Cytokine 113 (2019) 68–73

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

# Anti-inflammatory effects of the phosphodiesterase type 4 inhibitor CHF6001 on bronchoalveolar lavage lymphocytes from asthma patients

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ARTICLE INFO

*Keywords:*
Asthma
PDE4
Corticosteroid
Lymphocyte
Bronchoalveolar lavage

ABSTRACT

*Background:* Lymphocytes play a key role in asthma pathophysiology, secreting various cytokines involved in chronic inflammation. CHF6001 is a highly potent and selective phosphodiesterase type 4 (PDE4) inhibitor designed for inhaled administration and has been shown to reduce the late asthmatic response. However, the effect of PDE4 inhibition on the different cytokines produced by lung lymphocytes from asthma patients has not been examined.

*Methods:* This study investigated the anti-inflammatory effects of CHF6001 and the corticosteroid, 17-BMP, on T-cell receptor (TCR) stimulated Th1, Th2 and Th17 cytokine release from bronchoalveolar lavage (BAL) cells from mild (n = 12) and moderate asthma (n = 12) patients.

*Results:* CHF6001 inhibited IFNγ, IL-2 and IL-17, but not IL-13, secretion from both mild and moderate asthma patient BAL cells; there was a greater effect on IFNγ and IL-2 than IL-17. The corticosteroid inhibited all four cytokines from both patient groups, but was less effective in cells from more severe patients. CHF6001 had a greater inhibitory effect on IFNγ and IL-2 than 17-BMP.

*Conclusion:* The PDE4 inhibitor CHF6001 had a greater effect on Th1 cytokines from TCR-stimulated BAL cells than corticosteroid. This pharmacological effect suggests the therapeutic potential for PDE4 inhibitors to be used in the subset of more severe asthma patients with increased airway levels of IFNγ.

## 1. Introduction

Cyclic 3′5-adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are key regulators of inflammation [1]. A decrease in intracellular cAMP and cGMP levels, following hydrolysis by phosphodiesterase (PDE) enzymes, promotes inflammatory responses [2]. The PDE4 subtype is selectively expressed in leukocytes, and selective PDE4 inhibition has anti-inflammatory effects in obstructive lung diseases [3]. The orally administered PDE4 inhibitor roflumilast reduces exacerbation rates in patients with chronic obstructive pulmonary disease (COPD), and there is also evidence of clinical benefits in asthma [3–5]. 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 [6].

In an attempt to limit systemic exposure and the associated side effects, novel topical- PDE4 inhibitors have been developed to be administered directly into the lung by inhalation [7]. CHF6001, in particular, is a highly potent and selective PDE4 inhibitor designed for inhaled administration characterized by high lung retention and low plasma levels [8]. In vitro, CHF6001 displayed potent (subnanomolar $IC_{50}$ values) anti-inflammatory effects resulting in the inhibition of tumor necrosis factor-α release from human peripheral blood mononuclear cells (PBMCs), rhinovirus (RV1B)-induced cytokines release from bronchial epithelial cells, the activation of oxidative burst in neutrophils and eosinophils and the release of interferon-γ (IFNγ) from

*Abbreviations:* ACQ, Asthma control questionnaire; AP-1, Activator protein 1; BAL, Bronchoalveolar lavage; cAMP, Cyclic 3′5-adenosine monophosphate; cGMP, Cyclic guanosine monophosphate; COPD, Chronic obstructive pulmonary disease; FeNO, Forced exhaled nitric oxide (50 ml/sec); FEV1, Forced expiratory volume in 1 s; FVC, Forced vital capacity; GINA, Global initiative for asthma; GR, Glucocorticoid receptor; ICS, Inhaled corticosteroid; IFNγ, Interferon gamma; IL-, Interleukin; LLOQ, Lowest level of quantification; NFAT, Nuclear factor of activated T-cells; NF-κB, NF-kappaB; PBMC, Peripheral blood mononuclear cells; PDE4, phosphodiesterase 4; T2, Type 2 inflammation; TCR, T-cellreceptor; Th, T-helper

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https://doi.org/10.1016/j.cyto.2018.06.007
Received 22 November 2017; Received in revised form 9 May 2018; Accepted 7 June 2018
Available online 19 June 2018
1043-4666/ 

CD4(+) T-cells [9,10]. When administered intratracheally to rats, CHF6001 inhibited liposaccharide-induced pulmonary neutrophilia and leukocyte infiltration with an efficacy comparable to a high corticosteroid dose [9]. Intratracheal administration of CHF6001 to ovalbumin-sensitized Brown-Norway rats suppressed the antigen-induced lung function decline and eosinophilia [8]. It has been reported that CHF6001 reduces the late response to allergen challenge in patients with mild asthma, suggesting that the broad anti-inflammatory properties of CHF6001 may be beneficial in asthma [11].

Allergic inflammation in asthma is associated with increased production of T2 cytokines such as IL-5, IL-4 and IL-13 by different cell types, including lymphocytes [12]. There is also evidence that T-helper1, responsible for IL-2 and IFNγ secretion, and T-helper-17 cells, responsible for IL-17 secretion, are involved in more severe asthma by promoting neutrophilic airway inflammation [13].

It is noteworthy that cAMP is a negative regulator of T-cell activation and that PDE4 inhibitors suppress cytokine production from lymphocytes [14]. However, the T-cell subtype preference of PDE4 inhibitors is still controversial. Selective PDE4 inhibitors have been reported to inhibit the proliferation and IL-4 and IL-5 secretion by human allergen-specific Th2 lymphocytes in vitro [15]. On the other hand, other in vitro studies suggest that PDE4 inhibitors have complex inhibitory effects on Th1-mediated immunity at the concentration ranges achievable in vivo, whereas Th2-mediated responses are mostly unaffected or even enhanced [16]. To date, the effect of PDE4 inhibition on the different cytokines produced by lung lymphocytes from asthma patients has not been studied. To fill this gap, we have evaluated the anti-inflammatory effects of CHF6001 on lymphocyte derived cytokines from bronchoalveolar lavage (BAL) cells collected from patients with asthma.

## 2. Methods

### 2.1. Patients

Two groups of asthma patients were recruited for bronchoscopy; (1) 12 mild asthma patients not using inhaled corticosteroids (ICS) corresponding to GINA step 1 patients and (2) 12 asthma patients with moderate disease who were using ICS at a dose > 800 μg/day beclomethasone equivalent plus a long acting β2 agonist (LABA), and had suboptimal control with an asthma control questionnaire (ACQ) score > 1, corresponding to GINA step 4 patients. Six separate moderate asthma patients were recruited to donate blood. All subjects were never smokers. Subjects were excluded if there was any history of lung disease other than asthma. The study was approved by the local research ethics committee (NRES Committee North West – Greater Manchester South; REC Ref: 06/Q1403/156). All subjects provided written informed consent.

### 2.2. Cell collection and culture

BAL was collected from right and left upper lobes, with a maximum of 4 × 60 ml of pre-warmed 0.9% sterile saline being administered to each lobe. The BAL was filtered (100 μm filter, Becton Dickenson) and centrifuged (400g, 10 min at 4 °C). The cell pellet was resuspended in RPMI 1640 medium supplemented with 10% v/v fetal calf serum, with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. PBMCs were isolated using Ficol Paque (GE Healthcare, Little Chalfont, UK) and T-cells purified using EasySep™ Human T Cell Enrichment Kit (Stemcell Technologies, Cambridge, UK). Viable counts were performed using trypan blue and cell concentration adjusted to $1 \times 10^6$/ml. BAL differential cell counts were performed using Rapi-Diff II stain (Atom Scientific, Hyde, UK).

Mixed BAL cells, or isolated T-cells, were seeded at $1 \times 10^5$ cells/well in 96-well plates and treated with either the PDE4 inhibitor CHF6001 or the corticosteroid beclomethasone-17-monopropionate (17-BMP) (both supplied by Chiesi Farmaceutici S.p.A., Parma, Italy) for 1 h before addition of T-cell activation/expansion anti CD2/3/28 beads (Miltenyi Biotech, Bisley, UK) to induce a T-cell receptor (TCR) specific response. Cells were left for 24 h before supernatants were collected and frozen at −20 °C for subsequent cytokine analysis.

### 2.3. Cytokine analysis

IL-2, IL-13, IL-17 and IFNγ were measured in cell culture supernatants by "Ready-Set-Go!" ELISA (eBioscience, UK). Lower levels of quantification (LLOQ) for all assays were 4 pg/ml.

### 2.4. Statistical analysis

Data distribution was determined by Kolmogorov-Smirnov test. Clinical characteristics compared between groups by Student's T-test or Chi-square test. Absolute cytokine levels and percentage inhibition data were normally distributed. Analyses of drug effects, within a patient group, were assessed by 1-way ANOVA with Dunnett's post-hoc test against the stimulated control with no drug. Comparisons between patient groups and between CHF6001 and 17-BMP were assessed by 2-way ANOVA with Sidak post-hoc test between match drug concentrations. The effects of CHF6001 on isolated T-cells were assessed by T-test against stimulated control with no drug. Correlations between BAL lymphocyte proportions and stimulated cytokine levels were assessed by Spearman's rank. Mann Whitney test was used to compare lymphocyte proportions in BAL from mild and moderate asthma patients. All statistical analysis was performed using Prism 7.01 (http://www.graphpad.com).

## 3. Results

The clinical characteristics of the participants are shown in Table 1. The two patient groups had similar age, gender proportion and age of diagnosis. The moderate asthma patients had lower FEV1 % predicted, greater reversibility, worse asthma control and a greater proportion who had experienced an exacerbation treated with oral corticosteroids in the previous 12 months.

**Table 1**
Patient clinical features for BAL cell study.

| | Mild | Moderate |
|---|---|---|
| Number | 12 | 12 |
| Age | 37.7 ± 9.8 | 45.0 ± 12.5 |
| Gender; male/female | 9/3 | 6/6 |
| BMI | 27.6 ± 2.7 | 27.0 ± 4.2 |
| Age of diagnosis | 20.8 ± 13.2 | 17.1 ± 11.8 |
| Patients with an exacerbation in previous 12 months | 0 | $4^{\#}$ |
| SABA users | 12 | 12 |
| LABA users | 0 | $12^{\#\#\#}$ |
| ICS users | 0 | $12^{\#\#\#\#}$ |
| Total Daily Dose BDP Equivalent | 0 | $1380.0 \pm 511.6^{***}$ |
| FeNO (ppb) | 45.8 ± 34.9 | 21.6 ± 20.1 |
| Pre FEV1 % | 93.3 ± 11.1 | $66.2 \pm 16.9^{***}$ |
| Pre Ratio | 71.6 ± 9.8 | $60.3 \pm 9.5^{**}$ |
| Reversibility % | 8.9 ± 7.7 | $18.4 \pm 12.3^{*}$ |
| ACQ | 0.8 ± 0.5 | $2.0 \pm 0.9^{***}$ |
| Skin Prick Test | n = 11 | n = 11 |

Data is presented as mean ± sd. Abbreviations: BMI: Body mass index; SABA: short bronchial beta agonists; ICS: inhaled corticosteroid; BDP: beclomethasone dipropionate; FeNO: Forced exhaled nitric oxide (50 ml/sec); FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; ACQ: asthma control questionnaire. Comparisons between mild and moderate were by T-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) or Chi-square test ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$).

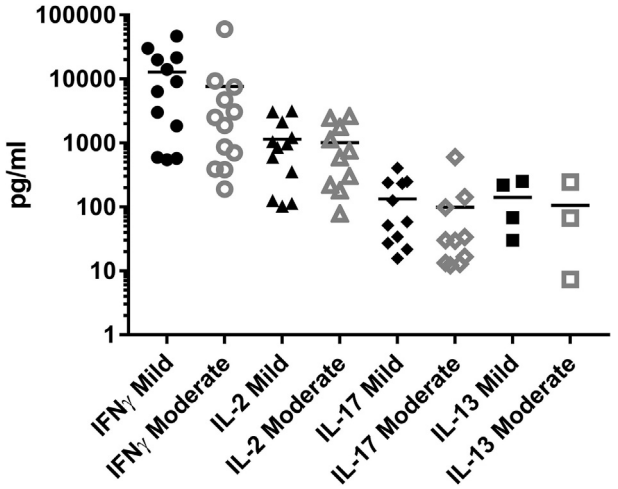


**Fig. 1.** TCR-induced cytokine levels in BAL cells from mild and moderate asthma patients. Cells were stimulated with CD2/3/28 activation beads for 24 h. IFNγ, IL-2, IL-17 and IL-13 levels were measured by ELISA. Bars illustrate mean cytokine levels. Comparisons between mild and moderate patients were by T-Test; all non-significant.

### 3.1. TCR activation of BAL cells

BAL cells from mild (n = 12) and moderate (n = 12) asthma patients were stimulated; there was increased IFNγ, IL-2 and IL-17 secretion in cells from all mild asthma patients, while cells from two moderate asthma patients did not respond to the stimulus. Due to limited cell numbers, IL-13 was only assessed in cell cultures from n = 7 and n = 6 mild and moderate asthma patients respectively, with IL-13 levels above the LLOQ observed in n = 4 and n = 3 mild and moderate asthma patients respectively. Cytokine levels post-stimulation were similar between the two groups (Fig. 1). Levels of all cytokines were below the LLOQ in unstimulated cells. Sufficient cell yields were obtained that allowed BAL differential cell counts for 9 subjects. The proportion of lymphocytes in the BAL correlated with TCR stimulated IFNγ (rho = 0.73, p = 0.033) and IL-2 (rho = 0.82, p = 0.035) levels, but not IL-17 (rho = 0.13, p = 0.77).

### 3.2. Effects of 17-BMP and CHF6001 on TCR response in BAL cells

CHF6001 inhibited TCR-induced IFNγ, IL-2 and IL-17 release from both mild and moderate asthma patients (Fig. 2; individual patient data illustrated in Supplementary Fig. 1). The effects of CHF6001 were similar in mild and moderate asthma. CHF6001 caused greater inhibition of IFNγ and IL-2 than IL-17, in cells from both patient groups (Supplementary Fig. 2).

17-BMP inhibited TCR-induced IFNγ, IL-2 and IL-17 from both patient groups (Fig. 2; individual patient data illustrated in Supplementary Fig. 3), with the highest concentration tested (100 nM) having less inhibitory effects on IFNγ and IL-2 release in cells from moderate compared to mild asthma patients ($p < 0.05$ for both cytokines; Fig. 3), while the effect of 17-BMP on IL-17 production was similar in the two groups. In cells from mild asthma patients, 17-BMP had a greater effect on IFNγ than IL-17 (Supplementary Fig. 2). In moderate asthma patients, 17-BMP had a similar effect on all three cytokines.

CHF6001 had a lower $IC_{50}$ value than 17-BMP in relation to IL-2 and IFNγ release in cells from both mild and moderate asthma patients (Fig. 4), with significantly greater effects on both cytokines at 0.1–10 nM in cells from mild asthma patients and at all concentrations tested in cells from moderate asthma patients. 17-BMP had greater effects than CHF6001 on IL-17 secretion from mild asthma cells at 10 and 100 nM ($p < 0.001$ for both concentrations), while there was no difference in moderate asthma cells. $IC_{50}$ and $EC_{50}$ values for both CHF6001 and 17-BMP are summarised in Table 2.

Due to the smaller data sets, IL-13 results for mild and moderate asthma were combined to allow comparisons between CHF6001 and 17-BMP (see Supplementary Fig. 4). CHF6001 had limited effects on IL-13 secretion, while over 60% inhibition was observed with 100 nM 17-BMP.

In the experiments described above, a T-cell specific stimulant was used to initiate inflammatory responses in a mixed BAL cell population. To demonstrate that CHF6001 had a direct effect on TCR stimulation, T-cells were isolated from the blood of six moderate asthma patients. In these cells, 100 nM CHF6001 reduced IFNγ and IL-2 levels by 43% (p = 0.012) and 54% (p = 0.003), respectively.

## 4. Discussion

PDE4 inhibition with CHF6001 suppressed TCR-stimulated IFNγ, IL-2 and IL-17 release in BAL cells from both mild and moderate asthma patients. Furthermore, the effect of CHF6001 on IFNγ and IL-2 was greater than the corticosteroid 17-BMP. These results provide evidence of the anti-inflammatory potential of CHF6001 on lung cells from patients with asthma.

T-cell receptor induced activation and proliferation of T-cells is enhanced by a range of co-stimulatory receptors, the most prominent being CD28. Stimulation of CD28 activates PDE4, with the resulting hydrolysis of cAMP increasing NF-κB, AP-1 and NFAT induced pro-inflammatory responses [17]. CHF6001 and other PDE4 inhibitors have previously been shown to inhibit cytokine release in TCR-stimulated blood lymphocytes [9,18,19]. Lung lymphocytes from patients with obstructive lung disease differ from cells in the peripheral circulation, and so the magnitude of drug effect is likely to differ between lymphocytes from different anatomical sites. We have previously shown anti-inflammatory effects of PDE4 inhibitors on CD8(+) cells from COPD patients [14]. The current study now demonstrates that CHF6001 inhibits lung lymphocyte cytokine production in asthma patients, with approximately 80% inhibition observed for IL-2 and IFNγ. Such effects occurred at low nanomolar/subnanomolar concentrations, a finding consistent with the on-target potency of CHF6001 [9].

IL-17 may play a role in airway neutrophilia [20] that is insensitive to corticosteroid treatment. The effect of CHF6001 on IL-17 from BAL cells was modest, with only 29% inhibition in moderate asthma cells. This suggests that IL-17 production in these cells is less dependent on cAMP signalling compared to, for example, IL-2 and IFNγ.

IL-13 plays a key role in mucous hypersecretion and fibrosis [21] and IL-13-dependent genes, such as periostin and serpinB2, have been used as biomarkers of Type-2 inflammation in asthma [22]. Although IL-13 production was not inhibited by CHF6001, these results should be interpreted with caution, as there were fewer samples in this analysis and data from mild and moderate asthma patients were pooled. Furthermore, other cell types such as bronchial epithelia are known to be important sources of IL-13 production [23]. IL-4 is another important Th2 cytokine in asthma biology [24], however it was not assessed in this study because 24 h TCR-stimulation is not optimal for IL-4 production [25]. As CHF6001 may affect IL-13 and IL-4 differently, the kinetics of TCR-induced IL-4 production should be taken into account for future in vitro PDE4 inhibitor studies.

A recent study in patients with mild asthma showed that CHF6001 inhibited the late asthmatic response following allergen challenge [26]. This suggests that CHF6001 does inhibit human allergic airway inflammation through inhibition of pathways that trigger inflammation such as eosinophilic recruitment, where a non-significant trend to inhibition was observed. It has been reported that CHF6001 inhibits allergen-induced eosinophilia in rats, providing further evidence for the effects of this drug on type-2 inflammation in asthma [8].

We have previously shown that BAL cells from more severe asthma patients are less responsive to corticosteroids compared to those from mild patients [27,28]. This study reconfirms this result in a new cohort

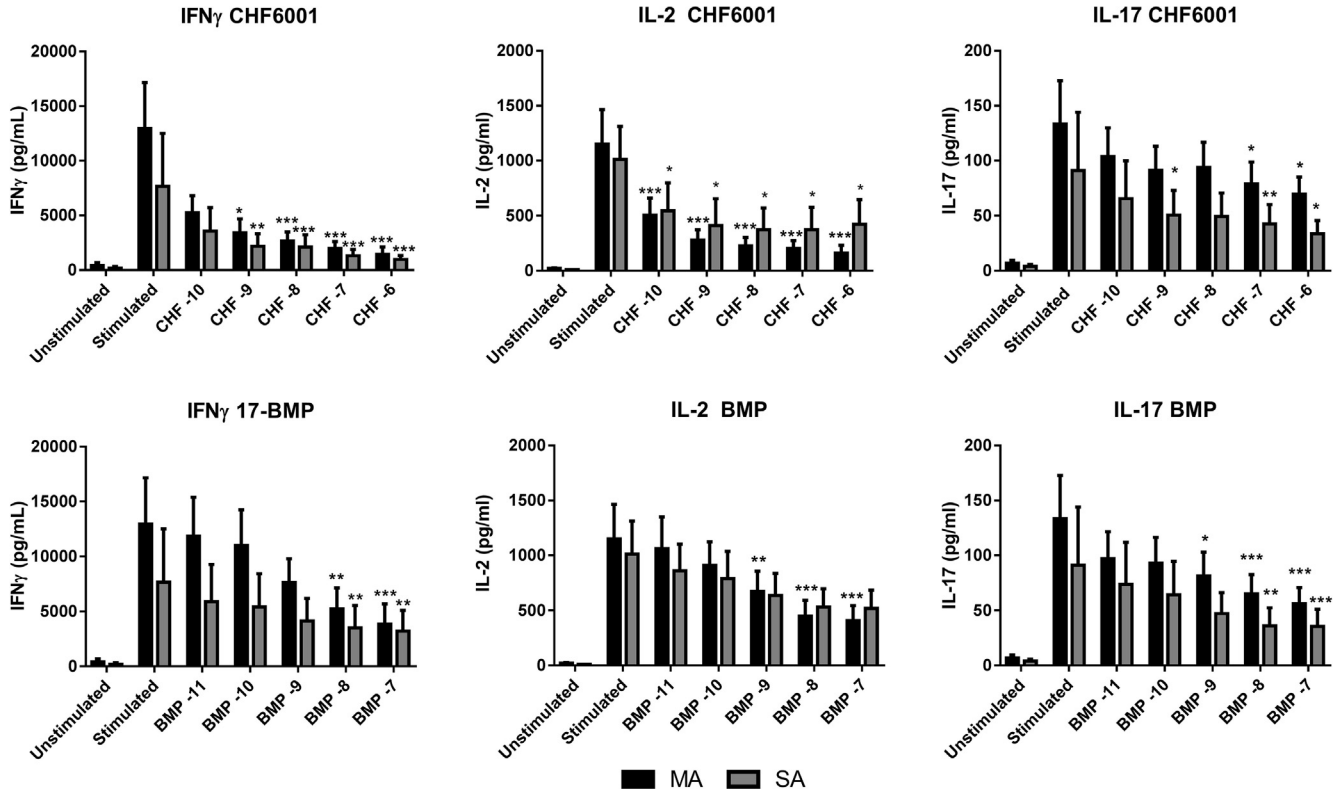


**Fig. 2.** The Effects of CHF6001 and 17-BMP on TCR-stimulated cytokine release in BAL cells from mild (n = 12) and moderate (n = 10) patients. BAL cells were treated with CHF6001 or 17-BMP for 1 h prior to TCR stimulation. IFNγ, IL-2 and IL-17 levels were measured by ELISA. CHF6001 and 17-BMP effects were analysed by 1-way ANOVA with Dunnett's posthoc test against the no drug stimulated control: *p < 0.05; **/## p < 0.01; ***/### p < 0.001.

of patients. Changes in T-cell numbers are probably not the reason for these differences in corticosteroid sensitivity. Although we did not have enough BAL differential cell counts from this study to robustly analyse differences between asthma groups, we have looked at other bronchoscopy data from our laboratory [29]; analysis of historically collected BAL differential cell counts from 42 mild and 23 moderate to severe asthma patients showed no difference in lymphocyte proportions between the two groups (median lymphocyte percentage: mild = 1.6%; moderate = 1.5%, p = 0.51).

Corticosteroid insensitivity has been reported in blood T-cells from corticosteroid resistant compared to corticosteroid sensitive asthma patients [30,31], which may be due to increased levels of the negative glucocorticoid receptor (GR) regulator GRβ in corticosteroid resistant asthma patients [30,32]. Other possible mechanisms for corticosteroid insensitivity in asthma have been proposed, including decreased function of HDAC2 causing decreased GR function [33]. There is a need to further investigate mechanisms of corticosteroid sensitivity in airway lymphocytes from asthma patients. The isolation of lymphocytes from BAL would be ideal for this purpose, but previous attempts to extract sufficient numbers of these cells from BAL has proved difficult [28].

Unlike 17-BMP, CHF6001 had a similar effect in cells from both mild and moderate asthma patients. Furthermore, CHF6001 had a greater effect than 17-BMP on BAL cell IFNγ and IL-2 secretion. Th1 inflammation, measured by increased airway IFNγ levels, occurs in 50% of severe asthma patients despite high dose corticosteroid treatment [34]. Oral corticosteroid usage and emergency department visits are increased in this IFNγ-high population [35]. Animal asthma models have shown that IFNγ activation of macrophage induces prolonged and corticosteroid insensitive airway hyper-responsiveness [36] and *in vitro* studies using human airway cells have demonstrated that IFNγ-induced inflammatory responses are corticosteroid-insensitive [37–39]. A small study in psoriasis has recently shown that IFNγ-producing lymphocyte levels were reduced following 6 months PDE4 treatment [40]. These studies provide a rationale for targeting IFNγ-high asthma patients with PDE4 inhibitors.

Although the sample size for this study was modest, the number of subjects recruited was typical for bronchoscopy studies investigating the pharmacological effects of drugs [28,41]. The limited cell numbers obtained by bronchoscopy means that it was not possible to measure a wide range of inflammatory mediators.

In conclusion, the main finding of this study is that the PDE4 inhibitor CHF6001 had a greater effect than corticosteroids on the Th1 response after TCR-stimulation of BAL cells in asthma. This pharmacological effect suggests the therapeutic potential for PDE4 inhibitors to be used in the subset of asthma patients with increased airway levels of IFNγ.

## 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; REC Ref: 06/Q1403/156) 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.

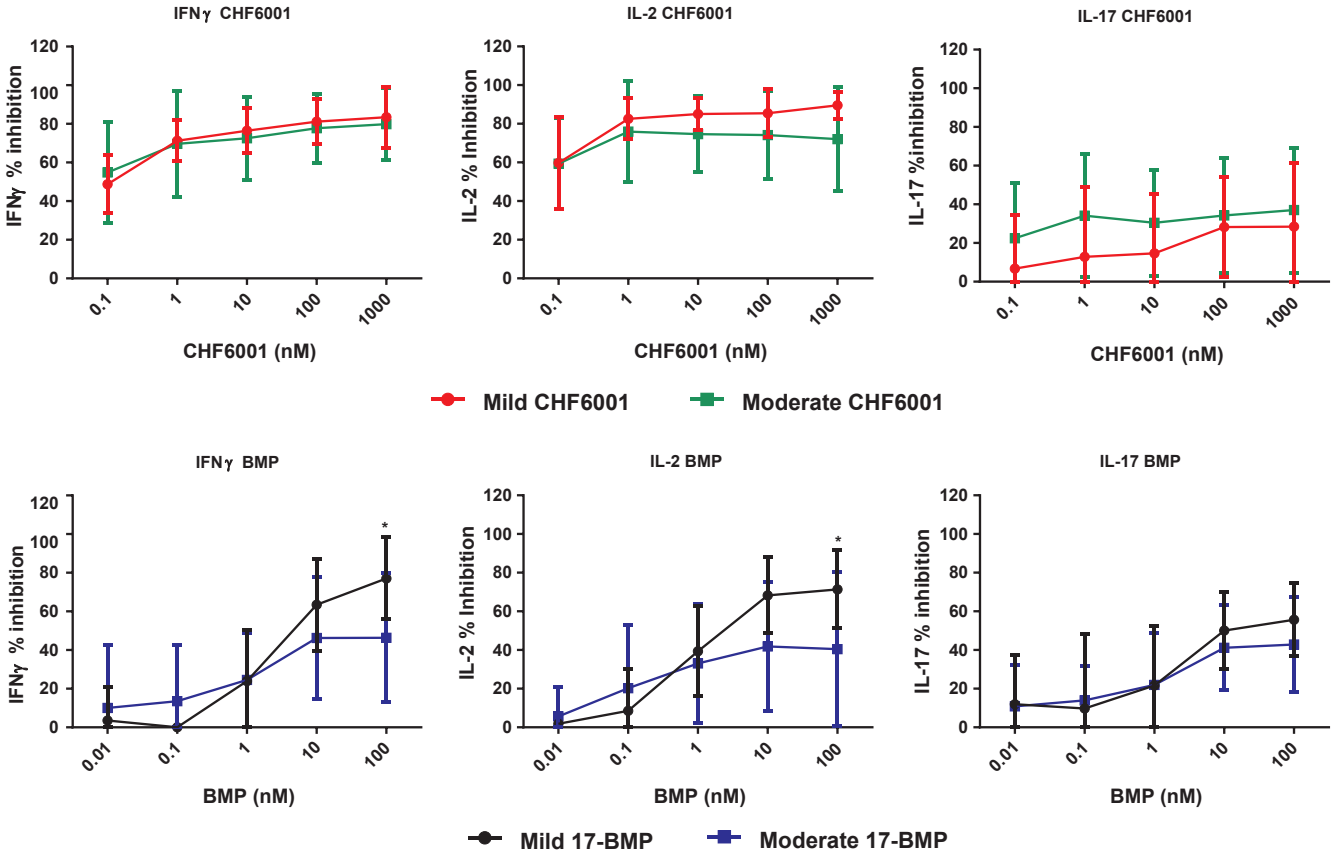


**Fig. 3.** Comparison of the effects of CHF6001 and 17-BMP between mild (n = 12) and moderate (n = 10) asthma cells. BAL cells were treated with CHF6001 or 17-BMP for 1 h prior to TCR stimulation. Cytokine levels were measured by ELISA. Comparisons between SNA and M-SA were by 2-way ANOVA with Sidak posthoc test between match drug concentrations: *p < 0.05.

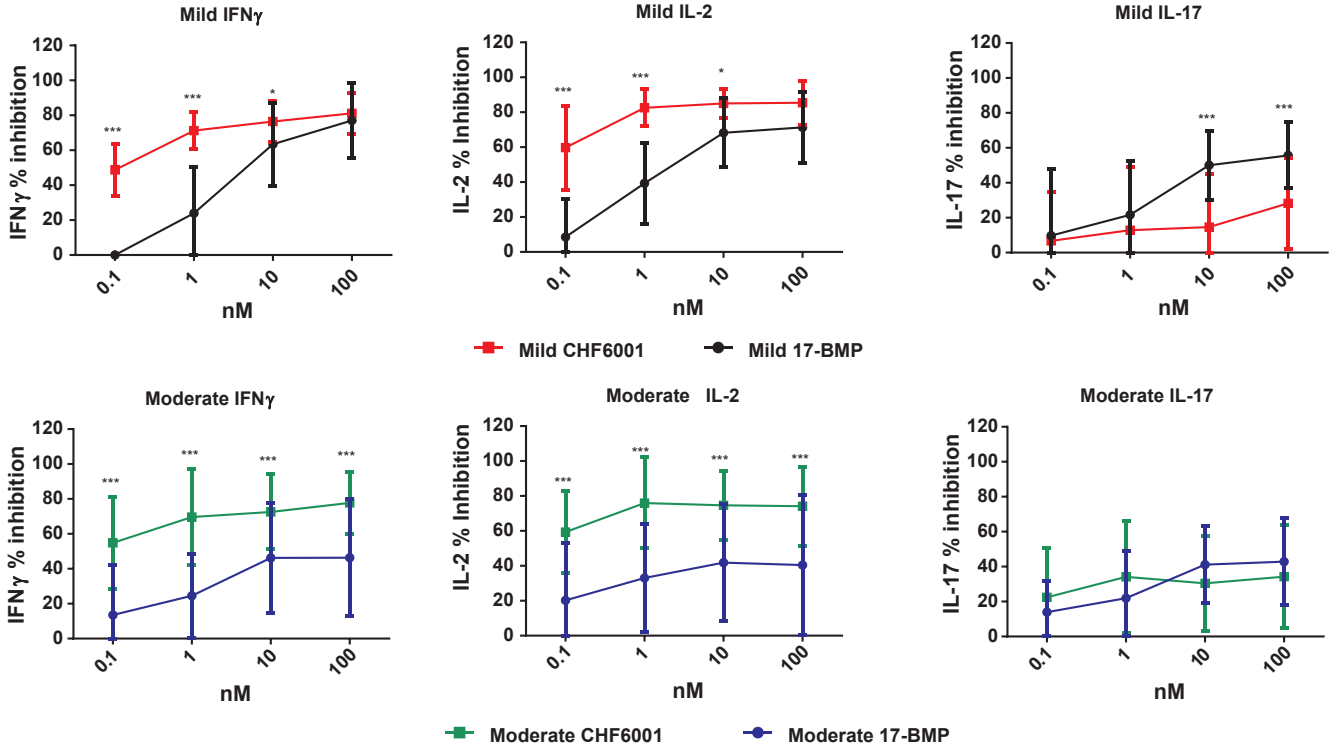


**Fig. 4.** Comparison between the effects for CHF6001 and 17-BMP. BAL cells from mild (n = 12) and moderate (n = 10) asthma were treated with CHF6001 or 17-BMP for 1 h prior to TCR stimulation. Cytokine levels were measured by ELISA. Comparisons between CHF6001 and 17-BMP were by 2-way ANOVA with Sidak posthoc test between matching drug concentrations: *p < 0.05; **p < 0.01; ***p < 0.001.

**Table 2**
$IC_{50}$ values for CHF6001 and 17-BMP as calculated from their effects on TCR-induced cytokine release from BAL cells. Values are in nM concentrations.

| BAL Cell Data | | Mild | Moderate |
|---|---|---|---|
| IFNγ | CHF6001 | 0.107 | 0.054 |
| | 17-BMP | 4.01 | > 100 |
| IL-2 | CHF6001 | 0.065 | < 0.1 |
| | 17-BMP | 1.780 | > 100 |
| IL-17 | CHF6001 | > 1000 | < 0.1 |
| | 17-BMP | 9.732 | > 100 |

### 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. MK, FF, GV and MC are employees of Chiesi Farmaceutici S.p.A. TS and LH declare that they have no competing interests.

### 5.4. Funding

The authors would like to acknowledge the North West Lung Centre Charity, Manchester, UK and Chiesi Farmaceutici S.p.A, Italy for financially supporting this project.

### 5.5. Authors' contributions

TS, MK, FF, GV, MC and DS all made substantial contributions to the conception and design of the study. TS, MK and LH were involved in the acquisition of data. All authors were involved in the analysis and interpretation of the results. TS drafted the article, with all other authors revising it critically for important intellectual content. All authors approved the final version of the manuscript prior to submission.

## Acknowledgements

This report is independent research supported by National Institute for Health Research South Manchester Respiratory and Allergy Clinical Research Facility at Manchester University NHS Foundation Trust (South). The views expressed in this publication are those of the author (s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health. In addition we would like to thank all study participants for their contribution.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.06.007.

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