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The modulatory effects of the PDE4 inhibitors CHF6001 and roflumilast in alveolar macrophages and lung tissue from COPD patients

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ABSTRACT

Background: We compared the anti-inflammatory effects of phosphodiesterase type 4 (PDE4) inhibitor roflumilast with CHF6001, a novel PDE4 inhibitor designed for inhaled administration, using human alveolar macrophages (AM) and lung tissue explants models.

Methods: AM from 13 chronic obstructive pulmonary disease (COPD) patients and 10 smoking controls and lung tissue from 7 COPD patients were stimulated with LPS following preincubation with roflumilast (0.000001–10 μM), CHF6001 (0.000001–0.1 μM), or vehicle. After 24 h, supernatants were analysed for cytokines by ELISA. The effects of both compounds on the phosphorylation and cellular localisation of cAMP response element binding protein (CREB) were assessed by immunofluorescence and Western blot analysis. Extracted RNA was used for quantitative PCR analysis of PDE4 A, B and D mRNA.

Results: PDE4 A, B and D expression were increased in alveolar macrophages and lung tissue of COPD patients compared to controls. Roflumilast and CHF6001 significantly reduced TNF-α production in AM and lung tissue. CHF6001 was more potent than roflumilast with lower EC_{50}s of 0.02, 0.01 and 0.31 nM compared to 0.87, 0.47 and 10.8 nM in respective samples. PDE4 inhibition also inhibited secretion of the chemokines CCL2 and CCL4 from macrophages. Both compounds increased nuclear levels of phosphorylated CREB.

Conclusion: PDE4 inhibitors caused a robust anti-inflammatory effect on TNF-α production from COPD AM, with inhibition of selective chemokines also observed. CHF6001 caused more potent inhibition of TNF-α production from COPD AM and lung tissue compared to roflumilast.

1. Background

Airway inflammation in chronic obstructive pulmonary disease (COPD) involves a network of different immune cells, including macrophages which secrete a broad range of inflammatory mediators and proteases. Cyclic adenosine monophosphate (cAMP) is a second messenger that negatively regulates many cellular immune responses [1]. Increased cAMP levels in macrophages decrease inflammatory cytokine release, reduce the activity of reactive oxygen species and inhibit phagocytosis [2]. Phosphodiesterase (PDE) enzymes metabolise cAMP [3]; the PDE4 isoenzyme is expressed in immune cells, and reduces cAMP levels thereby activating inflammatory responses [4–6]. There are four subtypes of the PDE4 enzyme; animal studies have shown PDE4 A, B and D but not C to be involved in cytokine production [7–9]. Furthermore, macrophages and monocytes express subtypes A, B and D but have little to no expression of subtype C. Using semiquantitative polymerase chain reaction (PCR), it has been reported that the gene expression levels of PDE4 A4 but not PDE4 B or PDE4 D are upregulated in COPD alveolar macrophages compared to controls [10], but no recent data with more quantitative technology have been published.

The orally administered PDE4 inhibitor roflumilast reduces exacerbation rates in severe COPD patients with chronic bronchitis [11–13]. However, the clinical dosage and efficacy of the only oral PDE4 inhibitor currently approved for COPD treatment (roflumilast) is limited by target-related side effects, such as nausea, diarrhoea and weight loss that make it intolerable for some patients [14].

With the aim of limiting systemic exposure and the associated side effects, novel PDE4 inhibitors to be administered directly into the lung by inhalation have been developed [15]. CHF6001, in particular, is a highly potent and selective PDE4 inhibitor designed for inhaled

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administration characterized by high lung retention, low plasma levels and potent effects in vivo rodent models in suppressing pulmonary inflammation [16]. CHF6001 is 7- and 923-fold more potent than other PDE4 inhibitors roflumilast and cilomilast, respectively, in inhibiting PDE4 enzymatic activity (IC_{50} = 0.026 ± 0.006 nM). CHF6001 inhibits PDE4 isoforms A-D with equal potency, showed an elevated ratio of high-affinity rolipram binding site versus low-affinity rolipram binding site (i.e., > 40) and displays > 20,000-fold selectivity versus PDE4 compared with a panel of several PDEs [15]. In vitro, CHF6001 displays potent (subnanomolar IC_{50} values) anti-inflammatory effects resulting in the inhibition of tumor necrosis factor (TNF)-α release from human peripheral blood mononuclear cells, rhinovirus (RV1B)-induced cytokines release from bronchial epithelial cells [17], the activation of oxidative burst in neutrophils and eosinophils and the release of interferon-γ (IFN-γ) from CD4(+) T- cells [15].

Given that the novel PDE4 inhibitor CHF6001 is currently undergoing phase 2b clinical trials (clinicaltrials.gov/ct2/show/NCT02986321) in COPD patients as an inhaled agent, we sought to compare it's anti-inflammatory effects with roflumilast by using COPD alveolar macrophages and whole tissue explants cultured *ex-vivo*. We also used quantitative PCR to determine PDE4 subtype expression in COPD alveolar macrophages and whole tissue, in order to verify previous findings [10] that used semi-quantitative methods.

2. Methods

2.1. Study subjects

114 patients undergoing surgical resection for suspected or confirmed lung cancer were recruited for different experiments; the demography of these subjects is shown in Table 1, and the number of samples used in each experiment is stated in the results text. COPD was diagnosed using GOLD criteria [18]. Controls were either smokers (S) with normal lung function or lifelong non-smokers (NS). All subjects gave written informed consent. This research was approved by the local research ethics committee (South Manchester Research Ethics Committee).

2.2. Macrophage culture

Alveolar macrophages were isolated from resected lung tissue as previously described [19]. To measure cytokine production, macrophages were stimulated for 24 h with LPS (1 μg/ml) after a 1 h pre-incubation with roflumilast or CHF6001 [reconstituted in dimethyl sulphoxide (DMSO)] at concentrations stated in the results text or matched DMSO vehicle control. ELISAs for TNF-α, interleukin-6 (IL-6), CXCL8, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL9 (MIG) and CXCL10 (IP-10) (R&D Systems, Abingdon, UK) and multiplex analysis for IL-1ra, IL-10, G-CSF, IFN-γ and CCL5 (RANTES) (Bio-Rad, UK) were performed on supernatants as per manufacturers' instructions. Cells were lysed for RNA extraction or Western blot analysis (see on-line supplement).

Table 1
Lung subject demography.

	NS	*S*	*COPD*
Sex (M/F)	5/13	13/16	31/23
Age (years)	67 (38–82)	65 (39–80)	67 (50–76)
FEV_1	2.17 ± 0.7	2.35 ± 0.6	1.80 ± 0.5
FEV_1 predicted	102 ± 24	93 ± 17	69 ± 14
FEV_1/FVC %	73 ± 9.5	75 ± 7.9	61 ± 9.6
Pack year history	0 (0–1)	32 (12–119)	50 (10–1204)
ICS use (%)	0	0	33
Current smokers (%)	0	93	76

Data presented as mean ± SD or median (range). Forced expratory volume (FEV), forced vital capacity (FVC), inhaled corticosteroids (ICS).

2.3. Whole lung tissue culture

Lung tissue (prepared as previously described [20] and in the on-line supplement) was stimulated for 24 h with LPS (1 μg/ml) after a 1 h pre-incubation with roflumilast (0.005–10 μM) or CHF6001 (0.000001–0.1 μM) [reconstituted in DMSO] or matched DMSO vehicle control. Supernatants were stored at −20 °C until analysis by ELISA and multiplex as already described.

2.4. Quantitative PCR

Extracted RNA was used for PCR analysis of PDE4 A, PDE4 B, PDE4 D (Thermo Scientific), TNF-α, CXCL8, (Applied Biosystems) expression using Taqman gene expression assays as described in the on-line supplement.

2.5. Cellular fractionation

Alveolar macrophages were harvested and washed with PBS. Cells were lysed and separated into cytoplasmic and nuclear extracts by using NE-PER kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents (NER and CER), Thermo Scientific). 100 μl of CER was added to cells and vortexed vigorously 15 s, then incubated on ice for 10 min. 5.5 μl of ice cold CER II was added to cell lysates, vortexed twice for 5 s followed by 5 min maximum speed spin. Supernatants were transferred into new tubes as cytoplasmic extraction. Insoluble pellet which contained nuclei were suspended into 50 μl NER buffer, followed by vortexing for 15 s every 10 min for a total of 40 min. Nuclear lysates were centrifuged at maximum speed for 10 min. Immediately the supernatants (nuclear extract) were transfered into new tubes.

2.6. Western blot analysis

Western blot analysis was performed using the following antibodies; rabbit anti-β actin (Abcam, Cambridge, UK), rabbit anti-phospo-NF-κB p65, total-cAMP response element binding protein (CREB) antibody, phospho-CREB primary antibody, cyclophilin A, histone-3 (Cell Signaling technology, MA, USA) and horseradish peroxidise-conjugated goat anti-rabbit (DakoCytomation, California, USA) (see on-line supplement).

2.7. Immunofluorescence

Alveolar macrophages from COPD patients were treated with and without roflumilast (0.1-1 μM) or CHF6001 (0.1μM) for 15 mins. Cells were washed in PBS before staining with anti-human phospho-CREB primary antibody (Cell Signalling) and detected with Alexa-488 conjugated goat-anti rabbit antibody (Invitrogen). Cells were counterstained with DAPI, analysed by microscope (Nikon ECLIPSE 80i) and images were taken using Image-Pro Plus (MediaCybernetics).

2.8. Statistical analysis

Normality was assessed using the Kolmogorov-Smirnov test. All ELISA and multiplex data that were normally distributed were compared between all subject groups using one-way ANOVA followed by unpaired t-tests. Data were compared within subject groups using repeated measures ANOVA followed by Dunnett's multiple comparisons tests. Percentage inhibition of cytokines TNF-α, IL-6 and CXCL8 between compounds were compared using two-way ANOVA followed by paired t-tests. All ELISA and multiplex data that were not normally distributed were compared between all subject groups using Kruskal-Wallis followed by Mann-Whitney tests. Data were compared within subject groups using Friedman followed by Dunn's multiple

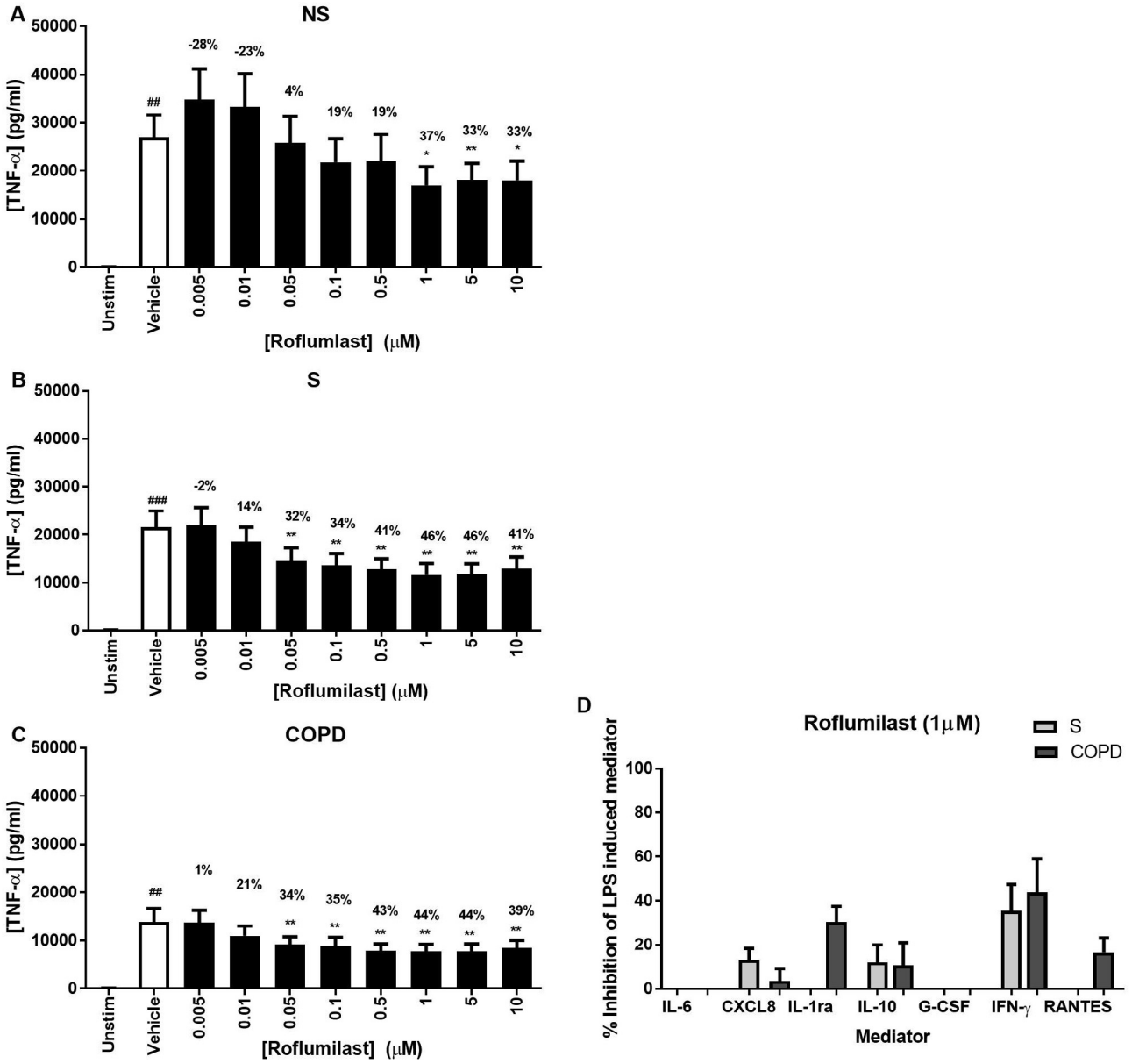

Fig. 1. The effects of roflumilast on LPS stimulated mediators in alveolar macrophages. Alveolar macrophages were treated with roflumilast (0.005–10 μM or 1 μM A-C and D respectively) or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. Culture supernatants were analysed for TNF-α (A-C), IL-6, CXCL8, IL-1ra, IL-10, G-CSF, IFN-γ and RANTES (D). Data shown are mean ± SEM of TNF- α production (A-C) from 6 non-smoking controls (NS), 11 smoking controls (S) and 11 COPD patients. Percentage inhibitions of LPS stimulated TNF-α are shown above each bar. D shows mean ± SEM of percentage inhibition of LPS stimulated mediators by roflumilast from 10 S and 9 COPD patients. ##, ### = significantly above unstimulated control ($p < 0.01$ and $p < 0.001$ respectively). *, ** = significant inhibition below vehicle control ($p < 0.05$, and $p < 0.01$ respectively).

comparisons tests. PDE4 subtype mRNA expression data were not normally distributed. Data were compared between all subject groups using Kruskal-Wallis followed by Mann-Whitney tests. All mRNA expression data were normally distributed and compared within groups using repeated measures ANOVA followed by paired t-tests. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of roflumilast on alveolar macrophage cytokine production

3.1.1. ELISA analysis

LPS significantly increased TNF-α production above basal levels in alveolar macrophages from 11 COPD patients, 11 S and 6 NS (Fig. 1) with no significant difference between groups ($p > 0.05$). Roflumilast inhibited TNF-α production, with maximal effects (defined as the lowest concentration at which the top of the inhibition curve is reached) observed at 1 μM; 37% in NS, 46% in S and 44% in COPD patients (Fig. 1A-C), with no difference between the groups at any concentration (ANOVA $p > 0.05$ at each concentration).

As roflumilast had maximal inhibitory effectiveness on TNF-α at 1 μM, then the effects of 0.1 and 1 μM on LPS stimulated IL-6 and CXCL8 production were investigated; Fig. 1D (1 μM) and Supplement Table 2 (both 0.1 and 1 μM) show that roflumilast had no effect on these cytokines in either COPD patients or S. There were insufficient supernatants left to perform IL-6 or CXCL8 analysis on NS samples.

3.1.2. Multiplex analysis

LPS increased IL-1ra, IL-10, G-CSF, IFN-γ and CCL5 production in alveolar macrophages from 9 COPD patients and 10 S (Supplement

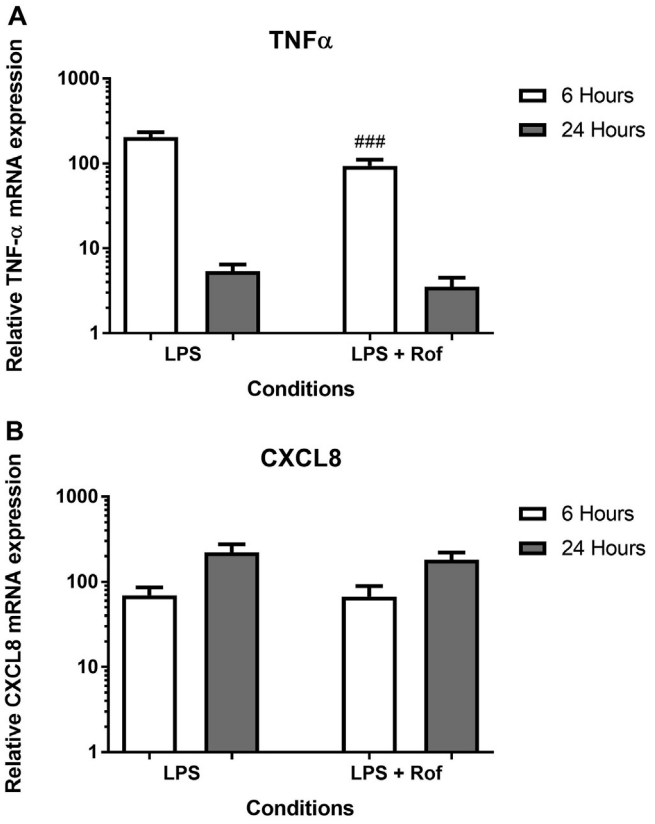

Fig. 2. The effects of roflumilast on LPS stimulated TNF-α and CXCL8 mRNA expression. Alveolar macrophages from 8 patients of mixed demographies (4 smokers 4 COPD patients) were treated with roflumilast (1 μM) or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) or unstimulated for 6 and 24 h. mRNA was extracted for PCR analysis of TNF-α (A) and CXCL8 (B). Data shown are mean ± SEM relative expression of TNF-α (A) and CXCL8 (B) mRNA. ### = Significant difference in relative mRNA expression compared to LPS stimulated without roflumilast ($p < 0.001$).

Table 1). Roflumilast did not inhibit the production of any of these proteins (Fig. 1D -1 μM and Supplement Table 2–0.1–1 μM).

3.1.3. Cytokine gene expression

LPS increased TNF-α and CXCL8 mRNA expression in alveolar macrophages from 8 patients (COPD; n = 4, S; n = 4) (Fig. 2); TNF-α was upregulated at 6 h, returning to baseline levels at 24 h, while CXCL8 expression was upregulated at both timepoints. Roflumilast significantly reduced TNF-α expression at 6 h ($p < 0.001$) but had no effect on CXCL8 expression at either time point. There were no differences between the effects of roflumilast in S and COPD patients, so the pooled data is shown in Fig. 2.

3.2. Effects of roflumilast on whole lung tissue cytokine production

3.2.1. ELISA analysis

LPS significantly increased TNF-α, IL-6 and CXCL8 production in whole lung tissue from 9 COPD patients and 10 S (Fig. 3A, 3B and Supplement Table 3). Roflumilast caused concentration dependent inhibition of TNF-α in both groups, with the maximal inhibitory effect reached at a concentration of 5 and 0.5 μM in COPD patients and S (73% and 73% inhibition respectively); The effects in COPD patients and S were similar ($p > 0.05$ at each concentration).

Fig. 3C and Supplement Table 4 show that roflumilast (1 μM and 0.1–1 μM respectively) did not significantly reduce LPS stimulated IL-6 or CXCL8 production in S or COPD patients.

3.2.2. Multiplex analysis

LPS increased production of all mediators apart from CCL-5 above basal levels in whole lung tissue from 7 COPD patients and 7 S (See online supplement and Supplement Table 3). Roflumilast (1 μM) significantly inhibited IFN-γ in S ($p < 0.05$) (Fig. 3C), and also inhibited IFN-γ and IL-10 production at 0.1 μM in COPD patients (Supplement Table 4).

3.3. Anti-inflammatory effects of CHF6001 compared to roflumilast

3.3.1. Cytokines

As TNF-α was the cytokine most sensitive to the effects of PDE4 inhibition, we compared roflumilast to CHF6001 using this endpoint in LPS stimulated alveolar macrophages from 13 COPD patients and 10 S. The effects of CHF6001 and roflumilast are shown in Fig. 4. The maximum inhibitory effect of CHF6001 was reached at 0.001 μM (35% and 42% inhibition in S and COPD patients respectively) with no differences between groups at any concentration (ANOVA $p > 0.05$). The inhibitory effect of CHF6001 was significantly greater than roflumilast at concentrations ≤ 0.01 and ≤ 0.001 μM in COPD and S respectively. CHF6001 was more potent than roflumilast with lower EC_{50}s of 0.02 and 0.01 nM compared to 0.87 and 0.47 nM in COPD patients and S respectively. The maximal effect of CHF6001 was similar to roflumilast in both groups, although the maximal effect was achieved at a 100 times higher concentration for roflumilast (0.1 μM).

The effects of CHF6001 and roflumilast on TNF-α production from LPS stimulated whole lung tissue from 7 COPD patients are shown in Fig. 5. The maximum effect of CHF6001 (approximately 70% inhibition) was reached at 0.01 μM. The maximal inhibitory effect of roflumilast was similar, but reached at a 100-fold higher concentration (1 μM). The inhibitory effect of CHF6001 was significantly greater than roflumilast at 0.001 – 0.01 μM. CHF6001 was more potent than roflumilast with a lower EC_{50} of 0.3 compared to 10.6 nM.

3.3.2. Chemokines

In a further 10 COPD patients we compared the effects of roflumilast to CHF6001 on LPS induced chemokine release from alveolar macrophages. LPS induced significant production of CCL2, CCL3, CCL4, CXCL9 and CXCL10. Both drugs reduced CCL-2 production, although only CHF6001 reached statistical significance with inhibition up to 60% observed (Fig. 6A). Both drugs had modest (< 40% inhibition), but statistically significant effects on CCL4 production (Fig. 6C). Neither drug had any significant effect on LPS induced CCL3, CXCL9 or CXCL10 production (Supplement Fig. 1A-C).

LPS stimulation of whole lung tissue from 10 COPD patients significantly induced CCL2 and CCL4; neither drug had any effect on these chemokines (Fig. 6C-D). CCL3, CXCL9 and CXCL10 were not significantly induced by LPS stimulation (Supplement Fig. 1D-F).

3.4. The effect of roflumilast on LPS-induced phosphorylation of p65

LPS increased the expression of phospho-p65 in alveolar macrophages from 3 COPD patients and 3 smokers; Supplement Fig. 2 is a representative Western blot. Maximum phospho-p65 expression was at 30–60 mins. Roflumilast did not alter the LPS-induced phosphorylation of p65 – see densitometry in Supplement Fig. 2 which shows the combined data from the 6 patients.

3.5. The effect of PDE4 inhibition on CREB nuclear localisation

Unstimulated macrophages showed staining for phosphorylated CREB (green) in either the cytoplasm, the nucleus (counter stained with DAPI-blue) or in both the cytoplasm and nucleus (Fig. 7A); staining was predominantly cytoplasmic only. Roflumilast (0.1 and 1 μM) or CHF6001 (0.1 μM) treatment for 15 min significantly increased the percentage of cells with phosphorylated CREB staining in the nucleus

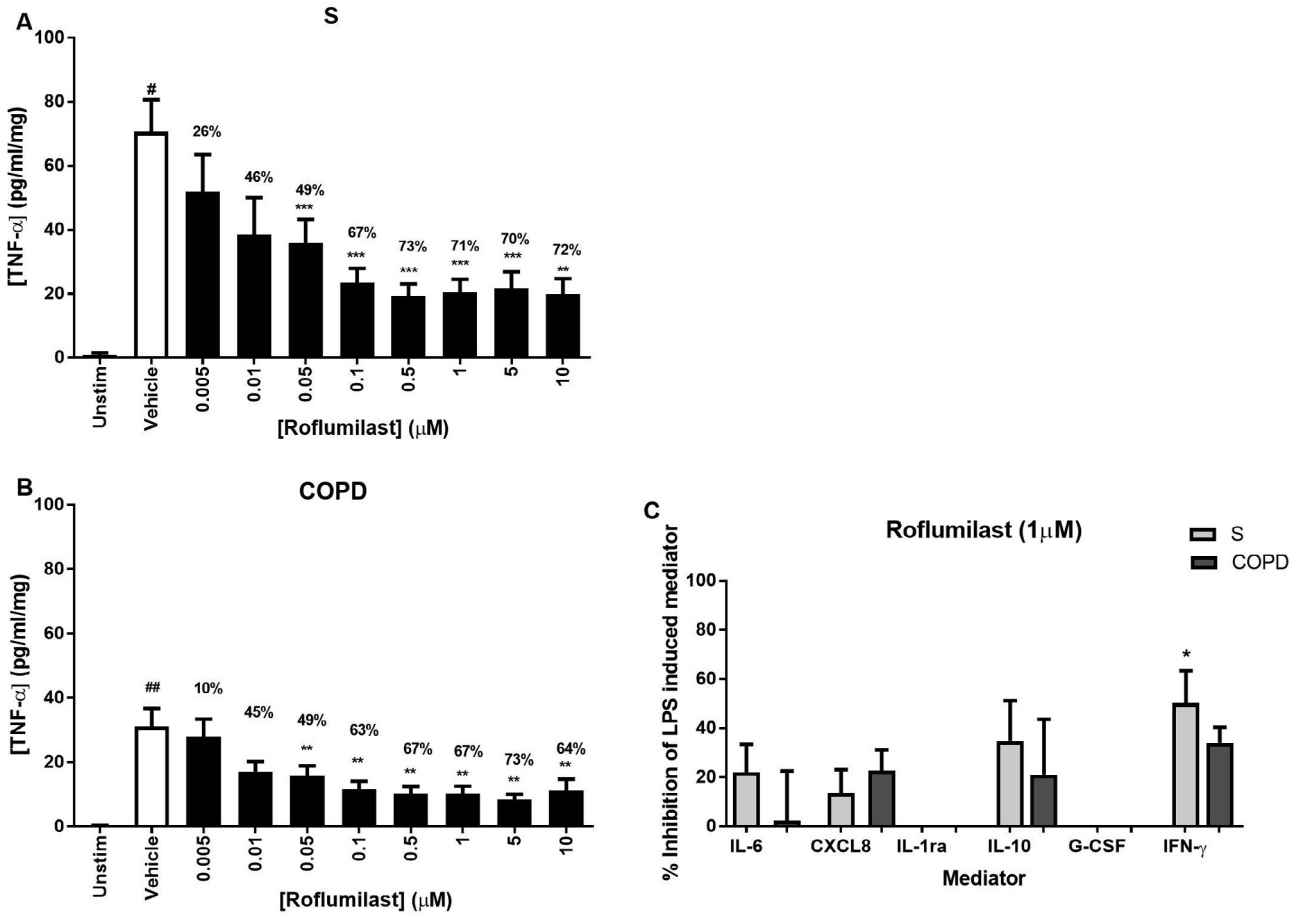

Fig. 3. The effects of roflumilast on LPS stimulated mediators in whole lung tissue. Lung tissue were treated with roflumilast (0.005–10 μM or 1 μM A-B and C respectively) or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. Culture supernatants were analysed for TNF-α (A-B), IL-6, CXCL8, IL-1ra, IL-10, G-CSF, and IFN-γ (C). Data shown are mean ± SEM of TNF- α production from 10 smokining controls (S) and 9 COPD patients (A-B). Percentage inhibitions of LPS stimulated TNF-α are shown above each bar. C shows mean ± SEM of percentage inhibition of LPS stimulated mediators by roflumilast from 10 S and 9 COPD patients. #, ## = significantly above unstimulated control ($p < 0.05$ and $p < 0.01$ respectively). *, **, *** = significant inhibition below vehicle control ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively).

only or in both the cytoplasm and nucleus (Fig. 7B-D); representative images of 4 experiments are shown in Fig. 7E.

Western blot analysis showed an increase in total CREB protein in the nuclear fraction of alveolar macrophages treated with roflumilast (0.1 and 1 μM) or CHF6001 (0.1 μM) for 15 min compared to untreated cells (Fig. 8B). There was also a decrease in total CREB in the cytoplasmic fraction (Fig. 8A). Roflumilast and CHF6001 also increased phosphorylated CREB levels in the nuclear fraction compared to controls, reaching significance for CHF6001 0.1 μM and roflumilast 1 μM ($p < 0.05$ for both comparisons).

3.6. PDE4 gene expression

3.6.1. Alveolar macrophages

The mRNA levels of PDE4 subtypes A, B and D were significantly increased in macrophages from patients with COPD (n = 11) compared to NS (n = 8) (Fig. 9 A-C). PDE4 subtype mRNA levels were numerically increased in COPD patients compared to S (n = 8), with this difference reaching statistical significance for PDE4 A ($p = 0.04$) but not PDE4 B ($p = 0.055$) or D ($p = 0.13$). PDE4 A was the most abundant subtype in macrophages, with PDE4 D being least abundant.

3.6.2. Whole lung tissue

The mRNA levels of PDE4 subtypes B and D were significantly increased in whole lung tissue from patients with COPD (n = 10) compared to NS (n = 10); $p = 0.02$ and $p = 0.04$ respectively (Fig. 9 D-F). There were no differences between groups for PDE4 A mRNA levels. The PDE4 subtypes were expressed at similar levels in whole lung tissue.

4. Discussion

Pharmacological inhibition of PDE4 reduced TNF-α production from alveolar macrophages and whole lung tissue from COPD patients and controls. CHF6001 was more potent compared to roflumilast for TNF-α inhibition. We also demonstrated inhibition of the chemokines CCL2 and CCL4 from alveolar macrophages by both PDE4 inhibitors. Overall, these results indicate selective inhibition of LPS induced innate immune inflammatory mediators in COPD lung macrophages and tissue.

We also demonstrate that PDE4 inhibition activates CREB but has no effect on NF-κB translocation in COPD alveolar macrophages, and that PDE4 A, B and D gene expression levels are increased in alveolar macrophages and whole lung tissue from COPD patients compared to controls. These experiments support the case for the pharmacological targeting of PDE4 in COPD, as there is increased expression of this PDE isoform in COPD lungs. Furthermore, the CREB results demonstrate the ability of both roflumilast and CHF6001 to modulate the activity of the PDE4 pathway in alveolar macrophages.

TNF-α is capable of playing different roles in COPD inflammation,

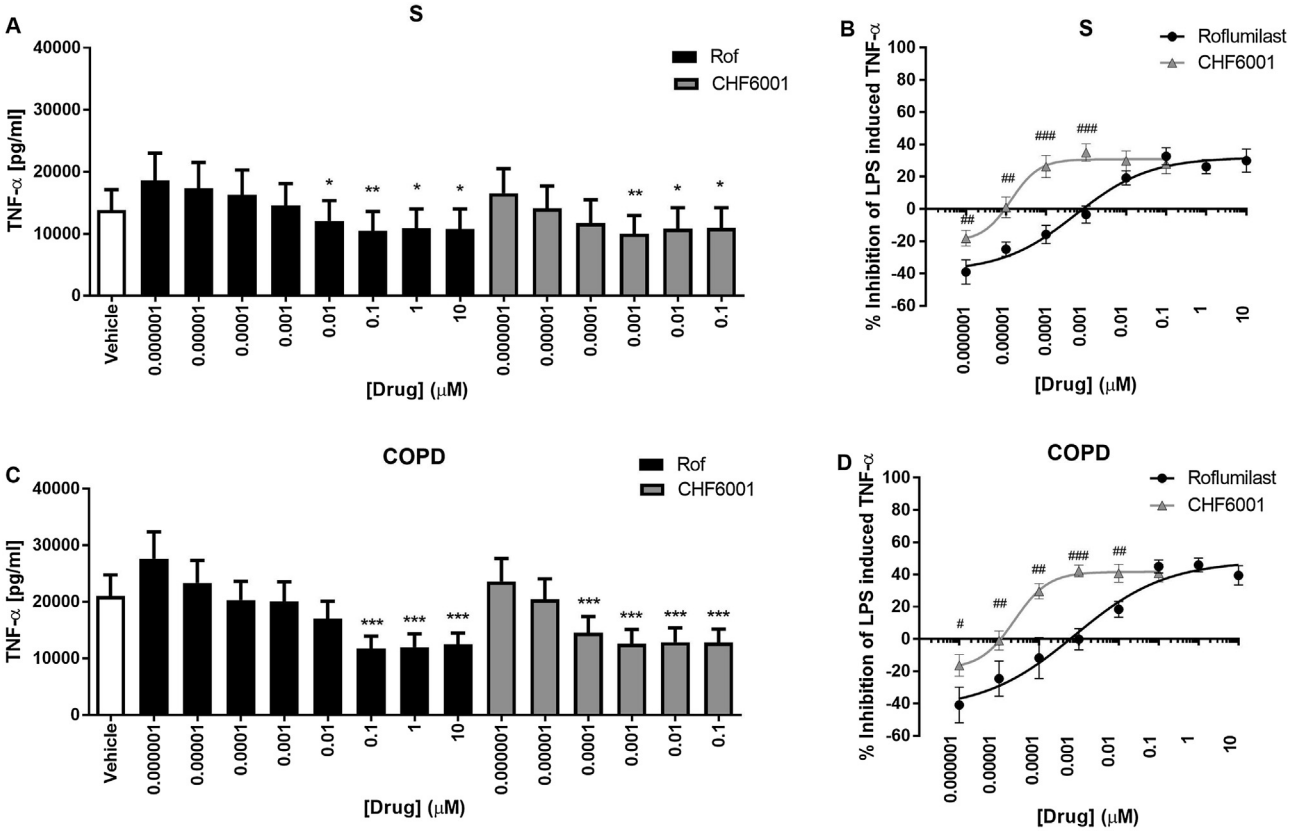

Fig. 4. The effects of roflumilast and CHF6001 on LPS induced TNF-α in alveolar macrophages. Alveolar macrophages from 11 smoking controls (S) (A-B) and 13 COPD patients (C-D) were treated with roflumilast (0.000001–10 μM), CHF6001 (0.000001–0.1 μM), or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. Culture supernatants were analysed for TNF-α. Data shown are mean ± SEM absolute concentrations (A and C) and percentage inhibition (B and D) of TNF-α production. *, **, *** = significantly below vehicle control ($p < 0.05$, 0.01, 0.001 respectively). #, ##, ### = significant inhibition above co-responding concentration of roflumilast ($p < 0.05$, 0.01, 0.001 respectively).

as it is associated with muscle disease, while there are increased levels of sputum TNF-α during acute exacerbations [21]. The production of TNF-α from human lung tissue appears to be an important regulator for the secretion of other inflammatory cytokines, as experiments using LPS stimulated human lung explants have shown that antibody blockade of the effects of TNF-α inhibits the release of IL-6 and CXCL8 [22]. The selective effect of PDE4 inhibition on TNF-α production from COPD alveolar macrophages and lung tissue may therefore have important further downstream anti-inflammatory effects on a range of cytokines and chemokines. This may be an important mechanism that contributes to the effect of PDE4 inhibitors on exacerbation prevention.

CHF6001 more potently inhibited TNF-α production compared to roflumilast. The same maximal effect ("efficacy") was observed for both drugs. The implications of these findings are that CHF6001 achieves the same pharmacological effects on lung cells as roflumilast but at lower concentrations. The use of roflumilast in clinical practice is limited by side effects due to systemic exposure after oral administration. CHF6001 has been developed for inhaled delivery in order to restrict

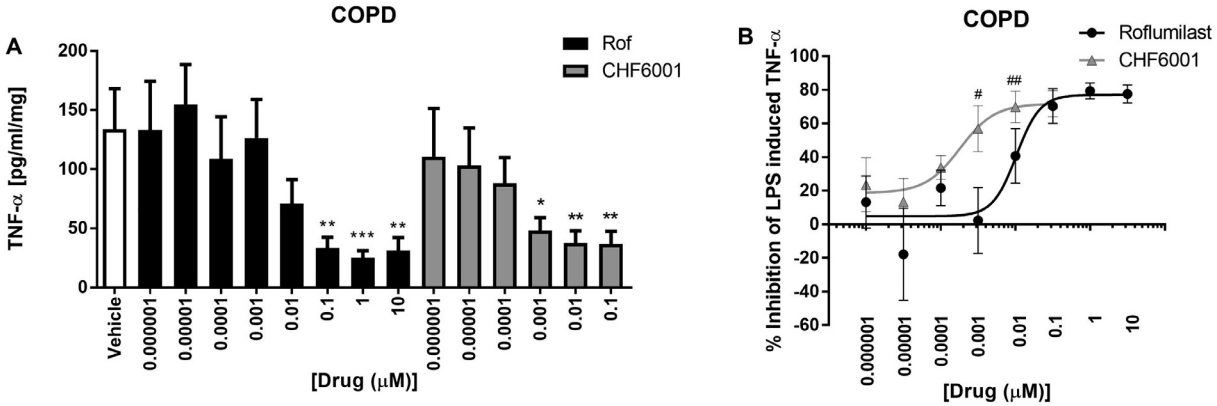

Fig. 5. The effects of roflumilast and CHF6001 on LPS induced TNF-α in whole lung tissue. Whole lung tissue from 7 COPD patients was treated with roflumilast (0.000001–10 μM), CHF6001 (0.000001–0.1 μM), or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. Culture supernatants were analysed for TNF-α. Data shown are mean ± SEM absolute concentrations (A) and percentage inhibition (B) of TNF-α production. *, **, *** = significantly below vehicle control ($p < 0.05$, 0.01, 0.001 respectively). #, ## = significant inhibition above co-responding concentration of roflumilast ($p < 0.05$, 0.01 respectively).

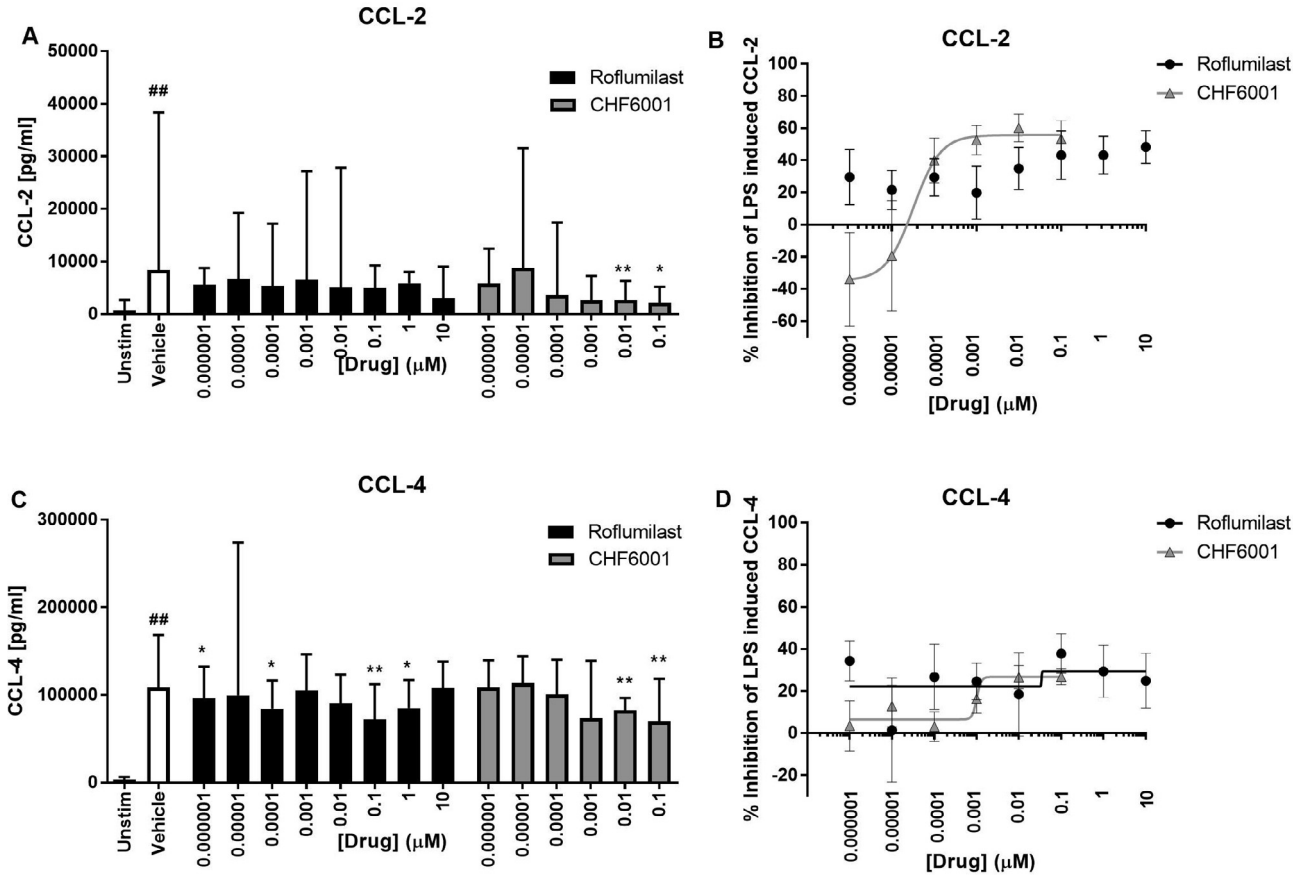

Fig. 6. The effects of roflumilast and CHF6001 on LPS induced CCL2 and CCL4 in alveolar macrophages. Alveolar macrophages from 10 COPD patients were treated with roflumilast (0.000001–10 μM), CHF6001 (0.000001–0.1 μM), or vehicle control (DMSO) for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. Culture supernatants were analysed for CCL2 and CCL4. Data shown are median ± interquartile range absolute concentrations (A and C) and mean ± SEM percentage inhibition (B and D) of CCL2 or CCL4 production. # = significantly above unstimulated control ($p < 0.05$). *, ** = significantly below vehicle control ($p < 0.05$, 0.01 respectively).

systemic exposure, and the low concentrations needed for pharmacological activity in the lungs further reduce the potential for side effects due to systemic exposure.

In response to the extracellular environment macrophages exhibit a degree of plasticity [23] which can be described by the M1/M2 model of macrophage polarisation [23]. M1 macrophages have pro-inflammatory and cytotoxic properties, and M2 macrophages have anti-inflammatory and tissue repair functions [24]. This model however is simplistic, as macrophages may have both M1 and M2 characteristics, and Chana *et al* reported that COPD macrophages can have both M1 and M2 properties [25]. Furthermore, distinct subpopulations of macrophages exist in the lower airways of COPD patients and controls [19]; Small interstitial macrophages that are pro-inflammatory, small alveolar macrophages that are highly phagocytic and large alveolar macrophages with low pro-inflammatory and phagocytic ability have been identified [19].

LPS stimulation of COPD alveolar macrophages mimics the pro-inflammatory effects of bacteria in the lungs. LPS stimulation of TLR4 activates signalling cascades including NF-κB and MAP kinases, increasing inflammatory mediator secretion. LPS stimulation also increases PDE4 activity and subsequent cAMP hydrolysis in human alveolar macrophages. PDE4 inhibitors reduce LPS stimulated TNF-α production close to baseline levels from human peripheral blood monocytes [9]. In contrast, it has previously been shown that roflumilast inhibits LPS stimulated TNF-α production by only approximately 40% in human lung macrophages, with no effect on CXCL8 production [26]. This may be due to the down regulation of PDE4 during monocyte to macrophage differentiation. We also observed similar partial inhibition of TNF-α production from COPD and control alveolar macrophages, and no effect on CXCL8 production. Similarly, roflumilast inhibited TNF-α but not CXCL8 at the level of transcription.

It has been reported that roflumilast increased CREB phosphorylation in LPS stimulated PBMCs at a concentration that also inhibited TNF-α production [27]. We also show that CHF6001 and roflumilast increased phosphorylation of CREB in alveolar macrophages and inhibited TNF-α production. There was no suppression of other cytokines, suggesting that CREB activation in alveolar macrophages has a specific effect on TNF-α transcription. CREB is a transcription factor involved in cell differentiation, proliferation and macrophage survival [28,29]. cAMP causes CREB activation through protein kinase A (PKA). Activated CREB associates with CREB-binding protein (CBP) or p300, and subsequently binds to cAMP-responsive elements (CREs) to initiate transcription [30,31]. CBP/p300 is a co-factor for optimal NF-κB activation of target genes [32,33] including TNF-α [34]. Activated CREB may deplete the amount of available CBP/p300, thereby leading to inhibition of NF-κB activity [28,35]; this may explain the selective effect of roflumilast on TNF-α.

Roflumilast inhibited LPS induced activation of NF-κB in the murine macrophage cell line RAW268.7 cells [36]. We did not observe any modulation of the phosphorylation of the p65 subunit, which stimulates transcriptional activity, nuclear localisation of the NF-κB complex, by roflumilast in human alveolar macrophages. It cannot be ruled out the possibility that roflumilast could modulate NF-κB activity through other mechanisms. Moreover, results from cell lines or animal cells may not reflect human lung cells.

Buenestado et al. (2012) [26] showed that roflumilast inhibited the

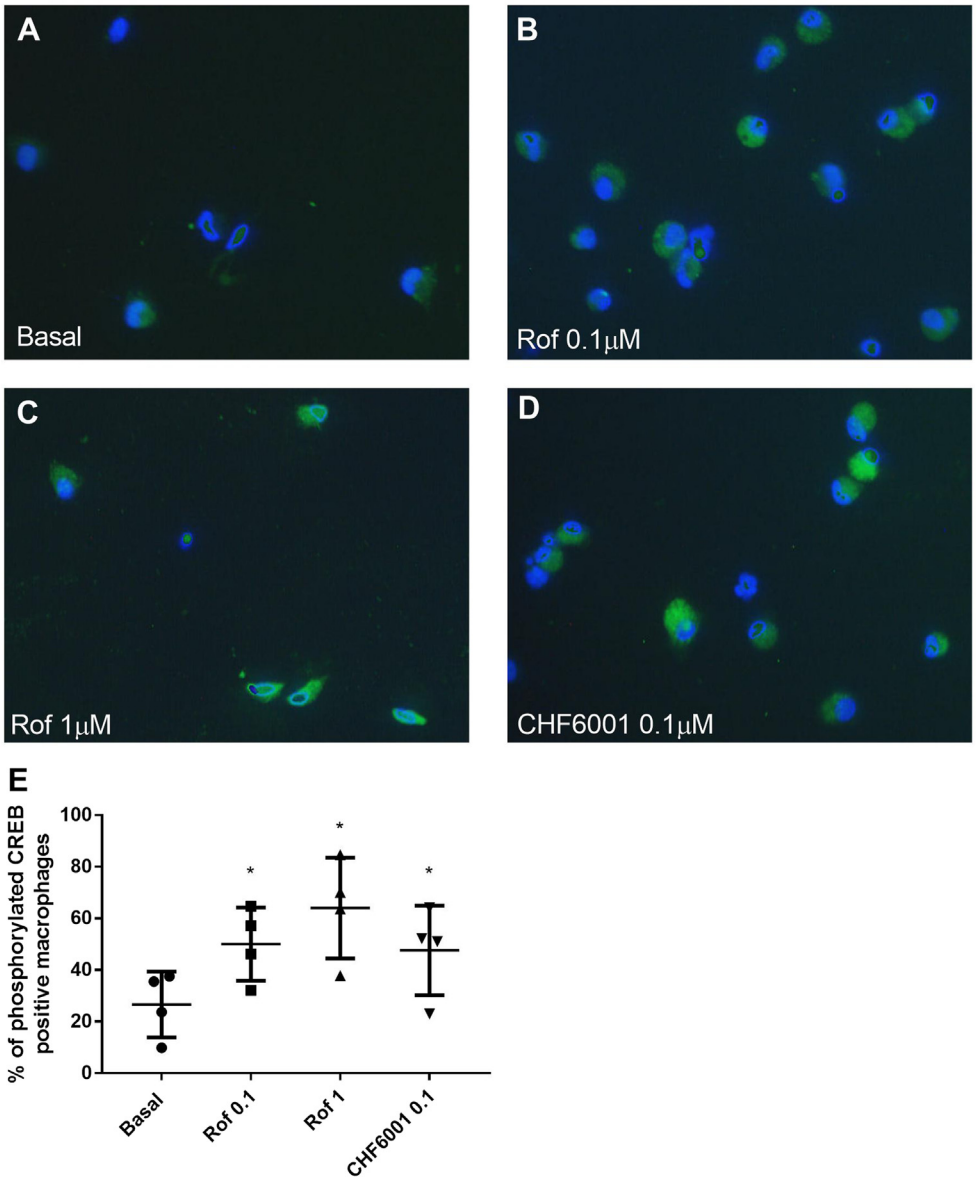

Fig. 7. The effects of roflumilast and CHF6001 on phosphorylation of CREB in alveolar macrophages. Alvolar macrophages from 4 COPD patients were treated with vehicle control (DMSO) (A) or roflumilast (0.1 or 1 μM) (B and C respectively) or CHF6001 (0.1 μM) (D) for 15 mins. Data shown are representative images of alveolar macrophages stained for phosphorylated CREB protein (green) and counterstained with nuclear specific stain DAPI (blue). The percentage of macrophages positive for phosphorylated CREB was calculated. E shows percentages for individual patients (n = 4). * = statistically above vehicle control ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production of the chemokines CCL2, CCL3, CCL4 and CXCL10 from human alveolar macrophages. We also showed inhibition of both CCL2 and CCL4 in alveolar macrophages with maximum inhibition levels comparable to those reported by Buenestado et al (~60% and ~30% for CCL2 and CCL4 respectively). We did not observe inhibition of CCL3 and CXCL10; this may be due to methodological differences such as the higher LPS concentration in the present study. In whole lung tissue, none of these chemokines were inhibited, but we found some inconsistent evidence of an inhibitory effect on IFN-γ, and IL-10 production e.g. there was significant inhibition of IFN-γ using roflumilast 0.1 μM but not 1 μM. These inconsistent results are likely to be due to a modest anti-inflammatory effect coupled with methodological variability that occurs when using COPD lung tissue i.e. the nature of the tissue cannot be harmonised in different experiments. Nevertheless, PDE4 inhibitors, including CHF6001 and roflumilast, are known to reduce IFN-γ production in CD4+ T cells and whole blood cultures [37,38], and our results demonstrate the same effect in lung tissue.

Previous studies using monocyte derived macrophages have used exogenous PGE2 to increase cAMP production in order to study the effects of PDE4 inhibitors. This is not necessary when using human alveolar macrophages, as LPS stimulation increases COX-2 mRNA expression and thus upregulates the production of endogenous prostanoids including PGE2. Buenestado et al. (2012) [26] were consequently able to study the effects of roflumilast on LPS stimulated alveolar macrophages without using exogenous PGE2, and we adopted the same approach. We have assessed PDE4 inhibitor effects in the TLR4 pathway; it would be valuable to investigate the effects using other relevant stimuli such as cytokines and other TLR agonists.

The gene expression levels of PDE4 A, PDE4 B and PDE4 D in alveolar macrophages and whole lung tissue from COPD patients were increased compared to controls. Barber el al. (2004) [10] showed increased expression of PDE4 A only in COPD patients compared to controls, using semi-quantitative PCR. The difference between the studies is likely to be due to the increased sensitivity afforded by quantitative real-time PCR methodology used here. Barber et al reported that PDE4 A was the most abundant subtype in alveolar macrophages, which we also observed. However, the whole lung tissue experiments showed similar expression levels of the three subtypes, probably due to the contribution of other PDE4 expressing cell types.

The PDE4 inhibitors both had greater effects on TNF-α production in the whole tissue model compared to alveolar macrophages. This difference could be due to the effect of roflumilast on lung cells other

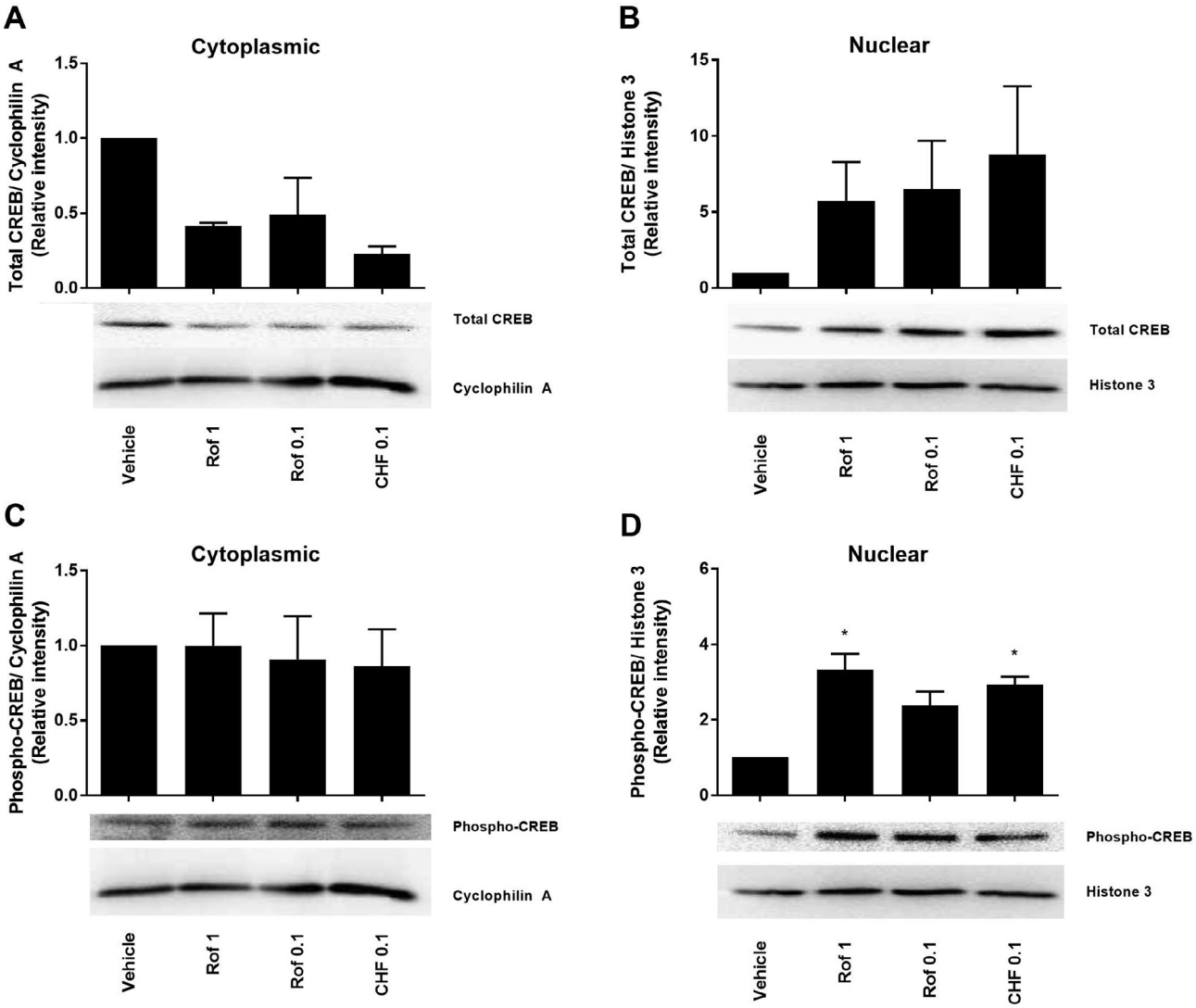

Fig. 8. The effects of roflumilast and CHF6001 on cellular localisation of CREB and phosphorylated CREB in alveolar macrophages. Alveolar macrophages from 3 COPD patients were treated with roflumilast (0.1 or 1 μM) or CHF6001 (0.1 μM) for 15 mins or vehicle control (DMSO). Cytoplasmic (A and C) and nuclear (B and D) cellular fractions were obtained and analysed for Total CREB (A-B) and phosphorylated CREB (C-D) levels by Western blot analysis. Band density was normalised to cyclophillin A for cytoplasmic or histone-3 for nuclear fractions. Representative blots are shown under corresponding conditions. Data presented as Mean ± SEM (n = 3). * = significance above vehicle control (DMSO) in nuclear fraction ($p < 0.05$).

than alveolar macrophages in the whole tissue model. The expression of PDE4 subtypes may be important in this respect. The reduction of TNF-α production by PDE4 inhibitors is correlated to inhibition of PDE4 A and PDE4 B but not PDE4 D [9] in human peripheral blood monocytes. However, only PDE4 B inhibition reduces TNF-α production in mouse macrophages [39]. These findings suggest a dominant role for PDE4 B in the anti-inflammatory effects of roflumilast in macrophages. PDE4 B expression in alveolar macrophages was lower than PDE4 A, whereas this difference was not observed in whole tissue; lower PDE4 B expression may be responsible for a reduced effect of roflumilast on TNF-α production in macrophages.

In summary, PDE4 expression is increased in alveolar macrophages and lung tissue of COPD patients compared to controls. Roflumilast and CHF6001 showed a selective effect on LPS induced cytokine and chemokine production, with TNF-α production in particular being sensitive to inhibition. This observation may be important clinically in exacerbations, given the role of TNF-α in amplifying the innate immune response. The finding that the anti-inflammatory effects of CHF6001, which is currently undergoing clinical trials in COPD patients, are obtained at low sub-nanomolar concentrations underscore the potential of such agent as inhaled anti-inflammatory drug in COPD.

5. Declarations

5.1. Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local research ethics committee (NRES Committee North West – Greater Manchester South; reference 03/SM/396) and subjects provided written informed consent.

5.2. Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

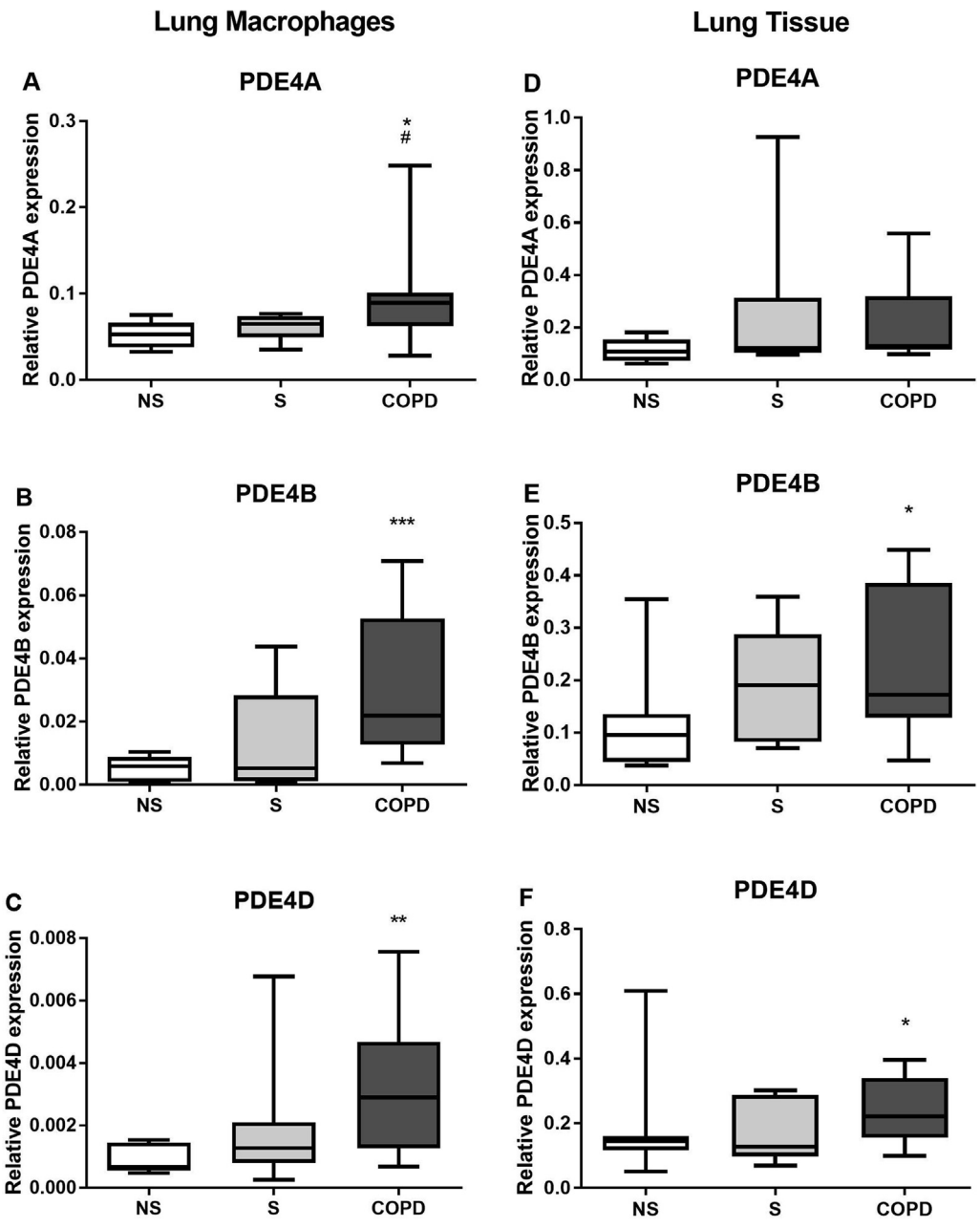

Fig. 9. PDE4 A, B and D mRNA expression. The expression of PDE4 A (A and D), PDE4 B (B and E) and PDE4 D (C and F) mRNA in alveolar macrophages from 8 non-smoking controls (NS), 8 smoking controls (S) and 11 COPD patients (A-C) and whole lung tissue from 10 non-smoking controls (NS), 10 smoking controls (S) and 10 COPD patients (D-F) . Data shown are median ± range of relative PDE4 A, B or D expression levels. Kruskal-Wallis followed by Mann-Whitney tests were performed to determine differences in expression levels between subject groups. *, **, *** = Significantly increased expression above NS ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively). # = Significantly increased expression above S ($p < 0.05$).

5.3. Competing interests

DS has received sponsorship to attend international meetings, honoraria for lecturing or attending advisory boards and research grants from various pharmaceutical companies including Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, Genentech, GlaxoSmithKline, Glenmark, Johnson and Johnson, Merck, NAPP, Novartis, Pfizer, Skypharma, Takeda, Teva, Therevance and Verona. GV, MC and FF work for Chiesi Pharmaceuticals. SL, AM, JL, AH and CB have no competing interests.

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Authors' contributions

Conception and design: SL, GV, MC, FF, and DS; Analysis and interpretation: SL, AM, AH, CB, JL, FF and DS; Drafting the manuscript for important intellectual content: SL, FF, GV, MC and DS.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154739.

References

[1] K. Tasken, E.M. Aandahl, Localized effects of cAMP mediated by distinct routes of protein kinase A, Physiol. Rev. 84 (1) (2004) 137–167.

[2] C.H. Serezani, M.N. Ballinger, D.M. Aronoff, M. Peters-Golden, Cyclic AMP: master regulator of innate immune cell function, Am. J. Respir. Cell Mol. Biol. 39 (2) (2008) 127–132.

[3] V. Boswell-Smith, C.P. Page, Roflumilast: a phosphodiesterase-4 inhibitor for the treatment of respiratory disease, Expert Opin. Investig. Drugs 15 (9) (2006) 1105–1113.

[4] N.A. Jones, V. Boswell-Smith, R. Lever, C.P. Page, The effect of selective phosphodiesterase isoenzyme inhibition on neutrophil function in vitro, Pulm. Pharmacol. Ther. 18 (2) (2005) 93–101.

[5] D.C. Grootendorst, S.A. Gauw, R.M. Verhoosel, P.J. Sterk, J.J. Hospers, D. Bredenbroker, T.D. Bethke, P.S. Hiemstra, K.F. Rabe, Reduction in sputum neutrophil and eosinophil numbers by the PDE4 inhibitor roflumilast in patients with COPD, Thorax 62 (12) (2007) 1081–1087.

[6] R. Hidi, S. Timmermans, E. Liu, C. Schudt, G. Dent, S.T. Holgate, R. Djukanovic, Phosphodiesterase and cyclic adenosine monophosphate-dependent inhibition of T-lymphocyte chemotaxis, Eur. Respir. J. 15 (2) (2000) 342–349.

[7] J. Selige, A. Hatzelmann, T. Dunkern, The differential impact of PDE4 subtypes in human lung fibroblasts on cytokine-induced proliferation and myofibroblast conversion, J. Cell Physiol. 226 (8) (2011) 1970–1980.

[8] D. Peter, S.L. Jin, M. Conti, A. Hatzelmann, C. Zitt, Differential expression and function of phosphodiesterase 4 (PDE4) subtypes in human primary CD4+ T cells: predominant role of PDE4D, J. Immunol. 178 (8) (2007) 4820–4831.

[9] C.D. Manning, M. Burman, S.B. Christensen, L.B. Cieslinski, D.M. Essayan, M. Grous, T.J. Torphy, M.S. Barnette, Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B, Br. J. Pharmacol. 128 (7) (1999) 1393–1398.

[10] R. Barber, G.S. Baillie, R. Bergmann, M.C. Shepherd, R. Sepper, M.D. Houslay, G.V. Heeke, Differential expression of PDE4 cAMP phosphodiesterase isoforms in inflammatory cells of smokers with COPD, smokers without COPD, and non-smokers, Am. J. Physiol. Lung Cell Mol. Physiol. 287 (2) (2004) L332–L343.

[11] P.M. Calverley, K.F. Rabe, U.M. Goehring, S. Kristiansen, L.M. Fabbri, F.J. Martinez, Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials, M, M.s. groups, Lancet 374 (9691) (2009) 685–694.

[12] S.I. Rennard, P.M. Calverley, U.M. Goehring, D. Bredenbroker, F.J. Martinez, Reduction of exacerbations by the PDE4 inhibitor roflumilast–the importance of defining different subsets of patients with COPD, Respir. Res. 12 (2011) 18.

[13] E.D. Bateman, K.F. Rabe, P.M. Calverley, U.M. Goehring, M. Brose, D. Bredenbroker, L.M. Fabbri, Roflumilast with long-acting beta2-agonists for COPD: influence of exacerbation history, Eur. Respir. J. 38 (3) (2011) 553–560.

[14] P. Rogliani, L. Calzetta, M. Cazzola, M.G. Matera, Drug safety evaluation of roflumilast for the treatment of COPD: a meta-analysis, Expert Opin. Drug Safety 15 (8) (2016) 1133–1146.

[15] N. Moretto, P. Caruso, R. Bosco, G. Marchini, F. Pastore, E. Armani, G. Amari, A. Rizzi, E. Ghidini, R. De Fanti, C. Capaldi, L. Carzaniga, E. Hirsch, C. Buccellati, A. Sala, C. Carnini, R. Patacchini, M. Delcanale, M. Civelli, G. Villetti, F. Facchinetti, CHF6001 I: a novel highly potent and selective phosphodiesterase 4 inhibitor with robust anti-inflammatory activity and suitable for topical pulmonary administration, J. Pharmacol. Exp. Ther. 352 (3) (2015) 559–567.

[16] G. Villetti, C. Carnini, L. Battipaglia, L. Preynat, P.T. Bolzoni, F. Bassani, P. Caruso, M. Bergamaschi, A.R. Pisano, V. Puviani, F.F. Stellari, V. Cenacchi, R. Volta, V. Bertacche, V. Mileo, V. Bagnacani, E. Moretti, P. Puccini, S. Catinella, F. Facchinetti, A. Sala, M. Civelli, CHF6001 II: a novel phosphodiesterase 4 inhibitor, suitable for topical pulmonary administration–in vivo preclinical pharmacology profile defines a potent anti-inflammatory compound with a wide therapeutic window, J. Pharmacol. Exp. Ther. 352 (3) (2015) 568–578.

[17] M.R. Edwards, F. Facchinetti, M. Civelli, G. Villetti, S.L. Johnston, Anti-inflammatory effects of the novel inhaled phosphodiesterase type 4 inhibitor CHF6001 on virus-inducible cytokines, Pharmacol. Res. Perspect. 4 (1) (2016) e00202.

[18] C.F. Vogelmeier, G.J. Criner, F.J. Martinez, A. Anzueto, P.J. Barnes, J. Bourbeau, B.R. Celli, R. Chen, M. Decramer, L.M. Fabbri, P. Frith, D.M. Halpin, M.V. Lopez Varela, M. Nishimura, N. Roche, R. Rodriguez-Roisin, D.D. Sin, D. Singh, R. Stockley, J. Vestbo, J.A. Wedzicha, A. Agusti, Global strategy for the diagnosis, management, and prevention of chronic obstructive lung disease 2017 report: GOLD executive summary, Eur. Respir. J. 49 (3) (2017).

[19] J.A. Dewhurst, S. Lea, E. Hardaker, J.V. Dungwa, A.K. Ravi, D. Singh, Characterisation of lung macrophage subpopulations in COPD patients and controls, Sci Rep 7 (1) (2017) 7143.

[20] A. Pomerenke, S.R. Lea, S. Herrick, M.A. Lindsay, D. Singh, Characterization of TLR-induced inflammatory responses in COPD and control lung tissue explants, Int. J. Chron. Obstruct. Pulmon. Dis. 11 (2016) 2409–2417.

[21] S.D. Aaron, J.B. Angel, M. Lunau, K. Wright, C. Fex, N. Le Saux, R.E. Dales, Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 163 (2) (2001) 349–355.

[22] T.L. Hackett, R. Holloway, S.T. Holgate, J.A. Warner, Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study, Respir. Res. 9 (2008) 47.

[23] P.J. Murray, J.E. Allen, S.K. Biswas, E.A. Fisher, D.W. Gilroy, S. Goerdt, S. Gordon, J.A. Hamilton, L.B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F.O. Martinez, J.L. Mege, D.M. Mosser, G. Natoli, J.P. Saeij, J.L. Schultze, K.A. Shirey, A. Sica, J. Suttles, I. Udalova, J.A. van Ginderachter, S.N. Vogel, T.A. Wynn, Macrophage activation and polarization: nomenclature and experimental guidelines, Immunity 41 (1) (2014) 14–20.

[24] F.O. Martinez, A. Sica, A. Mantovani, M. Locati, Macrophage activation and polarization, Front. Biosci. 13 (2008) 453–461.

[25] K.K. Chana, P.S. Fenwick, A.G. Nicholson, P.J. Barnes, L.E. Donnelly, Identification of a distinct glucocorticosteroid-insensitive pulmonary macrophage phenotype in patients with chronic obstructive pulmonary disease, J. Allergy Clin. Immunol. 133 (1) (2014) 207-16 e1-11.

[26] A. Buenestado, S. Grassin-Delyle, F. Guitard, E. Naline, C. Faisy, D. Israel-Biet, E. Sage, J.F. Bellamy, H. Tenor, P. Devillier, Roflumilast inhibits the release of chemokines and TNF-alpha from human lung macrophages stimulated with lipopolysaccharide, Br. J. Pharmacol. 165 (6) (2012) 1877–1890.

[27] S.L. Tannheimer, E.A. Sorensen, A.C. Haran, C.N. Mansfield, C.D. Wright, M. Salmon, Additive anti-inflammatory effects of beta 2 adrenoceptor agonists or glucocorticosteroid with roflumilast in human peripheral blood mononuclear cells, Pulm. Pharmacol. Ther. 25 (2) (2012) 178–184.

[28] A.Y. Wen, K.M. Sakamoto, L.S. Miller, The role of the transcription factor CREB in immune function, J. Immunol. 185 (11) (2010) 6413–6419.

[29] J.M. Park, F.R. Greten, A. Wong, R.J. Westrick, J.S. Arthur, K. Otsu, A. Hoffmann, M. Montminy, M. Karin, Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis–CREB and NF-kappaB as key regulators, Immunity 23 (3) (2005) 319–329.

[30] A.J. Shaywitz, M.E. Greenberg, CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals, Annu. Rev. Biochem. 68 (1999) 821–861.

[31] B. Mayr, M. Montminy, Transcriptional regulation by the phosphorylation-dependent factor CREB, Nat. Rev. Mol. Cell Biol. 2 (8) (2001) 599–609.

[32] R. Medzhitov, T. Horng, Transcriptional control of the inflammatory response, Nat. Rev. Immunol. 9 (10) (2009) 692–703.

[33] S. Ghosh, M.S. Hayden, New regulators of NF-kappaB in inflammation, Nat. Rev. Immunol. 8 (11) (2008) 837–848.

[34] E.Y. Tsai, J.V. Falvo, A.V. Tsytsykova, A.K. Barczak, A.M. Reimold, L.H. Glimcher, M.J. Fenton, D.C. Gordon, I.F. Dunn, A.E. Goldfeld, A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo, Mol. Cell Biol. 20 (16) (2000) 6084–6094.

[35] G.C. Parry, N. Mackman, Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription, J. Immunol. 159 (11) (1997) 5450–5456.

[36] H.J. Kwak, J.S. Song, J.Y. Heo, S.D. Yang, J.Y. Nam, H.G. Cheon, Roflumilast inhibits lipopolysaccharide-induced inflammatory mediators via suppression of nuclear factor-kappaB, p38 mitogen-activated protein kinase, and c-Jun NH2-terminal kinase activation, J. Pharmacol. Exp. Ther. 315 (3) (2005) 1188–1195.

[37] A. Hatzelmann, C. Schudt, Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro, J. Pharmacol. Exp. Ther. 297 (1) (2001) 267–279.

[38] D. Claveau, S.L. Chen, S. O'Keefe, D.M. Zaller, A. Styhler, S. Liu, Z. Huang, D.W. Nicholson, J.A. Mancini, Preferential inhibition of T helper 1, but not T helper 2, cytokines in vitro by L-826,141 [4-[2-(3,4-Bisdifluromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan- 2-yl)-phenyl]-ethyl]3-methylpyridine-1-oxide], a potent and selective phosphodiesterase 4 inhibitor, J. Pharmacol. Exp. Ther. 310 (2) (2004) 752–760.

[39] S.L. Jin, L. Lan, M. Zoudilova, M. Conti, Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages, J. Immunol. 175 (3) (2005) 1523–1531.