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Lung epithelial-C/EBPβ contributes to LPS-induced inflammation and its suppression by formoterol

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ABSTRACT

The inflammatory processes associated with pulmonary disorders remains incompletely understood. CCAAT/enhancer-binding protein (C/EBP)β is implicated in inflammatory lung disorders as well as in β_2-adrenoceptor signaling. We hypothesized that C/EBPβ in the lung epithelium contributes to lipopolysaccharide (LPS)-induced airway neutrophilia and expression of neutrophil chemoattractant chemokine (C-X-C) motif ligand (CXCL)1, as well as the suppressive effects of long-acting β_2-agonists (LABAs) and glucocorticoids (GCs).

To investigate this, mice with a lung epithelial-specific deletion of C/EBPβ (*Cebpb*$^{\Delta LE}$) and control littermates (*Cebpb*$^{fl/fl}$) were pre-treated with a LABA, formoterol and/or a GC, budesonide, and challenged with LPS. Inflammatory cell recruitment in bronchoalveolar lavage (BAL) fluid and pulmonary expression of inflammatory mediators were investigated. In addition, the ability of formoterol to increase C/EBP transactivation was assessed *in vitro*.

LPS-challenged *Cebpb*$^{\Delta LE}$ mice exhibited fewer BAL neutrophils and lower pulmonary expression of CXCL1 *versus* *Cebpb*$^{fl/fl}$ mice. Suppression of LPS-induced neutrophilia by formoterol was impaired in *Cebpb*$^{\Delta LE}$ mice and *Cxcl1* expression was increased. However, suppression of the neutrophilia by budesonide with/without formoterol was preserved. Further studies indicated that C/EBP transactivation was increased by the cAMP elevating agent forskolin and formoterol in a β_2-adrenoceptor dependent manner.

Thus, C/EBPβ in the lung epithelium contributes to LPS-induced CXCL1 expression and airway neutrophilia as well as to the suppressive effects of formoterol. Reduced C/EBPβ activity, observed in smokers with chronic obstructive pulmonary disease, may impair the responsiveness to LABAs when used without GCs.

1. Introduction

Pulmonary inflammation is an intricate part of obstructive pulmonary disorders and contributes to their pathogenesis [1]. Our understanding of the inflammatory processes that drive pathogenesis are, however, still inadequate. Lipopolysaccharide (LPS) induces a pulmonary inflammation characterized by neutrophil recruitment to the airways [2]. Inflammatory mediators such as the neutrophil chemoattractant chemokine (C-X-C motif) ligand (CXCL)1, a functional murine homologue of interleukin (IL)-8 [3,4], and the inflammatory cytokine IL-6 are central in pulmonary inflammation [5]. Both CXCL1 and IL-6 are proposed to be regulated by the lung-enriched transcription factor CCAAT/enhancer-binding protein (C/EBP)β [6–8]. It has also been reported that β_2-adrenergic agonists, which are mainstay in treatment of inflammatory lung diseases, activate C/EBPα *in vitro* [9,10]. It is, however, presently unknown whether C/EBPβ contributes to the regulation of CXCL1 in the lung following LPS exposure, or whether long-acting β_2-agonists (LABAs) activate C/EBPs in the airway epithelium. On the other hand, glucocorticoids (GCs) are also central in treatment of inflammatory lung diseases and have been demonstrated

Abbreviations: BAL, bronchoalveolar lavage; BUD, budesonide; C/EBP, CCAAT/enhancer-binding protein; CXCL1, chemokine (C-X-C motif) ligand 1; COPD, chronic obstructive pulmonary disease; DMSO, dimethylsulphoxide; FM, formoterol; GC, glucocorticoid; HPRT1, hypoxanthine phosphoribosyltransferase 1; IL, interleukin; LABA, long-acting β_2-agonist; LPS, lipopolysaccharide.

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to increase the DNA-binding activity of C/EBPβ by post-translational modification in bronchial epithelial cells [11,12]. Also, the ability of GCs to spare or enhance expression of host defense molecules has been suggested to be dependent on C/EBPβ [13]. Previous studies have identified diverse roles for C/EBPs in regulating gene expression in different cell types [14], however, our knowledge on C/EBPβ in inflammatory responses especially *in vivo* still remains unsophisticated, warranting further investigations. In the current *in vivo* study, we have addressed the hypothesis that lung epithelial-C/EBPβ contributes to LPS-induced neutrophilia and expression of inflammatory mediators, as well as to the suppressive effects of LABAs and GCs.

2. Material and methods

2.1. Animals

Animal housing, generation of mice with a lung epithelial-specific deletion of C/EBPβ (*Cebpb*$^{\Delta LE}$ mice) by *SFTPC*-Cre mediated excision, genotyping and lacZ staining are described elsewhere [15]. *Cebpb*$^{\Delta LE}$ mice, or littermate controls (*Cebpb*$^{fl/fl}$) were pre-treated with 3 mg/kg, formoterol (FM), budesonide (BUD), BUD + FM combined (AstraZeneca, Lund, Sweden) or vehicle alone (PBS containing 10% dimethyl sulfoxide (DMSO), Sigma–Aldrich, St. Louis, MO) by intra-peritoneal injection, as effective pulmonary drug deposition is difficult to achieve with inhalation in mice [16]. After 1 h, some mice were exposed through inhalation to 1 mg/ml *Pseudomonas aeruginosa* LPS as described in the Supplementary material. Bronchoalveolar lavage (BAL), total cell and differential cell counts in BAL fluid were performed as described in the Supplementary material. The animal studies were approved by the Northern Stockholm animal welfare ethics committee.

2.2. Sample analysis

RNeasy Mini (Qiagen, Hilden, Germany) and RNA-to-cDNA high capacity kit (Applied Biosystems, Carlsbad, CA) were used to extract RNA from lung tissue and to transcribe RNA to cDNA, according to protocol provided by the manufacturers. Gene expression relative to hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) was determined by quantitative real-time PCR on an Applied Biosystems Prism 7700 sequence detector using ABI PRISM Sequence Detection System 1.9.1 (Applied Biosystems). Gene expression assays were purchased from Applied Biosystems and assessed in duplicates. Quantikine Colorimetric Sandwich ELISA (R&D Systems, Minneapolis, MN) was used to determine protein concentrations in BAL supernatants, according to the manufacturer's protocol.

2.3. Cell culture

Human bronchial epithelial (BEAS-2B, LGC Standards, Borås, Sweden) cells were maintained as previously described [17]. Cells were transfected with 0.75 μg *Cebp*-luc reporter plasmid (containing consensus C/EBP binding sites, as described elsewhere [18]) and stimulated with *P. aeruginosa* LPS, FM, with or without pre-treatment with propranolol (kindly donated by Dr. Lena Palmberg, Karolinska Institutet, Sweden) or with forskolin (Sigma–Aldrich) as described in the Supplementary material. Luciferase activity was analyzed with the Luciferase Assay Kit (BioThema, Handen, Sweden), according to protocol provided by the supplier.

2.4. Statistical analysis

Data was normalized as described in the Supplementary material. Statistical analysis was performed using the GraphPad Prism 5 software (GraphPadSoftware, La Jolla, CA), testing for treatment effects using Kruskal–Wallis analysis, Mann–Whitney test for pair-wise comparisons and Spearman's rank correlation test for correlation analysis. Differences were considered statistically significant at $p \leqslant 0.05$.

3. Results

3.1. Blunted LPS-induced pulmonary neutrophilia in *Cebpb*$^{\Delta LE}$ mice

To investigate the functional role of C/EBPβ in LPS-induced pulmonary inflammation *in vivo*, *Cebpb*$^{\Delta LE}$ mice and littermate *Cebpb*$^{fl/fl}$ mice were exposed to aerosolized *P. aeruginosa* LPS (Fig. 1A). Naïve, unchallenged *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice displayed comparable number of inflammatory cells in BAL. Following LPS challenge, total BAL cells were significantly increased only in *Cebpb*$^{fl/fl}$ mice ($p < 0.05$), while BAL neutrophils were increased in both *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice ($p < 0.05$, Fig. 1A). The recruitment of neutrophils was, however, significantly blunted in *Cebpb*$^{\Delta LE}$ mice, with 65% fewer neutrophils compared to *Cebpb*$^{fl/fl}$ mice ($p < 0.05$).

3.2. Blunted LPS-induced *Cxcl1* and *Il6* expression in *Cebpb*$^{\Delta LE}$ mice

To further characterize the blunted neutrophilia in *Cebpb*$^{\Delta LE}$ mice, the expression of the neutrophil chemoattractant *Cxcl1* [3,4] and the inflammatory cytokine *Il6* [5] was assessed. *Cxcl1* and *Il6* were up-regulated in both *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice following LPS challenge ($p < 0.05$, Fig. 1B and C). Expression of *Cxcl1* correlated positively with the number of neutrophils in the airways of both types of mice ($r = 0.96$, $p < 0.0001$), corroborating the neutrophil attractant function. In line with the blunted LPS-induced neutrophilia in *Cebpb*$^{\Delta LE}$ mice, reduced induction of *Cxcl1* (70%, $p < 0.05$) and *Il6* (40%, $p = 0.064$) was observed in these mice compared to *Cebpb*$^{fl/fl}$ mice (Fig. 1B and C). Lower protein concentration of CXCL1 (73%) was also observed in LPS-challenged *Cebpb*$^{\Delta LE}$ mice compared to *Cebpb*$^{fl/fl}$ mice ($p < 0.05$, Fig. 1D).

3.3. LPS-induced expression of *Cebpb* and *Nfkbia*

We further assessed whether the pulmonary expression of *Cebpb* is affected by LPS challenge and found a trend towards elevated *Cebpb* expression in *Cebpb*$^{fl/fl}$ mice ($p = 0.11$, Fig. 2A). A trend for activation of C/EBP reporter gene activity by LPS was observed in cultured BEAS-2B cells ($p = 0.097$, Fig. 2B), suggesting that LPS may have potential to activate C/EBPs in the lung epithelium. In addition, we investigated the expression of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor (*Nfkbia*), a downstream effector gene of nuclear factor (NF)κB [19], and found that it was equally induced by LPS in *Cebpb*$^{fl/fl}$ and *Cebpb*$^{\Delta LE}$ mice ($p < 0.05$, Fig. 2C). Thus, the activity of NFκB, which is central in inflammatory responses, is suggestively similar in both types of LPS-challenged mice.

3.4. Impaired suppression of neutrophils by formoterol in *Cebpb*$^{\Delta LE}$ mice

The role of C/EBPβ in suppressing inflammatory cell accumulation in mice pre-treated with FM, BUD or FM + BUD before LPS challenge was then investigated (Fig. 3 and Supplementary Fig. S1). The 62% suppression of total BAL cells by FM ($p < 0.01$) and 41% suppression by BUD ($p < 0.01$) in *Cebpb*$^{fl/fl}$ mice was impaired in *Cebpb*$^{\Delta LE}$ mice (Fig. 3A). The 70% suppression of total BAL cells by FM + BUD in *Cebpb*$^{fl/fl}$ mice ($p < 0.05$) was in contrast retained in *Cebpb*$^{\Delta LE}$ mice ($p < 0.05$).

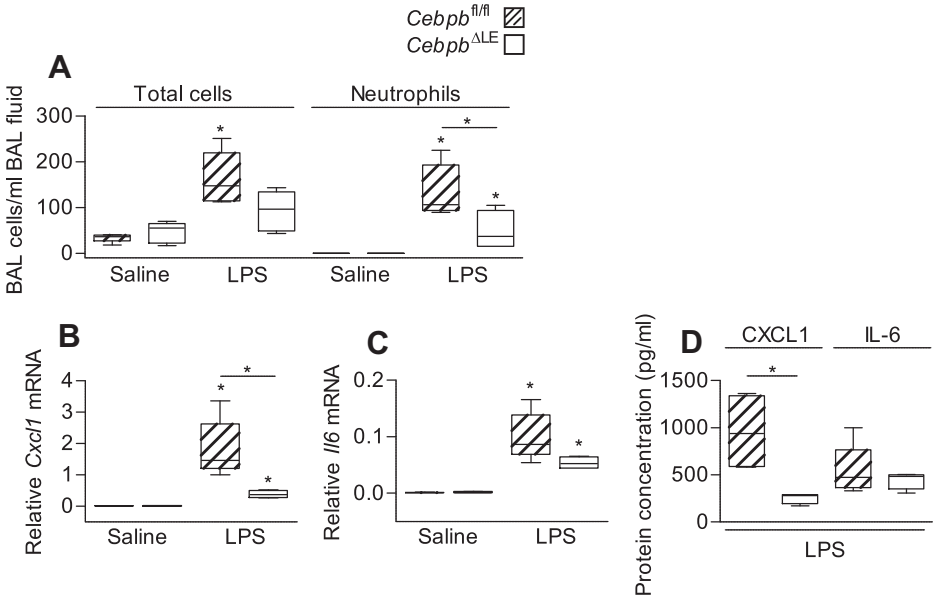

Fig. 1. Reduced LPS responses in $Cebpb^{\Delta LE}$ mice. $Cebpb^{\Delta LE}$ and $Cebpb^{fl/fl}$ mice were LPS-challenged, or mock-exposed to saline and sacrificed after 5 h. (A) Total cell and neutrophil concentration in bronchoalveolar lavage (BAL) fluid. Gene expression of (B) *Cxcl1*, (C) *Il6* relative to *Hprt1* in lung tissue homogenates and (D) protein concentration of CXCL1 and IL-6 in BAL supernatants. $n = 4–6$. * indicates $p < 0.05$ compared to mock-exposed animals or as indicated.

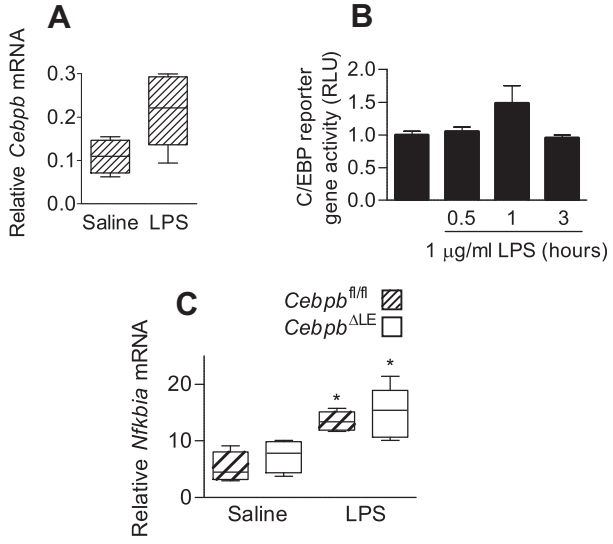

Fig. 2. Effect of LPS on *Cebpb* expression, C/EBP activity and *Nfkbia* expression. (A) *Cebpb* expression relative to *Hprt1* in lung tissue homogenates of LPS-challenged or mock-exposed to saline $Cebpb^{fl/fl}$ mice sacrificed after 5 h. $n = 4–6$. (B) BEAS-2B cells transfected with a C/EBP-luciferase construct exposed to 1 μg/ml *P. aeruginosa* LPS, or mock-exposed to cell media. Experiments were performed three times in triplicates ($n = 9$). RLU: Relative luciferase units. Bars represent means, error bars indicate SEM. (C) *Nfkbia* expression relative to *Hprt1* in lung tissue homogenates of LPS-challenged or mock-exposed to saline $Cebpb^{\Delta LE}$ and $Cebpb^{fl/fl}$ mice sacrificed after 5 h. * indicates $p < 0.05$ compared to mock-exposed animals.

Contrasting the 79% reduction in LPS-induced pulmonary neutrophilia by FM in $Cebpb^{fl/fl}$ mice ($p < 0.01$), FM failed to significantly suppress neutrophil accumulation in $Cebpb^{\Delta LE}$ mice (Fig. 3B). BUD on the other hand significantly ($p < 0.01$) suppressed neutrophil recruitment in both types of mice and the reduction was even greater in $Cebpb^{\Delta LE}$ mice (94% vs. 68% suppression, $p < 0.01$). Treatment with FM + BUD suppressed neutrophil accumulation almost completely in both types of mice ($p < 0.01$). The suppression by FM + BUD was significantly greater than suppression by BUD alone ($p < 0.05$) in $Cebpb^{fl/fl}$ mice and FM alone in both types of mice ($p < 0.01$).

3.5. Impaired suppression of LPS-induced Cxcl1 expression by formoterol in $Cebpb^{\Delta LE}$ mice

We subsequently analyzed the outcome of FM treatment on the expression of CXCL1 and IL-6 (Fig. 3C–F and Supplementary Figs. S2–S4). The LPS-induced expression of *Cxcl1* was significantly inhibited by FM (42% reduction, $p < 0.05$) in $Cebpb^{fl/fl}$ mice (Fig. 3C). In contrast, FM significantly increased expression of LPS-induced *Cxcl1* by 53% in $Cebpb^{\Delta LE}$ mice ($p < 0.05$). FM also tended to increase the protein concentration of CXCL1 in $Cebpb^{\Delta LE}$ mice ($p = 0.064$, Fig. 3E). Increased expression of LPS-induced *Il6* (43%, $p < 0.05$) and IL-6 protein (97%, $p < 0.05$, Fig. 3D and F) by FM was in similarity observed only in $Cebpb^{\Delta LE}$ mice. Of note, FM significantly suppressed *Nfkbia* expression in LPS-challenged $Cebpb^{fl/fl}$ mice ($p \leqslant 0.05$, Supplementary Fig. S2L) and a suppressive trend was observed in $Cebpb^{\Delta LE}$ mice ($p = 0.095$), suggesting that FM represses NFκB activity.

The effects of BUD treatment with/or without FM on CXCL1 and IL-6 expression was then analyzed. The LPS-induced expression of *Cxcl1* was significantly ($p < 0.01$) reduced by 88% and 66% in $Cebpb^{fl/fl}$ mice pre-treated with BUD with or without FM, respectively (Fig. 3C), and a tendency for repression of CXCL1 protein was observed by FM + BUD in these mice ($p = 0.064$, Fig. 3E). In $Cebpb^{\Delta LE}$ mice, the suppression of *Cxcl1* by BUD was preserved but suppression by FM + BUD only approached statistical significance ($p = 0.056$). Significant suppression of *Il6* by 87–93% was observed in $Cebpb^{fl/fl}$ mice treated with BUD with/without FM (Fig. 3D), and this was also observed at protein level (Fig. 3F). Significant suppression of IL-6 at both mRNA and protein level by BUD with/without FM was also observed in $Cebpb^{\Delta LE}$ mice although mRNA suppression was blunted ($p < 0.05$) as compared to $Cebpb^{fl/fl}$ mice. The effects of FM, BUD and FM + BUD on gene expression of *Cxcl2*, *Tnfa*, *Il1b*, *Csf2*, *Ptgs2*, *Nos2*, *Saa3*, *Scgb1a1*, and *Sftpa1* are shown in Supplementary Figs. S2 and S3.

3.6. Formoterol activates C/EBPs in bronchial epithelial cells

To gain further understanding of the impaired suppressive effects of FM in $Cebpb^{\Delta LE}$ mice, we investigated whether FM

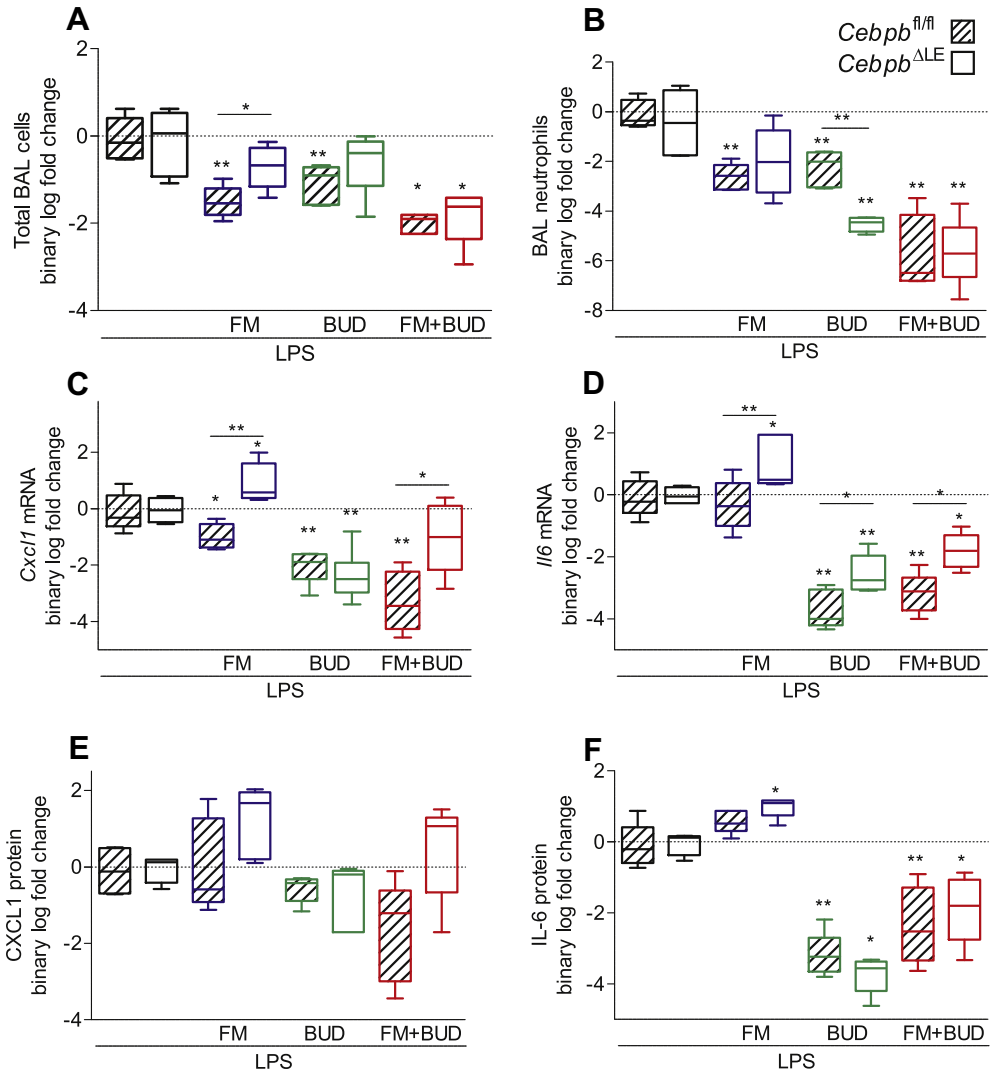

Fig. 3. Impaired suppression of LPS-induced neutrophilia, *Cxcl1* and *Il6*/IL-6 by formoterol in *Cebpb*$^{\Delta LE}$ mice. *Cebpb*$^{\Delta LE}$ and *Cebpb*$^{fl/fl}$ mice were pre-treated with 3 mg/kg formoterol (FM) and/or budesonide (BUD) or drug vehicle 1 h before LPS challenge and sacrificed 5 h after LPS challenge. Binary logarithm of fold change obtained after drug treatments *versus* vehicle control treatment for (A) total cells and (B) neutrophils in bronchoalveolar lavage (BAL) fluid, (C) *Cxcl1* and (D) *Il6* relative to *Hprt1* in lung tissue homogenates and (E) CXCL1 and (F) IL-6 protein concentration in BAL supernatants of *Cebpb*$^{\Delta LE}$ and *Cebpb*$^{fl/fl}$ mice. $n = 4–6$. * indicates $p < 0.05$, ** $p < 0.01$ compared to LPS-exposed and vehicle-pre-treated animals or as indicated.

directly affects C/EBP transactivation in lung epithelial cells. To examine this, BEAS-2B cells transfected with a C/EBP–luciferase reporter construct were exposed for 1 h to FM with/without LPS or FM with propranolol, or to forskolin (Fig. 4). FM with/without LPS significantly induced C/EBP transactivation compared to control cells ($p < 0.05$ and $p < 0.01$, respectively), and the effect of FM was reversed by pre-treatment with the β-adrenoceptor antagonist propranolol, indicating that the effect of FM on C/EBP activity is mediated by β_2-adrenoceptors. As β_2-adrenoceptor signaling involves cAMP [20], we investigated the effect of the cAMP elevating agent forskolin on C/EBP transactivation. Forskolin significantly increased C/EBP dependent transactivation ($p < 0.01$), which implicates cAMP in the β_2-adrenoceptor dependent activation of C/EBPs.

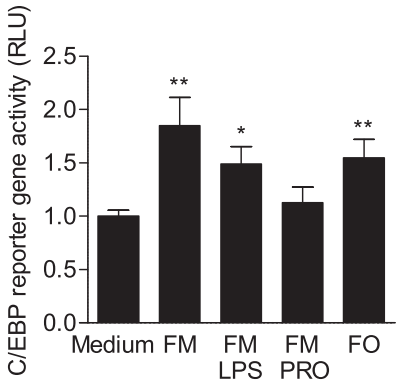

Fig. 4. Formoterol activates C/EBP reporter gene in bronchial epithelial cells. BEAS-2B cells transfected with a C/EBP-luciferase reporter construct exposed for 1 h to 10 nM formoterol (FM) with/without 1 μg/ml LPS, FM with 1 h pre-treatment with 10 μM propranolol (PRO), to 10 μM forskolin (FO) or to mock-exposed to medium. Experiments were performed two–three times in triplicates ($n = 6–9$). RLU: Relative luciferase units. Bars represent means, error bars indicate SEM. * indicates $p < 0.05$, ** $p < 0.01$ compared to mock-exposed cells.

4. Discussion

We herein demonstrate that C/EBPβ in the lung epithelium is involved in LPS-induced expression of CXCL1 *in vivo* and neutrophil accumulation. Inflammatory mediators such as CXCL1 and IL-6 play important roles in pulmonary inflammation and lung disor-

ders, such as chronic obstructive pulmonary disease (COPD) [5]. Both CXCL1 and IL-6 have previously been suggested to be regulated by C/EBPβ [6–8], although the role of C/EBPβ in the regulation of these mediators during LPS-induced pulmonary inflammation *in vivo* has not been investigated. LPS challenge induced the expression of both *Cxcl1* and *Il6*, and the expression of *Cxcl1* was positively correlated to number of airway neutrophils. Thus, the induction of *Cxcl1* may contribute to neutrophil recruitment, as supported by the known neutrophil chemoattractant role of CXCL1 [4]. In C/EBPβ deficient mice, the induction of *Cxcl1* by LPS was significantly blunted and such a trend was also observed for *Il6*. These findings, together with the involvement of C/EBPs in regulating lung specific genes with immunomodulatory functions, such as Clara cell secretory protein [7,17], suggests that C/EBPβ could play a role in pulmonary host defenses.

In line with earlier studies demonstrating stimulated expression of some inflammatory mediators by β_2-adrenoceptor agonists [10,21–24], the expression of *Cxcl1* and *Il6* was significantly increased by FM in $Cebpb^{\Delta LE}$ mice at baseline (Supplementary Fig. S3A and C) and after LPS challenge. In LPS-challenged $Cebpb^{fl/fl}$ mice, however, *Cxcl1* was significantly suppressed by FM while *Il6* was unaffected. These results show that although FM, as other β_2-adrenoceptor agonists, such as salmeterol and salbutamol [21], may increase the pulmonary baseline expression of some inflammatory genes, it has suppressive effects on LPS-induced neutrophilic inflammation when C/EBPβ expression is preserved. In addition, the reduced LPS-induced *Nfkbia* expression by FM support that NFκB activity is suppressed by FM, as previously proposed [25]. We furthermore present the first *in vivo* evidence of C/EBPβ involvement in mediating both the LPS-induced inflammation and the anti-inflammatory effects of β_2-adrenoceptor agonists. Although these effects are individually supported by previous *in vitro* studies [10–12,26], the underlying mechanism is unknown. Both FM alone, and together with LPS, increased C/EBP transactivation, indicating that the suppression of LPS induced inflammatory genes by FM is not an effect of decreased C/EBP activity. The pro- and anti-inflammatory functions of C/EBPβ may be theoretically explained by interactions with different transcription factors induced by diverse post-transcriptional modifications [27–29] such as phosphorylation [30], which could affect DNA-binding specificity. Supporting this, the expression of *Nfkbia* was induced by LPS challenge but suppressed by FM treatment. Hence, the activated C/EBPβ may interact with NFκB following LPS stimulation, in line with previous documentation of transcriptional cooperation between C/EBPβ and NFκB [31]. Following FM treatment, the frequency of NFκB and C/EBPβ interactions could be reduced as a result of decreased NFκB activity. This may enable C/EBPβ to interact with other proteins and bind to negative regulatory elements within the *Cxcl1* promoter, which reportedly contains C/EBP binding motifs [32]. Thus, we here report a possible mechanism by which LABAs suppress inflammatory responses by activating C/EBPs via the β_2-adrenoceptor and cAMP.

The expression of *CEBPB* is depressed among smokers [15] and the activity of C/EBPβ is reduced in smokers with COPD [8]. In spite of these findings, the functional role of C/EBPβ in inflammatory lung diseases remains poorly understood. Since the LPS-induced neutrophilia was impaired in $Cebpb^{\Delta LE}$ mice and neutrophils are central in bacterial elimination [33], reduced C/EBPβ signaling may contribute to impaired host defense responses and predispose the lungs to the reoccurring bacterial infections observed among COPD patients [34]. On the other hand, impaired neutrophilia could also be beneficial, as persisted neutrophil influx may contribute to COPD pathogenesis [33]. Thus, additional studies investigating the effect of C/EBPβ deletion in chronic inflammatory models, especially with bacterial infection, which are more relevant to COPD, are warranted.

To address the contribution of C/EBPβ in preventing LPS-induced neutrophil recruitment in a setting relevant to COPD maintenance therapy, pre-treatment with FM and BUD was performed in this study. Maintenance therapy with LABAs and GCs decreases the frequency and severity of COPD exacerbations [35,36], acute and sustained worsening of stable COPD that drive disease progression [1]. Considering that the largest portion of inhaled BUD is deposited in mouse gut [16], we used systemic drug administration. Relatively high doses were used since BUD is known to be efficiently inactivated in the liver in humans [37] and biotransformation of BUD is similar in mice and humans [38]. The liver metabolism of FM in mice is unknown, however, mouse lungs have lower proportion of β_2-adrenocoptors than human lungs [39,40] and are therefore less sensitive, which warrants a high dose.

To conclude, LPS-induced neutrophilia and CXCL1 expression were reduced in $Cebpb^{\Delta LE}$ mice. The suppression of pulmonary neutrophilia and CXCL1 by FM were impaired in $Cebpb^{\Delta LE}$ mice, while the suppression by BUD with/without FM was preserved. Thus, reduced anti-inflammatory responses to LABAs due to reduced C/EBPβ signaling may be largely compensated by addition of a GC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.096.

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