Journal of Cystic Fibrosis 19 (2020) 823–829

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

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

Original Article

Anti-inflammatory effects of lenabasum, a cannabinoid receptor type 2 agonist, on macrophages from cystic fibrosis

Abdullah A. Tarique[a], Tama Evron[b], George Zhang[b], Mark A. Tepper[b], Mohammed M. Morshed[a], Isabella S.G. Andersen[a], Nelufa Begum[a], Peter D. Sly[a,*], Emmanuelle Fantino[a]

[a] *Child Health Research Centre (CHRC), The University of Queensland, Brisbane, Australia*
[b] *Corbus Pharmaceuticals, Inc., Norwood, MA, USA*

ARTICLE INFO

Article history:
Received 16 December 2019
Revised 12 February 2020
Accepted 24 March 2020
Available online 6 May 2020

Keywords:
Cystic fibrosis
Macrophage responses
Macrophage polarization
Cannabinoid 2 receptor (CB2)
CB2 agonists
Phagocytosis
Endocytosis
Anti-inflammatory

ABSTRACT

Background: Lenabasum is an oral synthetic cannabinoid receptor type 2 agonist previously shown to reduce the production of key airway pro-inflammatory cytokines known to play a role in cystic fibrosis (CF). In a double-blinded, randomized, placebo-control phase 2 study, lenabasum lowered the rate of pulmonary exacerbation among patients with CF. The present study was undertaken to investigate anti-inflammatory mechanisms of lenabasum exhibits in CF macrophages.

Methods: We used monocyte-derived macrophages (MDMs) from healthy donors ($n = 15$), MDMs with CFTR inhibited with C-172 ($n = 5$) and MDMs from patients with CF ($n = 4$). Monocytes were differentiated to macrophages and polarized into classically activated (M1) macrophages by LPS or alternatively activated (M2) macrophages by IL-13 in presence or absence of lenabasum.

Results: Lenabasum had no effect on differentiation, polarization and function of macrophages from healthy individuals. However, in CF macrophages lenabasum downregulated macrophage polarization into the pro-inflammatory M1 phenotype and secretion of the pro-inflammatory cytokines IL-8 and TNF-α in a dose-dependent manner. An improvement in phagocytic activity was also observed following lenabasum treatment. Although lenabasum did not restore the impaired polarization of anti-inflammatory M2 macrophage, it reduced the levels of IL-13 and enhanced the endocytic function of CF MDMs. The effects of lenabasum on MDMs with CFTR inhibited by C-172 were not as obvious.

Conclusion: In CF macrophages lenabasum modulates macrophage polarization and function *in vitro* in a way that would reduce inflammation *in vivo*. Further studies are warranted to determine the link between activating the CBR2 receptor and CFTR.

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1. Introduction

Progressive destructive inflammation is the hallmark of cystic fibrosis (CF) lung disease; with an exaggerated neutrophilic response to infective and/or inflammatory stimuli [1–3] a major factor in the onset and progression of bronchiectasis [4–6]. Neutrophils, airway epithelial cells and macrophages are the key players in CF lung inflammation. During pulmonary infection, neutrophils infiltrate first, followed by circulating monocytes that mature into inflammatory monocyte-derived macrophages (MDMs) [7]. Macrophages are known to play crucial roles in initiation and resolution of pulmonary inflammation via pro-(M1) or anti- (M2) inflammatory phenotypes, respectively [8,9]. At the early stage of inflammation, the M1 macrophage phenotype activated via the classical pathway promotes inflammation by releasing pro-inflammatory mediators, such as, TNF-α, IL-6, IL-8 and engages in killing and clearance of bacteria by phagocytosis [10]. Later in the inflammatory process, inflammation resolving M2 macrophages release anti-inflammatory cytokines, such as, IL-13, CCL17, CCL18 and clear apoptotic cells by endocytosis [10]. A dynamic equilibrium of M1 and M2 macrophages is therefore required to respond during inflammatory condition and to maintain tissue homeostasis. We have previously shown specific defects in CF MDMs that favour an exaggerated inflammatory responses and limit inflammation resolution [11].

Cannabinoids are the pharmacologically active components of *Cannabis sativa.* They bind to cannabinoid (CB) receptors, CB

* Corresponding author.
E-mail address: p.sly@uq.edu.au (P.D. Sly).

https://doi.org/10.1016/j.jcf.2020.03.015
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receptor 1 (CB1) and CB receptor 2 (CB2) to regulate various physiological processes, such as, pain control and inflammation [12]. CB1 is expressed predominantly by the cells in the central nervous system (CNS), whereas CB2 is preferentially expressed on immune cells (such as, T cells and macrophages) or structural cells (such as fibroblasts, muscle cells and endothelial cells) [13,14]. Lenabasum (previously known as anabassum or JBT-101, AJA, CT-3) is a synthetic cannabinoid that selectively binds as an agonist to CB2 and has no psychotropic effects [15]. Lenabasum promotes resolution of neutrophilic inflammation by inhibiting pro-inflammatory cytokine secretion (*i.e.* IL-6, MMP-3, CCL2) and by suppressing neutrophil migration in both animal and human models of colitis and rheumatoid arthritis [13,16,17]. In a human blister model, lenabasum enhanced release of pro-resolving lipid mediators including lipoxins (LXA_4, LXB_4) and resolvin D [17]. In a double-blinded, randomized, placebo-controlled phase 2 study, lenabasum demonstrated safety, tolerability and lowered the rate of pulmonary exacerbation in patients with CF regardless of CFTR mutation or colonizing bacteria [18]. However, the cellular mechanism of lenabasum in CF remains unknown.

The present study was undertaken to investigate the effects of lenabasum on differentiation and polarization of macrophages under normal conditions and in the absence of functional CFTR. We hypothesized that lenabasum would ameliorate macrophage-driven inflammation in CF in a CFTR mutation-independent fashion. We used our recently developed *ex vivo* model where peripheral blood monocytes were differentiated into unpolarized M0 macrophages, then polarized into the pro-inflammatory M1 or inflammation-resolving M2 phenotypes [10]. By using both healthy MDMs, MDMs with CFTR function inhibited, and MDMs obtained from patients with CF, we herein report that lenabasum down-regulated pro-inflammatory macrophage polarization and inflammatory mediator release in MDMs without functional CFTR. Although lenabasum did not induce macrophage polarization into the anti-inflammatory (M2) phenotype, it enhanced phagocytosis and endocytosis in MDMs without functional CFTR.

2. Methods

2.1. Study approval

The study was approved by the Queensland Children's Hospital (QCH) human ethics committee and the University of Queensland (HREC16/QRCH/379). All parents of children with CF gave written consent for their children to participate in this study.

2.2. Study participants

Buffy coats from healthy individuals ($n = 15$) aged 20–40 years were obtained from the Australian Red Cross Blood Service. Children with CF, aged 9–17 years, carrying at least one F508del allele were recruited from the CF clinic at QCH, Brisbane, Australia. At least half of the children had previous infection with *P. aeruginosa*. We previously observed no difference in macrophage function between adults and children [11]. Peripheral blood was collected during an annual review when the children were clinically stable.

2.3. Lenabasum

Lenabasum, supplied by the Corbus pharmaceutical (US) as powder, was initially dissolved in DMSO at 10 mM concentration and aliquots stored at −80°C. Dose responses of lenabasum on macrophage differentiation were carried out using 0.1, 0.3, 1, 3, 10 and 30μM of lenabasum. Trypan blue and 7-AAD staining followed by flow cytometry were performed to identify and count live cells following lenabasum treatment. 7-AAD is a DNA binding fluorescent dye that only stains dead cells.

2.4. Ex vivo *macrophage differentiation and polarization*

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or peripheral blood of patients with CF by density gradient centrifugation using lymphoprep (AxisShiled, UK). PBMCs were washed twice with phosphate-buffered saline (PBS) to remove serum and platelets. Monocytes were enriched by positive selection using $CD14^+$ magnetic microbeads following manufacturer's instructions (Miltenyi, Germany). Macrophage differentiation and polarization were performed as previously described [10]. Briefly, $CD14^+$ monocytes were differentiated into unpolarised macrophages (M0s) by 6-day stimulation with rhGM-CSF (50 ng/ml, Miltenyi, Germany) in RPMI-1640 supplemented with 10% heat inactivated FBS (LifeTech, USA), 1% Penicillin-streptomycin-amphotericin B (Lonza, USA). Medium was refreshed on day 3 with GM-CSF (50 ng/ml). M1 or M2 polarization was induced by *E. coli* LPS (20 ng/ml, Sigma) or rhIL-13 (20 ng/ml, ThermoFisher), respectively, for 2 days, respectively (Fig. 1). Lenabasum was added to the culture medium during macrophage differentiation (day 0 and 3) and before polarization (day 6).

2.5. CFTR inhibition

$CFTR_{inh}$-172 (3- [(3-trifluoromethyl) phenyl] – 5 - [(4-carboxyphenyl)methylene] – 2 – thioxo – 4 -thiazolidinone) (C-172 hereafter) is a potent inhibitor of CFTR chloride (Cl^-) channel function. $CFTR_{inh}$-172 targets CFTR protein and blocks Cl^-transport in epithelial cells as well as macrophages [19,20]. Monocytes from healthy donors were exposed to 10μM of C-172 (Sigma) during macrophage differentiation and polarization [11]. 10μM of C-172, sufficient to inhibit CFTR channel activity, was added every 24h in the culture medium [21,22].

2.6. Flow cytometry for analysing surface expression of receptors/markers

For flow cytometric analysis, cells were harvested by using TrypLE (Invitrogen, US), washed with PBS and incubated with Fc blocking solution (FBS,2%-BSA,0.1%-PBS). Fully mature (M0) macrophages were analysed for the surface expression of TLR4 and IL-13Rα1, essential for M1 and M2 polarization, respectively. After 2-day polarization, the percentage of $CD80^+$ M1 macrophages and that of $CD209^+$ M2 macrophages were analysed by using anti-human CD80 PE (BioLegend, US) and anti-human CD209 BB515 (BD, US). Corresponding isotype controls were included in every flow cytometry analysis. Data were acquired using BD LSR-Fortessa. 7-AAD (BD Biosciences, US) staining was performed to gate out dead cells. All analyses were performed on CD68+ cells on Flowjo (Tree Star, US).

2.7. Phagocytosis and endocytosis

Polarized human M1 and M2 macrophages are functionally phagocytic and endocytic, respectively [10]. Phagocytosis was determined by incubating M1 macrophages with pHrodo green *E. coli* bioparticles (LifeTech, US) at 37°C for 90 min. pHrodo dye fluoresces only in acidic environments, which mimic the phagolysosome *in vivo*. Macrophages those fluoresces were acquired on BD Fortessa. Endocytosis was determined by incubating M2 macrophages with AF647-labelled dextran (LifeTech, US) at 37°C for 90 min. Cells were then washed and acquired on BD Fortessa. Median fluorescence intensities (MFIs) were normalized to MFI of cells without bacteria or dextran (background) on Flowjo.

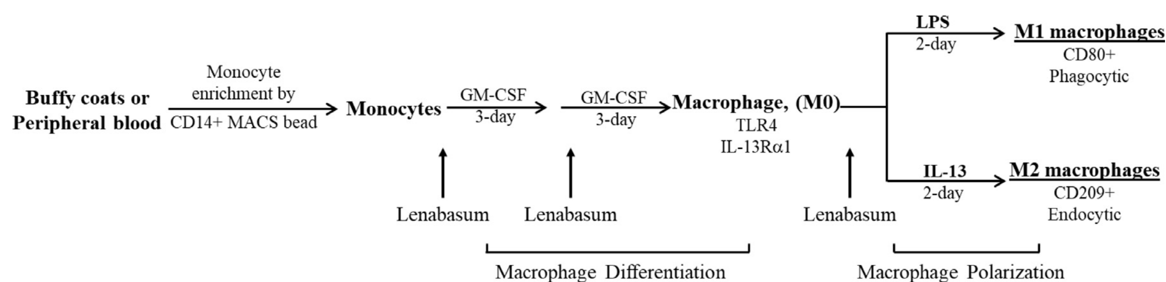

Fig. 1. Schematic representation of macrophage differentiation and polarization in the presence or absence of lenabasum [10]. Human CD14+ monocytes were differentiated into mature and unpolarised macrophages (M0) by rhGM-CSF (50 ng/ml). M0 macrophages were then polarized into classically-activated (M1) or alternatively-activated (M2) macrophages using *E. coli* LPS (20 ng/ml) and IL-13 (20 ng/ml) for 2 days, respectively. Lenabasum was added during differentiation (day 0, 3) and during polarization (day 6).

Phagocytic or endocytic index was calculated by normalizing corresponding MFI with either CD80+ or CD209+ cells respectively and expressed as percentage as previously reported by Hodge et al. [23].

2.8. Inflammatory mediators quantification

IL-6, IL-8, TNF-α and IL-13 were quantified in cell-free culture supernatant from M1 or M2 macrophages by alphaLISA (Perkin Elmer, US) following manufacturer instructions. The detection range of these assay kits was 4–30,000pg/ml.

2.9. Statistical analysis

Non-parametric hypothesis tests were performed to determine the statistical difference in inflammatory outcome variables among groups. For statistical comparison, the Wilcoxon rank-sum (Mann-Whitney) test was used for two groups and the Kruskal-Wallis rank test used for more than two groups. For more than two groups when the overall significance was observed, Dunn's multiple-comparison using a Bonferroni correction was used to determine pair-wise significance. Statistical significance was determined at the 0.05 level. Data are presented as median (25th-75th percentile) unless stated otherwise. All analyses were performed using GraphPad 7 (San Diego, CA).

3. Results

3.1. Lenebasum cytotoxicity

Cytotoxicity of lenabasum was tested by exposing monocytes from healthy individuals to increasing concentrations (0.1, 0.3, 1, 3, 10 and 30μM) of lenabasum during macrophage differentiation. Light microscopic observation revealed that cells treated with 30μM of lenabasum were dead by day-3. Cells treated with 10μM of lenabasum were comparatively smaller in size than vehicle control cells observed under light microscopy. In contrast, over 95% of the cells treated with $\leq$3μM of lenabasum were alive and appeared healthy. Flow cytometric analysis showed no significant difference between vehicle control cells and cells treated with $\leq$3μM of lenabasum following 7-AAD staining suggesting that 3μM of lenabasum was tolerated by the cells. A low (0.3µM) and a moderate dose (3µM) of lenabasum were chosen for subsequent experiments.

3.2. Minimal effect of lenabasum on the receptors required for macrophage polarization

Lenabasum treatment of MDMs derived from healthy individuals showed an overall effect on surface expression of TLR4 (p 0.0001) and IL-13Rα1 (p 0.02), however, there was no consistence dose-response effect when pairwise comparison was performed, suggesting that this was not a biologically significant effect (Fig. 2).

3.3. Lenabasum reduced M1 macrophage polarization and enhanced phagocytosis in the absence of functional CFTR

Lenabasum treatment of macrophages from healthy individuals did not influence the proportion of CD80+ M1 macrophages (Fig. 3A) or their phagocytic ability (Fig. 3B). When monocytes obtained from children with CF were used for macrophages differentiation and polarization, lenabasum 3 μM dose led a significant reduction in the percentage of CD80+ M1 macrophages compared to CF macrophages that didn't receive lenabasum (p 0.01) (Fig. 3A). When CFTR channel activity was physically inhibited by CFTR inhibitor (C-172), a decreasing trend in the proportion of CD80+ M1 macrophages was observed with lenabasum.

Phagocytic activity is defective in CF macrophages [11,19]. Lenabasum didn't show any effect on the phagocytic activity of LPS-stimulated M1 macrophages from healthy individuals or when CFTR channel activity was physically inhibited by C-172. However, phagocytic activity of CF macrophages was significantly increased improved following lenabasum treatment (p 0.0003) (Fig. 3B).

3.4. Lenabasum enhanced endocytosis without any increase in the proportion of CD209+ M2 macrophages in CF

Lenabasum showed no effect on the percentage of CD209+ M2 macrophages or their endocytic ability of MDMs from healthy individuals or when CFTR channel activity was physically inhibited by C-172 (Fig. 4A). The percentage of CD209+ M2 macrophages was neither restored nor improved when monocytes from children with CF were used. However, a significant increase in endocytic function of CF macrophages was observed with lenabasum (p 0.004) (Fig. 4B).

3.5. Lenabasum downregulated pro-inflammatory cytokine release from CF macrophages

Lenabasum showed no effect on cytokine release from macrophages from healthy individuals. However, a significantly reduction in the release of IL-8, TNF-α and IL-13 was observed significantly lowered in CF macrophages as well C-172 treated macrophages (Fig. 5) following lenabasum treatment. A decreasing trend was also observed on IL-6 release by CF MDMs though not significant (Fig. 5C).

4. Discussion

Data from the present study show that lenabasum has the potential to reduce macrophage-driven inflammation in CF. Lenaba-

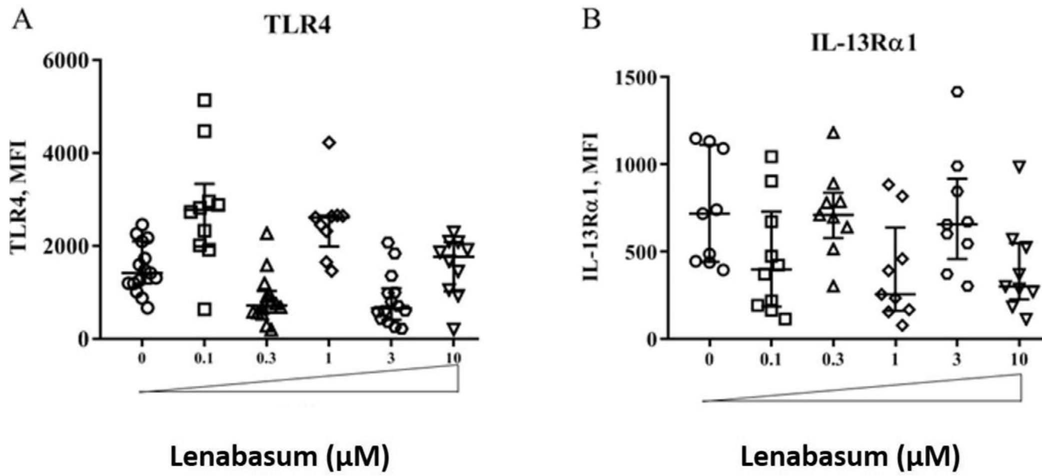

Fig. 2. Effects of lenabasum on the receptors required for M1 and M2 macrophage polarization. Monocytes from healthy individuals were stimulated with GM-CSF for 6-days to differentiate into unpolarised M0 macrophages in presence or absence of lenabasum. Surface expression of TLR4 (A) and IL-13Rα1 (B), required for M1 and M2 macrophage polarization respectively was measured by flow cytometry. Median fluorescence intensity (MFI) was measured using Flowjo. Each dot represents an individual data point. Data shown as median (25%, 75%).

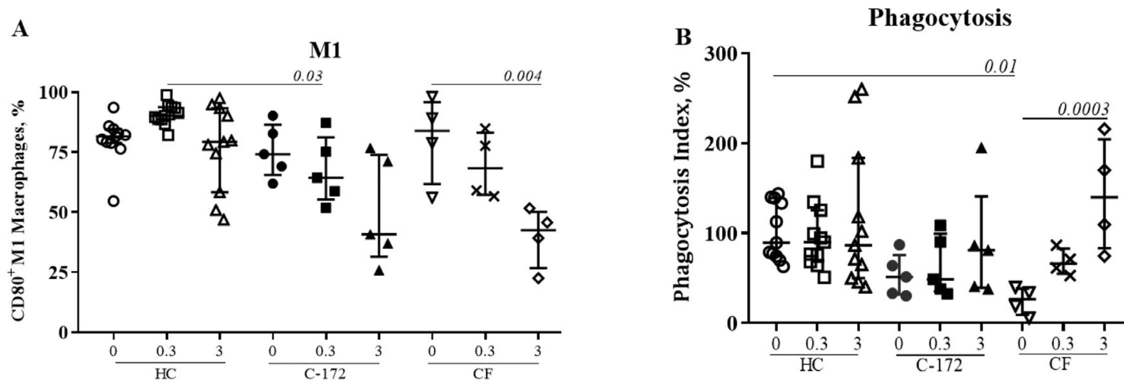

Fig. 3. Effects of lenabasum on M1 macrophage polarization their functions. Monocytes those were isolated from healthy individuals (HC) or from children with CF were stimulated with GM-CSF for 6-days to differentiate them into unpolarized M0 macrophages in presence or absence of lenabasum. Monocytes from HCs were exposed to CFTR inhibitor C-172. M1 polarization was induced by stimulating M0 macrophages using *E. coli* LPS (20 ng/ml). Population frequencies of CD80^{+} M1 (A) and their phagocytic ability (B) were assessed by flow cytometry. Phagocytosis of polarized M1 macrophages was analyzed by using pHrodo label *E. coli* bioparticle. Phagocytic index was calculated by normalizing the MFI with the number of CD80^{+} M1 macrophages. Each symbol represents an individual donor. Concentrations of lenabasum used were 0 (vehicle), 0.3 μM and 3.0 μM. Data are shown as median (25%, 75%).

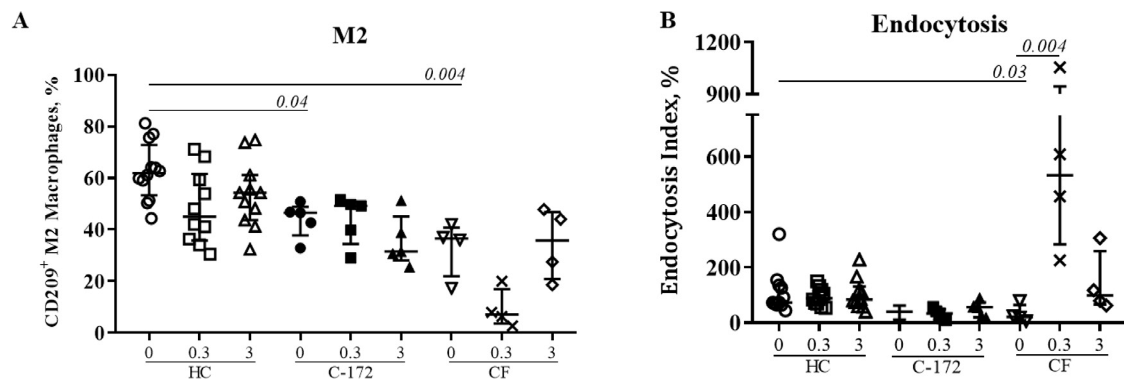

Fig. 4. Effects of lenabasum on M2 macrophage polarization their functions. Monocytes those were isolated from healthy individuals (HC) or from children with CF were stimulated with GM-CSF for 6-days to differentiate them into unpolarized M0 macrophages in presence or absence of lenabasum. Monocytes from HCs were exposed to CFTR inhibitor C-172. M2 polarization was induced by stimulating M0 macrophages using rhIL-13 (20 ng/ml). Population frequencies of CD209^{+} M2 (A) and their endocytic ability (B) were assessed by flow cytometry. Endocytosis of polarized M2 macrophages was analyzed by using AF647-labelled dextran (10KD). Endocytic index was calculated by normalizing the MFI with the number of CD209^{+} M2 macrophages. Each dot represents an individual donor. Concentrations of lenabasum used were 0 (vehicle), 0.3μM and 3.0μM. Data are shown as median (25%, 75%).

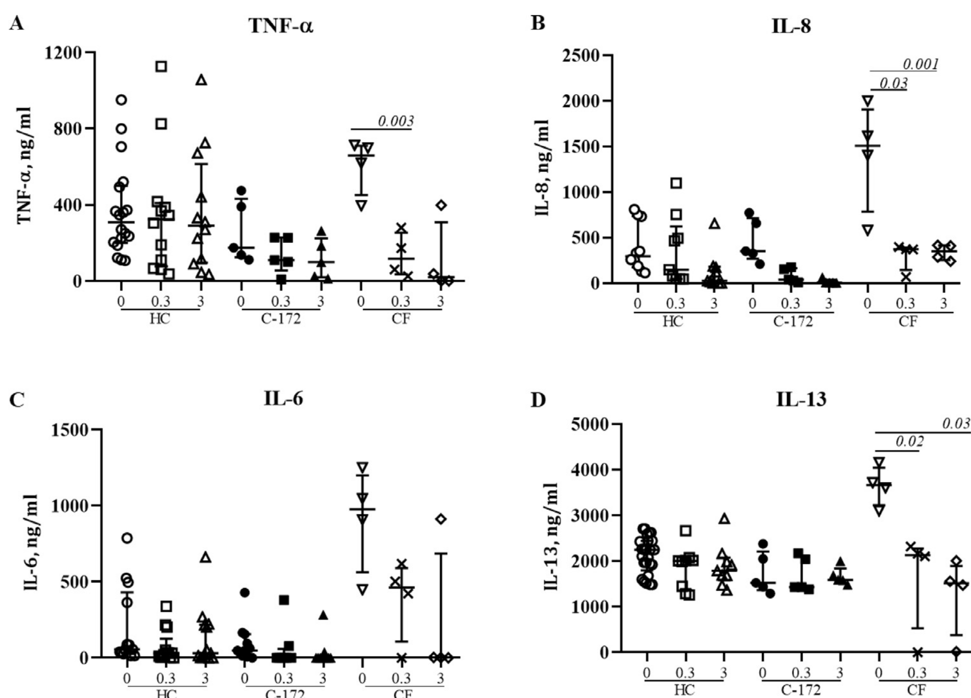

Fig. 5. Lenabasum downregulates inflammatory cytokines release by CF macrophages. Monocytes from healthy individuals (HCs) and patients with CF were differentiated to M0 macrophages and then induced to M1 and M2 polarization with LPS and IL-13 respectively in presence or absence lenabasum. Monocytes from HCs were exposed to CFTR inhibitor C-172. Release of TNF-α (A), IL-8 (B), IL-6 (C) by M1 macrophages and that of IL-13 (D) from M2 macrophages were quantified in cell free supernatant. Concentrations of lenabasum denoted as 0 (vehicle control), 0.3µM and 3.0µM. Each dot represents an independent donor. Data are shown as median (25%, 75%).

sum treatment led to a significant decrease in pro-inflammatory classical activation of macrophages and reduced release of inflammatory cytokines including IL-6, IL-8 TNF-α. However, lenabasum was unable to restore previously reported defective anti-inflammatory alternative activation of macrophage [11]. In addition, phagocytic activity of M1 (classical) and endocytic activity of M2 (alternative) macrophages were increased following lenabasum treatment of CF macrophages. The actions of lenabasum on macrophages with CFTR function inhibited by C-172 were less clear, questioning whether the improvements in CF macrophages were related to a CFTR-dependent mechanism. The anti-inflammatory actions of lenabasum was not seen in macrophages from healthy individuals, with functional CFTR.

Lenabasum has previously been reported as an anti-inflammatory agent that attenuates neutrophil migration and promotes resolution of inflammation in both animal and human models of skin blister, colitis, rheumatoid arthritis, systemic sclerosis and dermatomyositis [13,16,17,24–26]. Safety and tolerability of lenabasum was tested in a 16-week double blind, randomized and placebo-controlled study with 85 patients with CF patients with various CFTR mutations. Lenabasum or placebo was administered orally, initially 5 mg once daily for 4 weeks followed by 20 mg twice daily for a further 8 weeks. Clinical monitoring continued for 4 weeks after treatment cessation. Patients were then monitored for next 4 weeks for pulmonary exacerbation. Patients who received lenabasum had fewer acute pulmonary exacerbations during the study period [18]. No assessment of anti-inflammatory actions / effects were undertaken in this study.

The present study provides some insight into the cellular mechanism by which lenabasum blunts inflammatory responses of macrophages in CF. Lenabasum did not influence macrophage differentiation, polarization or function in healthy subjects, nor surface expression of TLR4. Anti-inflammatory effects of lenabasum were primarily observed in MDMs from patients with CF where functional CFTR was absent. Unlike our previous study [11] inhibiting CFTR function with the CFTR ion channel inhibitor C-172, the effects of lenabasum was less clear on these inhibited cells. In CF MDMs, lenabasum reduced the proportion of M0 macrophages polarizing into the M1 phenotype under the influence of LPS. The release of inflammatory mediators (IL-6, IL-8, TNF-α) in response to LPS was also significantly decreased by lenabasum. These findings are consistent with the reports from other studies using CB2 agonists. Du et al. recently reported that CB2 agonists attenuated inflammation during skin wound healing by inhibiting M1 macrophages in mice [12]. M1 associated markers and cytokines were significantly downregulated following CB2 agonist treatment. Rat models of neuro-inflammation show that a CB2 agonist down-regulated pro-inflammatory cytokine secretion by inhibiting the NFκB pathway [27]. LPS activates TLR4 signalling cascade which results in NFκB activation via ERK1/2 and IκBα, leading induction of a wide array of inflammatory genes. Phosphorylation of ERK1/2 and IκBα is critical for NFκB activation. Naguib et al. showed attenuated phosphorylation of ERK1/2 and IκBα along with blunted release of TNF-α in macrophages following CB2 agonist treatment [28]. Taken together with our observations, these data suggest that lowered M1 macrophage polarization along with reduced pro-inflammatory cytokine release in CF observed in this study may be due to inhibition of NFκB activation by lenabasum. However, why this occurs predominantly in the absence of CFTR in macrophages is unknown.

Macrophage phagocytosis is critical for pathogen killing and clearance from the tissues. CFTR has a direct link with macrophage phagocytosis [19]. A functional CFTR is required for acidification of phagolysosomal compartment and bacterial killing [19]. Inhibition of CFTR ion channel activity by C-172 also lowered phagocytic activity in macrophages [11]. In accordance to earlier study, we didn't observe any effect of lenabasum on phagocytosis of control and C-172 treated macrophages [29]. Enhancement of phagocytosis following lenabasum treatment was only observed in CF MDMs. This suggests that lenabasum may enhance phagocytosis via a

non-canonical pathway. $Ca^{2}+$ is known as second messenger for variety of cellular process including fusion of phagosomes with granules containing lytic enzymes [30,31]. Cannabinoids had been shown to elevate Ca^{2+} [32]. It is possible that enhanced phagocytosis was due to elevated Ca^{2+} ion because of lenabasum treatment. However, why this should occur in CF but not C-172 treat macrophages is not known.

In agreement with the mouse skin wound model study [12], we didn't observed any effect of lenabasum on polarization of M2 macrophages in any group. CB2 agonists has previously been shown to induce anti-inflammatory responses by upregulating the PI3K/Akt pathway [27,33]. PI3K-Akt signalling pathways are suppressed in CF macrophages [34]. A physical interaction of functional CFTR is required for activation of PI3K/Akt pathway [35]. This might be the underlying reason why anti-inflammatory M2 macrophage polarization was not induced in CF following lenabasum treatment. Viscomi et al. showed enhanced phosphorylation of Akt (p-Akt) following CB2 agonist treatment in cells from an inflammatory milieu, but not in cells under homeostatic conditions [33].

We acknowledge some limitations of our study. We used MDMs rather than alveolar macrophages (AMs). Elegant studies have demonstrated that AMs don't proliferate during infection [36,37], rather MDMs take control to initiate inflammation [38–41]. Following resolution of inflammation, MDMs persist in the lung and acquire transcriptional profiles similar to AMs [42,43]. These data suggest revision of the current consideration of AMs as sole macrophage population in the lungs. We did not study whether lenabasum influenced CFTR channel activity in macrophages. However, the differential effects on CF macrophages with absent CFTR and C-172 treated cells, suggests that lenabasum is not acting via improving CFTR channel function but may be modifying some non-channel CFTR function. Our CF study population was too small to shed light on interactions between lenabasum and specific CFTR mutations. We acknowledge that patients with CF were younger than the adult donors who provided cells for this study. We have previously shown that age was not a critical factor in macrophage function with macrophages from adults and children with CF behaving similarly [11].

In summary, lenabasum showed no effect on macrophage differentiation, polarization or function when monocytes from healthy individuals were used. However, in *ex vivo* differentiated macrophages from CF monocytes, lenabasum exerted its anti-inflammatory effects by reducing inflammatory M1 macrophage polarization and their cytokine secretion. Bacterial killing ability (phagocytosis) was enhanced in CF macrophages following lenabasum treatment. However, the anti-inflammatory M2 macrophage polarization in CF was not restored by lenabasum. Our data along with mouse and human studies related to inflammation [12,13,16,17,24–26] support lenabasum as a potential pharmacological options for patients with CF. An ongoing multicenter, double-blind, placebo-controlled Phase 2B clinical trial is assessing the effect of lenabasum on pulmonary exacerbation in 426 CF patients (NCT03451045).

Funding

The study was funded by Corbus Pharmaceuticals, Norwood, MA, USA in a form of investigator initiated mechanistic study grant.

Acknowledgement

We would like to thank the patients and their families for participating in this study and the two study-cohort coordinators including Joyce Cheney, Careana Moss, Nicholas Gailer, Andrea Beevers, Michelle Wood for enrolment of the participants and collection of blood samples. We would also like to thank the Red Cross Blood Service, Brisbane for providing buffy coats as per request.Conflict of interest:

The study was funded by Corbus Pharmaceuticals, Norwood, MA, USA in a form of investigator initiated mechanistic study grant.

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