, Ramji & Foka 2002). Therefore, it is tempting to speculate that C/EBPs can functionally replace each other with regard to activating gene transcription. In support of this concept, C/EBPs have been demonstrated to form heterodimers in all intrafamily combinations, and dimerization is required for DNA binding (Ramji & Foka 2002). C/EBPs also form protein–protein interactions with other transcription factors, including NFκB and NK2 homeobox 1 (Nkx2.1)/thyroid transcription factor (TTF)-1, additionally fine-tuning the regulation of target genes (LeClair *et al.* 1992, Hsu *et al.* 1994, Cassel *et al.* 2002). The synergistic activation of Clara cell secretory protein (CCSP/CC10) transcription by binding of

both C/EBPα and the lung-enriched transcription factor Nkx2.1/TTF1 to adjacent responsive elements (Cassel *et al.* 2002), but not by C/EBPβ or δ, provides an important clue to how differences in gene regulation may occur between different C/EBPs, even though they bind to virtually the same DNA sequences. C/EBP dimerization and interaction with other transcription factors is of particular interest for inflammatory and GC signaling, since different combinations may result in pro- or anti-inflammatory responses. Importantly, C/EBPs have been implicated in several inflammatory lung diseases (summarized in Table 1), including asthma, cystic fibrosis, pulmonary fibrosis, sarcoidosis, and COPD (Pittman *et al.* 1995, Nuthall *et al.* 1999, Roth *et al.* 2004, Overbergh *et al.* 2006, Borger *et al.* 2007, 2009, Didon *et al.* 2010, 2011), highlighting the need for further studies on the disease-specific role of these transcription factors.

C/EBPs in the lung

In addition to the two ubiquitously expressed family members C/EBPγ and ζ, C/EBPα, β, and δ are expressed in the lung (Cassel & Nord 2003), with C/EBPβ being the dominant DNA-binding C/EBP family member in the human airway epithelium (Didon *et al.* 2005) and in bronchial smooth muscle cells (Borger *et al.* 2007). Two C/EBPα polypeptides with different activation potential are produced from the same mRNA (the 42 kDa and the 30 kDa isoform; Ossipow *et al.* (1993)) and three C/EBPβ isoforms have been identified: the 38 kDa liver-enriched transcriptional AP (LAP1), the 35 kDa LAP2, and the 20 kDa liver-enriched transcriptional inhibitory protein (LIP). Since the transactivation domain is absent in the LIP protein, this isoform acts as a dominant negative inhibitor of C/EBP transactivation by forming non-functional heterodimers with other family members (Descombes & Schibler 1991, Ramji & Foka 2002). Thus, various combinations of isoforms with different transactivation potential could have a profound effect on the regulation of target genes.

Table 1 Function of lung-enriched C/EBPs in lung diseases and GC signaling

Gene	Biological role	Pulmonary disease	Role in GC signaling
C/EBPα	Inhibits proliferation and mediates differentiation	Asthma and non-small-cell lung cancer	Required for GC-induced transcription of p21 ^{WAF/Cip1} . Mediates GC-dependent differentiation via C/EBPβ and regulates constitutive expression of the cortisol activator 11β-HSD1
C/EBPβ	Supports proliferation, anti-apoptotic. Induced during acute-phase response, regulate inflammatory and innate immunity gene expression	COPD, chronic bronchitis and pulmonary fibrosis	Activated by GCs via post-transcriptional modification. Mediates GC effects on host-defense genes and pulmonary genes lacking GRE. Mediates GC suppression of inflammatory mediators (COX2 and IL6). Regulate GC-induced expression of phosphatase DUSP1 and constitutive and induced expression of cortisol activator 11β-HSD1
C/EBPδ	Supports proliferation. Role in acute-phase response	COPD	Upregulated by GCs in smooth muscle cells

11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; C/EBP, CCAAT/enhancer-binding protein; COPD, chronic obstructive pulmonary disease; DUSP1, dual specificity phosphatase; GC, glucocorticoid; GRE, glucocorticoid-responsive element.

Function of C/EBP α

A number of studies have indicated a role for C/EBP α in regulating proliferation in several organs, including the lung (Flodby *et al.* 1996, Diehl 1998, Loomis *et al.* 2007). Deletion and ectopic expression of C/EBP α in the lung have revealed a vital role in regulating proliferation also in the alveolar epithelium (Flodby *et al.* 1996, Sugahara *et al.* 2001, Basseres *et al.* 2006, Berg *et al.* 2006, Didon *et al.* 2010).

Proliferative control by C/EBP α

C/EBP α has been reported to control proliferation by several different mechanisms. Interaction occurs with two critical regulators of cell cycle progression, cyclin-dependent kinase (cdk) 2 and 4, that mediate growth arrest (Wang *et al.* 2001). C/EBP α also represses activation of E2F, which is necessary for passage through the cell cycle restriction point and is central in the regulation of proliferation (Hsu *et al.* 1994, Zaragoza *et al.* 2010). Also, a mechanism especially important for the anti-proliferative effect of GCs involves C/EBP α , discussed in the next paragraph.

Role of C/EBP α in GC effects on proliferation

While GCs induce apoptosis in cells of myeloid lineage, anti-apoptotic effects have become evident in lung epithelial cells (Wen *et al.* 1997, Pelaia *et al.* 2003, Saffar *et al.* 2011). The topical GC budesonide has a profound inhibitory effect on TGF- β -induced apoptosis in human bronchial epithelial cells, suggesting that inhaled GCs could protect the airway epithelium against injury in inflammatory lung diseases (Pelaia *et al.* 2003). Furthermore, GCs also have the ability to inhibit proliferation induced by acute lung injury (ozone) in bronchial epithelial cells (Salmon *et al.* 1998), and in lung-derived fibroblasts, GC inhibition of proliferation mediated by the cdk inhibitor p21^{Waf1/Cip1} is dependent on C/EBP α (Yang *et al.* 2008). In line with this concept, the anti-proliferative effect of GCs in lung mesenchymal cells is mediated via formation of a C/EBP α -GR complex (Rudiger *et al.* 2002), thus providing a mechanism by which C/EBP α mediates the anti-proliferative effects of GCs. The understanding of this mechanism was improved by studies in hepatic cells, from which it was postulated that cell cycle arrest by GC stimulation is dependent on GR interactions with C/EBP α , allowing for transcription of p21^{Waf1/Cip1} (Cram *et al.* 1998). In primary lung vascular smooth muscle cells and fibroblasts, it was subsequently determined that GCs induce C/EBP α -GR interactions and binding to a CCAAT motif present in the p21^{Waf1/Cip1} promoter. GREs are, however, not present in the p21^{Waf1/Cip1} promoter (Rudiger *et al.* 2002), indicating that CCAAT motifs are crucial. The GC budesonide also inhibits proliferation of bronchial smooth muscle cells, and the effect is suppressed by C/EBP α deletion (Roth *et al.* 2002). A following study revealed that GCs inhibited

proliferation in smooth muscle cells from healthy individuals, but not from asthmatics. Furthermore, C/EBP α expression is detected in healthy subjects, but not in smooth muscle cells from asthmatics (Roth *et al.* 2004). A more recent study has similarly shown that bronchial smooth muscle cells from asthmatics proliferate more than twofold faster and express lower levels of C/EBP α (Borger *et al.* 2009), supporting a functional role for C/EBP α in mediating the anti-proliferative effects of GCs. Conversely, smooth muscle cells from asthmatics transiently transfected with an expression vector for C/EBP α regain GC responsiveness (Roth *et al.* 2004). These data confirm that C/EBP α may be central for the anti-proliferative effects of GC treatment in asthma and that C/EBPs mediate the effects of GCs on GR-responsive genes lacking binding sites for the receptor. Furthermore, this mechanism appears to be important in the lung and could have a clinical impact on inflammatory lung diseases.

Role of C/EBP α in GC effects on differentiation

In addition to the anti-apoptotic effects, GCs have the ability to induce differentiation. In preadipocytes, this is thought to be partly mediated via acetylation of C/EBP β , leading to interaction with histone deacetylase 1 (HDAC1) and transcription of C/EBP α . This in turn facilitates transcription of genes associated with differentiation (Wiper-Bergeron *et al.* 2007). Similarly, GCs also induce hepatocyte differentiation in a C/EBP β -dependent manner (Al-Adsani *et al.* 2010).

Studies on lung development have revealed a critical role of GCs in the maturation of the lung. In support of this, the GR is highly expressed in the lung and airways (Adcock *et al.* 1996; www.nursa.org/10.1621/datasets.02001). In absence of GC signaling, mice die shortly after birth due to respiratory failure, as demonstrated in mice lacking the GR. The lungs of GR^{-/-} mice are highly immature and demonstrate increased proliferation of both mesenchymal and epithelial cells (Cole *et al.* 1995). In addition, antenatal GC administration is routinely used to decrease mortality in pre-term births. GCs increase lung volume, stimulate parenchymal maturation, increase surfactant production, and improve the response to surfactant treatment (Lyons & Garite 2002). Supporting the importance of GCs in lung development, GR^{-/-} mice show undifferentiated type I but not type II pneumocytes and reduced expression of pulmonary surfactant protein (SP)-A and SP-D (Cole *et al.* 1995, 2004), although no GRE has been found in any SP gene (Karin 1998). Moreover, mice with a mutation in the GR causing inability of GR to bind to DNA are viable and exhibit normal lungs, demonstrating that the impaired lung maturation caused by abolition of GR is not dependent on DNA binding (Reichardt *et al.* 1998).

Impaired lung development in mice with a lung epithelium-specific deletion of C/EBP α

Mice with a lung epithelium-specific deletion of C/EBP α (*Cebpa*^{ΔLE} mice) display impaired lung development

structurally recapitulating the GR^{-/-} mice with immature lungs and affected epithelial differentiation. Although a majority of animals succumb at birth, the surviving mice develop a severe pathological picture and gene expression profile similar to COPD (Didon *et al.* 2010), indicating a role for C/EBP α both in lung development and in lung pathology. The striking resemblance of the lung immaturity of *Cebpa*^{ΔLE} mice and GR^{-/-} mice suggests a relationship between these signaling pathways. Several lung-enriched genes lack GREs in their promoters, such as *CCSP/CC10*, the cytochrome P450 enzyme *CYP2B1*, and SPs, but are nevertheless regulated by GCs. Importantly, the GC regulation of both *CCSP/CC10* and *CYP2B1* genes is dependent on C/EBP motifs in the respective promoters and involves increased C/EBP activity (Berg *et al.* 2002, 2005).

C/EBP α and β mediate activation of cortisol

C/EBPs may also influence lung maturation and differentiation by affecting GC signaling through activation of cortisol. A CCAAT box motif has been identified in the promoter of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), an enzyme that converts inactive cortisol to active cortisone by reduction (Tannin *et al.* 1991, Sai *et al.* 2008). Cortisol activation by 11 β -HSD1 appears to be important for lung maturation, since 11 β -HSD1^{-/-} mice exhibit impaired lung development and depletion of pulmonary surfactant (Hundertmark *et al.* 2002). Mutation studies using different reporter gene constructs have confirmed that C/EBP α and C/EBP β are responsible for the constitutive expression and that C/EBP β is involved in TNF α , IL1 β , and cAMP-induced transcription of 11 β -HSD1 in several cell types, including pulmonary cells (Gout *et al.* 2006, Ignatova *et al.* 2009, Yang *et al.* 2009). Furthermore, GC-induced transcription of 11 β -HSD1 has been demonstrated to be dependent on C/EBP β activity in lung epithelial (A549) cells (Sai *et al.* 2008) and on C/EBP α in amnion fibroblast (Yang *et al.* 2007b). Thus, regulation of 11 β -HSD1 by C/EBPs may play a particularly important role in lung development, where GCs are required for alveolarization and lung maturation (Whitsett & Matsuzaki 2006).

Functions of C/EBP β and δ

In contrast to C/EBP α , C/EBP β and C/EBP δ support proliferation (McKnight 2001, Buck & Chojkier 2003) and C/EBP β can inhibit apoptosis (Buck & Chojkier 2003). Perhaps the most important role of these factors may, however, be the mediation of inflammatory responses.

Role in inflammatory and acute-phase responses

The importance of C/EBP β and δ in inflammatory responses has been highlighted in the hepatic acute-phase response induced by IL1 β , IL6, and GCs. C/EBP-binding motifs are

present in the promoters of many acute-phase responsive genes, e.g. α_1 -acid glycoprotein (AGP), serum amyloid (SA)A1, 2 and 3, complement C3, and C-reactive protein. C/EBP β transcriptional, isoform, and activity levels are modulated by inflammatory stimuli such as cytokines (i.e. tumor necrosis factor (TNF) α , IL1, and IL6) as well as lipopolysaccharide (LPS), indicating a role for C/EBP β in the acute-phase response (Poli 1998). In addition, several studies have demonstrated impaired host defenses in C/EBP β -deficient mice (Screpanti *et al.* 1995, Tanaka *et al.* 1997, Poli 1998).

C/EBP β and δ in inflammatory responses in the lungs

Despite the proposed function of promoting proliferation, C/EBP β and δ do not appear to play vital roles in baseline lung function or lung development, as findings in mice lacking C/EBP β and/or C/EBP δ indicate (Tanaka *et al.* 1995, 1997, Didon *et al.* 2011). However, similar to the observed effect in hepatic tissue, C/EBP β and δ are increased in the lung following inflammatory and acute-phase stimuli as well as acute lung injury (Akira *et al.* 1990, Poli *et al.* 1990, Poli 1998, Ramji & Foka 2002), suggesting a role in lung defenses. The reduced activity of C/EBP β in the airway epithelium of smokers with COPD (Didon *et al.* 2005) and lower expression of C/EBP δ in bronchial smooth muscle cells of COPD patients (Borger *et al.* 2007) are interesting, since these changes may act as feedback mechanisms and reduce inflammatory signaling. Although the precise role in chronic inflammatory diseases has not been determined, reduced C/EBP activity may decrease the regenerative capability of the lung epithelium, which may have a significant impact on smoking-related diseases.

C/EBPs have been suggested to regulate pulmonary expressed acute-phase proteins, such as SP-A and SP-D (McIntosh *et al.* 1996, Cassel & Nord 2003). In addition, early inflammatory mediators such as IL1 β , IL6, and TNF α , as well as the adhesion molecule ICAM1, all with functional C/EBP motifs, are also elevated upon acute lung injury (Chini *et al.* 1998, Poli 1998, Fan *et al.* 2001), indicating a link between C/EBP β and δ and these genes. Edwards *et al.* (2005) have demonstrated that IL1 β induction of IL8 in bronchial epithelial cells is dependent on both C/EBP β - and NF κ B-binding sites in the gene promoter. The inflammatory signaling pathways in which C/EBP β has been suggested to play a role are depicted in Fig. 1. C/EBP β has been linked to various pathogen-associated molecular patterns (PAMPs; Poli 1998, Ramji & Foka 2002, Barton *et al.* 2007, Im *et al.* 2009, Lu *et al.* 2009, Shin *et al.* 2010, Lorenz *et al.* 2011). Peptidoglycans (PNG) increase both transcription and activity of C/EBP β in macrophages (Hsu *et al.* 2010) and in lung epithelial cells, PNG, LPS, and double-stranded (ds)RNA induce or activate C/EBP β (Barton *et al.* 2007, AB Roos, L Didon & M Nord, unpublished observation). Taken together, these findings suggest that airway epithelial C/EBP β plays a potentially important role in signaling downstream of

Figure 1 C/EBPs are involved in multiple innate immunity signaling pathways. C/EBP β has been implicated in signaling pathways evoked by several inflammatory stimuli, including LPS that binds to TLR4; cytokines acting via their respective receptors; bacterial flagellin binding to TLR5; cAMP acting via G-coupled receptors and cigarette smoke as well as dsRNA and PNG that binds to TLR3 and the Nod1 receptor present on intracellular endosomes. C/EBPs are activated via post-transcriptional modification and hetero- or homodimerize. A number of enzymes have been demonstrated to activate C/EBPs by phosphorylating (p38, RSK, CaMKII, and PKC) the transcription factors. Some post-transcriptional modifications, including phosphorylation by PKA and PKC, reduce DNA-binding activity (enzymes indicated in red), and sumoylation leads to repression of COX2 transcription. Activated C/EBP β bind DNA and can induce transcription of pro-inflammatory genes. CaMKII, Ca²⁺/calmodulin-dependent protein kinase; C/EBP, CCAAT/enhancer-binding protein; dsRNA, double-stranded RNA; IL, interleukin; LPS, lipopolysaccharide; Nod, nucleotide-binding oligomerization domain-containing protein; PNG, peptidoglycan; PK, protein kinase; RSK, ribosomal S6 kinase; SUMO, small ubiquitin-related modifier; TLR, Toll-like receptor.

pathogen recognition receptors. In addition, the pulmonary activity and mRNA expression of C/EBP β are induced by influenza A infection in mice *in vivo* (Choi *et al.* 1996, AB Roos, L Didon & M Nord, unpublished observation), an induction that could be at least partly mediated via TLR3.

C/EBP β mediates the inflammatory response to LPS in the airway epithelium

We have recently observed that LPS-induced neutrophilic inflammation in the airways is partly C/EBP β dependent. Aerosolized LPS induces airway neutrophilia, which is one of the hallmarks of the inflammation in COPD and COPD exacerbations. Furthermore, bacterial colonization is found both in stable disease and during COPD exacerbations (Kharitonov & Sjobring 2007), indicating an important role for bacterial pathogens in disease progression. Mice lacking C/EBP β specifically in the lung epithelium (Cebp^{ΔLE} mice) challenged with aerosolized LPS display a blunted recruitment of neutrophils to the airways, together with blunted induction of several inflammatory mediators, including the neutrophil chemoattractant and functional murine homolog of IL8, growth-related oncogene (GRO) α . Thus, C/EBP β and the lung epithelium play central roles in the pulmonary inflammation induced by LPS *in vivo*, most likely by C/EBP β mediating LPS induction of the expression of pro-inflammatory genes, including neutrophil chemoattractants, in the lung epithelium (AB Roos, L Didon & M Nord, unpublished observation).

C/EBP β is necessary for the inflammatory response to cigarette smoke

Recent evidence also suggests that C/EBP β is essential for the acute inflammatory response to cigarette smoke. The inflammatory response to cigarette smoke is of particular interest, since this inflammation is at least partly GC insensitive (Marwick *et al.* 2009). C/EBP β mRNA levels are reduced in the bronchial epithelium of current as well as former smokers compared with never smokers. Furthermore, Cebp^{ΔLE} mice exposed to cigarette smoke display a blunted or impaired induction of inflammatory mediators and a severely impaired recruitment of neutrophils to the airways, compared with control littermates (Didon *et al.* 2011). Mechanistic *in vitro* studies confirmed an epithelial cell autonomous role for C/EBP β in the response to cigarette smoke. Thus, C/EBP β and the lung epithelium play vital roles also in the response to complex stimuli such as cigarette smoke. Furthermore, it has also been demonstrated that C/EBP β ^{-/-} mice exhibit decreased bleomycin-induced pulmonary fibrosis (Hu *et al.* 2007), linking C/EBPs to lung fibrosis.

C/EBPs as mediators of GC suppression of inflammatory gene expression

A novel transduction pathway for C/EBPs in mediating the immune-suppressive effects of GCs has slowly emerged. The first indications that C/EBPs could play a role in GC signaling came with the finding of a C/EBP-binding site in the proximal promoter of the herpes simplex thymidine kinase. Interestingly, this gene is induced by GCs but lacks GREs, and

in line with this, induction has been demonstrated to be independent of DNA binding of GR (Boruk *et al.* 1998).

GCs mediate effects by inducing DNA-binding activity of C/EBP β

GCs stimulate transcription of several GC target genes in the lung epithelium via increased DNA-binding activity of C/EBP β rather than via direct DNA binding of the GR (Berg *et al.* 2002). This phenomenon could explain how the expression of several lung-specific genes can be stimulated by GCs even though the promoters of these genes do not contain any functional GREs (Karin 1998). This is consistent with the previously mentioned knock-out mice lacking the GR that succumb at birth due to respiratory failure (Cole *et al.* 1995), unlike the mice with impaired ability of the GR to bind DNA, that exhibit normal lungs (Reichardt *et al.* 1998). We have found evidence pointing toward a scenario in which GCs activate C/EBP β via a rapid GR-dependent mechanism resulting in post-translational modification of the transcription factor (Fig. 2; Berg *et al.* 2005). Phosphorylation of Thr 235, which is one of several known phosphorylation sites regulating C/EBP β activity (Trautwein *et al.* 1993, 1994, Poli

1998, Buck *et al.* 2001a,b, Menard *et al.* 2002), is a likely target of this mechanism. In unpublished results utilizing gel shifts to investigate binding to different C/EBP-responsive elements, we have made findings suggesting that this activation targets C/EBP β toward promoters of GC-activated, anti-inflammatory genes (depicted in Fig. 3C), but not toward promoters of pro-inflammatory genes such as *IL6* (Fig. 3A; L Didon & M Nord, unpublished observation). Similarly, C/EBP δ appears to be induced by *in vitro* GC stimulation in cultured bronchial smooth muscle cells (Borger *et al.* 2007).

C/EBP mediation of GC stimulation of host defense molecules

The airway epithelium serves as a first line of defense to invading microorganisms, the primary cause of COPD, and asthma exacerbations (Schleimer 2004, Papi *et al.* 2006). In addition, the respiratory mucosa is exposed to the primary cause of COPD, namely cigarette smoke (Schleimer 2004). The airway epithelium is also involved in several processes of host defense, including barrier functions, inflammatory cell recruitment, and production of anti-microbial molecules (e.g. defensins, lysozyme, surfactants, and cathelicidins) through activation pattern recognition receptors, such as Toll-like receptors (TLRs) and Nod receptors (Sha *et al.* 2004). Innate immune responses can be enhanced by GCs, a mechanism that may be particularly important in inflammatory lung diseases with infectious components. Both the expression and the sensitivity of TLRs in epithelial cells are enhanced by GCs (Imasato *et al.* 2002, Shuto *et al.* 2002) and similar stimulation of collectins, SPs, and acute-phase proteins has been demonstrated (Schleimer 2004). Zhang *et al.* (2007) demonstrated that GCs enhanced dsRNA-stimulated expression of the acute phase genes C3, SAA, and CRP, in cultured primary bronchial epithelial cells and bronchial epithelial cell lines. In contrast, the expression of collectins, surfactants, anti-microbial molecules was spared, and cytokines as well as chemokines were inhibited. Furthermore, this sparing or enhancement by GCs was to a large extent dependent on C/EBP β . The authors propose that the GC-induced genes that were not dependent on C/EBP β expression may be regulated by C/EBP δ . We have recently observed a tendency towards stimulation of LPS-induced SAA3 expression by GCs and a significant stimulation by GCs with co-treatment with a long-acting β_2 agonist (formoterol), a stimulation that is dependent on C/EBP β (AB Roos, L Didon & M Nord, unpublished observation). This is also in agreement with studies demonstrating GC induction of human SAA1 (for a comprehensive review, see Thorn *et al.* (2003)).

Direct association between C/EBP β and the GR may explain this phenomenon. C/EBP β and the GR bind to overlapping and adjacent target sites in the promoter of gene encoding the GC-induced acute-phase reactant AGP (as most acute-phase genes induced by both inflammatory mediators and GCs; Fig. 4A). Mutation of the GRE site does not completely abrogate the stimulation of AGP by GCs, and

Figure 2 GCs increase DNA-binding activity of C/EBP β by post-translational modification. Activation increases transcription of lung-specific genes lacking GRE in the promoter. This activation may target C/EBP β toward promoters of GC-activated, anti-inflammatory genes, but not toward promoters of pro-inflammatory genes. C/EBP, CCAAT/enhancer-binding protein; GC, glucocorticoid; GRE, glucocorticoid-responsive element.

Figure 3 Different post-transcriptional modifications leading to binding of alternative C/EBP-responsive elements – a putative model for the dual role of C/EBPβ in inhibiting and mediating the inflammatory response. Inflammatory and anti-inflammatory (GC) stimuli may induce different post-transcriptional modifications (such as phosphorylations at different positions, sumoylations, or acetylations) and direct C/EBPβ to activate or repress inflammatory gene transcription or induce anti-inflammatory/host defense gene transcription. (A) Inflammatory signaling induces post-transcriptional modifications (indicated in purple) that activate C/EBPβ and promote binding to C/EBP-responsive elements stimulating transcription of pro-inflammatory genes. (B) GC-induced post-transcriptional modifications (indicated in red) direct C/EBPβ to bind negative regulatory DNA sequences (indicated in red), inhibiting gene transcription. (C) GCs induce post-transcriptional modification of C/EBPβ (indicated in blue) to target C/EBP-responsive elements of anti-inflammatory and/or host defense genes lacking GREs, not elements in promoters of inflammatory genes. C/EBP, CCAAT/enhancer-binding protein; GC, glucocorticoid; GRE, glucocorticoid-responsive element.

synergistic induction of AGP is still observed when GR or C/EBPβ lack either the DNA-binding or the transcriptional activation function (Alam *et al.* 1993, Nishio *et al.* 1993). This suggests that recognition of only the CCAAT motif is sufficient for partial induction and that protein–protein interaction is responsible for synergistic activation of AGP. Others have observed that peroxisome proliferator-activated receptor (PPAR)α interacts with C/EBPβ when stimulated and prevents GC stimulation of AGP (Mouthiers *et al.* 2005; Fig. 4A). The physical interaction of PPARα with C/EBPβ provides an example of the molecular mechanism of negative regulation of acute-phase protein gene expression by blocking C/EBPβ. Taken together, these findings demonstrate an important role for C/EBPs in GC modulation of the innate immune responses to PAMPs, a mechanism that could serve to explain the positive effect of GC treatment in COPD.

A role for C/EBPs in mediating GC suppression of inflammatory mediators

COX2 is involved in the stimuli-induced synthesis of prostanoids from arachidonic acid, and expression is increased in asthmatic airways. Thus, COX2 has been suggested to be important for disease pathogenesis (Park & Christman 2006). Similar to the C/EBP–GR interaction observed for AGP regulation, C/EBPβ and the GR physically interact and bind to the COX2 promoter upon GC treatment (Sun *et al.* 2008). In line with this, we have recently found that suppression of LPS-induced COX2 expression by the GC budesonide in the lung *in vivo* is reduced in mice lacking C/EBPβ in the airway epithelium. Furthermore, we also detected a blunted GC inhibition of LPS-induced IL6 expression in these mice (AB Roos, L Didon & M Nord, unpublished observation).

Figure 4 GC induction of different transcription factor interactions – a proposed model explaining C/EBPβ mediation of anti-inflammatory and pro-inflammatory signaling. C/EBP family members hetero- or homodimerize and also interact with other transcription factors including the GR. Anti-inflammatory signaling evoked by GCs may result in different types of dimers than inflammatory signaling. (A) GC signaling leads to C/EBP and GR binding of adjacent responsive elements of GRE- and C/EBP-responsive genes (e.g. AGP). PPARα can bind to C/EBPβ upon stimulation with PPARα agonists, and binding to C/EBP-responsive elements is prevented. (B) The activated GR binds C/EBPβ and prevents DNA binding to responsive elements and subsequent transcription (e.g. of the *IL1β* gene). (C) The GC receptor induces different C/EBP hetero- or homodimers and transcription of C/EBP-responsive genes. Different heterodimers may result in pro- or anti-inflammatory signaling. AGP, α1-acid glycoprotein; C/EBP, CCAAT/enhancer-binding protein; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; IL, interleukin; PPAR, peroxisome proliferator-activated receptor.

In support of this concept, others have suggested that the GR inhibits pro-inflammatory signaling by disrupting C/EBP binding to DNA elements in the *IL1β* promoter (Fig. 4B; Waterman *et al.* 2006). This indicates that the complex interactions between GCs and C/EBPs involve both inhibition of C/EBP-mediated pro-inflammatory signaling as well as activation. Taken together, these findings complement the previously observed sparing or enhancement of host defense molecules but suppression of inflammatory mediators, demonstrating that some suppressive effects of GCs on LPS-induced inflammatory mediator expression are partly dependent on C/EBPβ.

C/EBP regulation of MAPK phosphatase

C/EBPs also mediate GC-induced stimulation of anti-inflammatory responses. An example of this is the binding of C/EBPβ to the promoter region of MAPK phosphatase 1 (MKP1)/dual specificity phosphatase 1 (DUSP1), a phosphatase with no GR-binding sites in its promoter (Johansson-Haque *et al.* 2008). The crucial anti-inflammatory gene *DUSP1* is enhanced by GCs and mediates inhibition of ERK, JNK, and p38 MAPKs that influences the duration of

pro-inflammatory signaling (Kassel *et al.* 2001, Chen *et al.* 2002, Lasa *et al.* 2002, Engelbrecht *et al.* 2003, Hammer *et al.* 2006, Salojin & Oravec 2007). Johansson-Haque *et al.* (2008) showed that while the transactivation capacity of the GR is not required for DUSP1 induction by GCs, a C/EBP-binding site is necessary for the induction and proposed that activation involves a tethering mechanism with GR and C/EBPβ bound to the promoter (Fig. 4C). This demonstrates that C/EBPβ mediates the anti-inflammatory effects of GC by inducing DUSP1, possibly affecting GC suppression of inflammatory mediators such as COX2 and IL6 through inhibition of ERK, JNK, and p38 MAPKs.

Possible mechanisms of C/EBPs as mediators of both inflammatory responses and effects of GCs

One of the most intellectually challenging concepts of C/EBPs as mediators of GC effects is the involvement of C/EBPβ in mediating inflammatory responses as well as immunosuppressive effects of GCs. Such a dual role also seems to apply for β₂-adrenoreceptor agonists, which has anti-inflammatory effects on lung epithelial cells, which we

recently have found to be almost completely dependent on C/EBP β (AB Roos, L Didon and M Nord, unpublished observation). Involvement of C/EBP β in signaling downstream of the β_2 -adrenoreceptor has, in support of our findings, been demonstrated by others (Yin *et al.* 2006). The mechanisms underlying this dual-involvement of C/EBPs have, however, not been investigated. Finally, we will briefly consider the most probable concepts that could explain this dual role, namely post-transcriptional modifications, binding of different C/EBP-responsive elements, and interactions with other transcription factors.

Post-transcriptional modifications

Post-transcriptional modifications such as phosphorylations, SUMOylations, and acetylations have been shown to affect C/EBP β activity (Ramji & Foka 2002, Berberich-Siebelt *et al.* 2006, Cesena *et al.* 2008, Khanna-Gupta 2008). For instance, EGF stimulation *in vitro* has been shown to repress transcription of COX2 via sumoylation of C/EBP β (Wang *et al.* 2008), as opposed to the other documented role of C/EBP β in promoting COX2 transcription (Hsu *et al.* 2010). This provides a possible mechanism by which the function of C/EBP β could be altered from inflammatory to anti-inflammatory dependent on post-transcriptional modification. Some of these post-transcriptional modifications could direct the transcription factor to bind negative regulatory DNA sequences. Also, dual phosphorylations have been reported, indicating an additional means of fine-tuning or direction of transcriptional control (Tsukada *et al.* 2011). By binding negative regulatory C/EBP-responsive elements, C/EBP β with certain post-transcriptional modifications could inhibit gene transcription of inflammatory genes, as previously demonstrated (Fig. 3B; Kagan *et al.* 2003, Dong *et al.* 2010), as opposed to stimulating transcription of pro-inflammatory genes (Fig. 3A) or anti-inflammatory/host defense genes lacking GREs (Fig. 3C). As one of the C/EBP β isoforms, LIP, is dominant negative, it is also plausible that certain stimuli, such as GCs, increase the LIP/LAP ratio, thereby inhibiting pro-inflammatory gene expression. Whether this mechanism has any actual significance is, however, still unknown.

Binding of different C/EBP-responsive elements

One possibility is that inflammatory signaling leads to binding of C/EBPs to responsive elements in the promoters of pro-inflammatory genes and that C/EBP activity evoked by GCs promotes binding to responsive elements in anti-inflammatory genes, as we previously have detected in unpublished observations (see Fig. 3C). This may also apply to the mechanisms described by Zhang *et al.* (2007), where C/EBP β mediates the GC-induced increased expression of host defense genes. It is not known what distinguishes the responsive elements of inflammatory genes compared with anti-inflammatory/host defense genes or how C/EBPs may differentiate between them.

Interaction with other transcription factors

Since different C/EBP family members have been demonstrated to heterodimerize or interact with other transcription factors (Lee *et al.* 1997, Shuman *et al.* 1997, Boruk *et al.* 1998), different stimuli may result in alternative protein dimers. For instance, AP1, activating transcription factor (ATF)-2, and C/EBP α homodimers, but not C/EBP α -AP1 or C/EBP α -ATF2 heterodimers, bind GRE sequences. It has also been demonstrated that C/EBP α -AP1 heterodimers bind to different DNA sequences than the C/EBP α or AP1 homodimers (Shuman *et al.* 1997, Cai *et al.* 2007). Furthermore, the amount of C/EBP dimers containing C/EBP α is shifted to C/EBP β and C/EBP δ dimers during acute-phase reaction in the liver, indicating that interaction between C/EBPs could be stimuli specific (Poli 1998). In addition to heterodimerizations, C/EBP dimers have been shown to interact with several transcription factors from different families (for instance Nkx2.1/TTF1 and the GR itself) as discussed previously in this review. The efficiency of such protein-protein interactions could be affected by post-translational modifications of the C/EBP. C/EBPs could be targeted to certain promoter-binding sites, depending on protein-protein interactions with other transcription factors bound to adjacent binding sites. Thus, immunoregulatory stimuli may trigger different combinations of transcription factors, including C/EBPs. Therefore, C/EBPs could mediate both pro-inflammatory induction and immune suppressive effects of GCs. This mechanism may explain GC induction of genes lacking GRE in their promoters (Fig. 4C).

Conclusions

GCs are important for lung development and in the treatment of inflammatory lung diseases. However, a component of relative steroid resistance is present in many lung diseases, such as COPD. C/EBPs are intracellular transcription factors that play important roles in the inflammatory and protective functions in the lung, as well as during lung development and maturation. Additionally, C/EBPs have been implicated in several inflammatory lung diseases such as asthma and COPD. A number of studies have revealed a link between GCs and C/EBPs, including effects on lung maturation, control of proliferation, differentiation, and apoptosis as well as suppression of inflammation and induction of host defense molecules. Since emerging evidence supports a critical role of C/EBPs in inflammatory diseases, further studies more closely examining the role of C/EBPs in mediating the effects of GCs in the lung are called for, in particular since C/EBPs could contribute to the relative GC resistance observed in COPD.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Both authors contributed to the structure of the review. A B R did the literature search and prepared the initial draft. M N revised and finalized the manuscript together with A B R.

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