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Pharmacological characterisation of anti-inflammatory compounds in acute and chronic mouse models of cigarette smoke-induced inflammation

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Abstract

Background: Candidate compounds being developed to treat chronic obstructive pulmonary disease are typically assessed using either acute or chronic mouse smoking models; however, in both systems compounds have almost always been administered prophylactically. Our aim was to determine whether the prophylactic effects of reference anti-inflammatory compounds in acute mouse smoking models reflected their therapeutic effects in (more clinically relevant) chronic systems.

Methods: To do this, we started by examining the type of inflammatory cell infiltrate which occurred after acute (3 days) or chronic (12 weeks) cigarette smoke exposure (CSE) using female, C57BL/6 mice ($n = 7-10$). To compare the effects of anti-inflammatory compounds in these models, mice were exposed to either 3 days of CSE concomitant with compound dosing or 14 weeks of CSE with dosing beginning after week 12. Budesonide (1 mg kg^{-1} ; i.n., q.d.), roflumilast (3 mg kg^{-1} ; p.o., q.d.) and fluvastatin (2 mg kg^{-1} ; p.o., b.i.d.) were dosed 1 h before (and 5 h after for fluvastatin) CSE. These dose levels were selected because they have previously been shown to be efficacious in mouse models of lung inflammation. Bronchoalveolar lavage fluid (BALF) leukocyte number was the primary endpoint in both models as this is also a primary endpoint in early clinical studies.

Results: To start, we confirmed that the inflammatory phenotypes were different after acute (3 days) versus chronic (12 weeks) CSE. The inflammation in the acute systems was predominantly neutrophilic, while in the more chronic CSE systems BALF neutrophils (PMNs), macrophage and lymphocyte numbers were all increased ($p < 0.05$). In the acute model, both roflumilast and fluvastatin reduced BALF PMNs ($p < 0.01$) after 3 days of CSE, while budesonide had no effect on BALF PMNs. In the chronic model, therapeutically administered fluvastatin reduced the numbers of PMNs and macrophages in the BALF ($p \leq 0.05$), while budesonide had no effect on PMN or macrophage numbers, but did reduce BALF lymphocytes ($p < 0.01$). Roflumilast's inhibitory effects on inflammatory cell infiltrate were not statistically significant.

Conclusions: These results demonstrate that the acute, prophylactic systems can be used to identify compounds with therapeutic potential, but may not predict a compound's efficacy in chronic smoke exposure models.

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Background

Chronic obstructive pulmonary disease (COPD) is a leading cause of hospitalizations and death worldwide. The most common cause of COPD is chronic smoking, which elicits a repetitive inflammatory insult that is thought to lead to airway remodeling and, consequently, to the accelerated lung function decline that characterizes the disease. Unlike other chronic inflammatory airway diseases like asthma, there are currently no therapeutic approaches (e.g., glucocorticoids) that can attenuate the inflammation associated with COPD. This suggests that there is something different about the molecular mechanisms regulating the cigarette smoke-induced inflammation associated with the disease, which at present is not understood.

Preclinical *in vivo* models of cigarette smoke-induced lung inflammation are commonly used to investigate prospective disease mechanisms and evaluate the efficacy of candidate compounds. Exposure of laboratory animals to cigarette smoke can recapitulate many of the central features of COPD, including a slowly resolving and steroid-resistant inflammation, mucus production, airway remodeling, emphysema and changes in lung function [1-4]. Although these models use the primary etiological factor to mimic several COPD-like changes, it is difficult to determine how reliable these models are for predicting the therapeutic efficacy of candidate compounds. For instance, while steroids lack efficacy in both the preclinical models and the clinic, approaches aimed at neutralizing TNF- α work in the preclinical models, but do not work in the clinic. In the latter example, a possible reason for the lack of translation is that in the preclinical models genetically modified mice deficient for the TNF- α receptors were used and thus, in these animals the initiation of the inflammatory response to cigarette smoke exposure (CSE) was attenuated [5,6]. This was clearly a different situation to that in the clinic where an anti-TNF- α antibody lacked the ability to affect the progression of ongoing disease [7].

In most studies, compounds which have efficacy in acute systems also have efficacy in chronic models, too. The caveat to this is that most preclinical investigations have focused on characterizing the effects of candidate mechanisms under prophylactic conditions (using either GM mice or compounds) whether in acute or chronic CSE models [2,8-13]. Unfortunately, this approach does not closely resemble the clinical scenario where patients are treated after chronic lung inflammation has already developed. Additionally, the inflammatory response to CSE appears to be bi-phasic, with an initial neutrophilic infiltrate peaking within one week of exposures. This is subsequently followed by a more pronounced

inflammation after one month of CSEs with progressive increases in neutrophils, macrophages and lymphocytes migrating to the airways [1,14]. The different kinetics and types of infiltrate suggests that there are potentially different mechanisms driving the two phases of this response; thus, a compound's efficacy may be different in an acute, prophylactic (< one week) versus chronic, therapeutic (> one month) model. This concept is supported by the observation that TLR4 knockout mice are partially protected from developing lung inflammation after acute CSE, but were not protected after chronic CSEs [15].

As such, the aim of this study was to compare the prophylactic and therapeutic effects of three broad spectrum anti-inflammatory compounds in acute and chronic CSE models, respectively. We focused on three compounds with distinct mechanisms of action - a glucocorticoid (budesonide), a phosphodiesterase (PDE) 4 inhibitor (roflumilast) and a statin (fluvastatin). As one of the primary functions of preclinical disease models is to assess the potential efficacy of candidate compounds, ideally one would examine the same endpoints in the models as in the clinic. Typically, early proof-of-concept studies for COPD anti-inflammatory strategies in man assess inflammatory cell numbers in biofluids such as bronchoalveolar lavage fluid (BALF) or induced sputum, while longer term clinical studies examine changes in lung functioning. As the latter changes are difficult to model in small animals, we focused on assessing the effects of these anti-inflammatory compounds on CSE-induced changes in BALF inflammatory cell numbers.

Methods

Materials

C57BL/6 mice were obtained from Charles River UK. Budesonide [16,17-Butylidenebis(oxy)-11,21-dihydroxypregna-1,4-diene-3,20-dione] was purchased from Sigma. Roflumilast [3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy) benzami] and fluvastatin [(3R, 5S, 6E)-7-[3-(4-fluorophenyl)-1-(propan-2-yl)-1H-indol-2-yl]-3,5-dihydroxyhept-6-enoic acid] were made in-house (Novartis Institutes for Biomedical Research, Basel, Switzerland). University of Kentucky Research Cigarettes (brand 1R3F) were obtained from the University of Kentucky (Louisville, KY, USA).

Animal Maintenance Conditions

Female, C57BL/6 mice (16-20 g) were housed in rooms maintained at constant temperature ($21 \pm 2^\circ\text{C}$) and humidity ($55 \pm 15\%$) with a 12 h light cycle and 15 - 20 air changes per h. Ten animals were housed per cage containing two nest packs filled with grade 6 sawdust

(Datesand, Manchester, UK), nesting material (Enviro-Dri, Lilloco, UK), maxi fun tunnels and Aspen chew blocks (Lilloco, UK) to provide environmental enrichment. Animals were allowed food, RM1 Pellets, (SDS UK Ltd.) and water ad libitum.

Statement on Animal Welfare

Studies described herein were performed under a Project License issued by the United Kingdom Home Office and protocols were approved by the Local Ethical Review Process at Novartis Institutes for BioMedical Research, Horsham.

Cigarette smoke exposure methodology

Cigarette smoke and sham exposures were performed as previously described [10]. Mice were exposed to 4 cigarettes per exposure period, which we had previously shown to elicit a submaximal inflammatory response [10]. Sham, age- and sex-matched control animals were exposed to room-air pumped into the exposure chambers for the same duration of time (approximately 45 minutes per exposure period).

Comparing inflammatory cell infiltrate after acute or chronic CSE

Mice were exposed as described above once a day for either 3 days or 5 days per week for 12 weeks. Animals were sacrificed with an overdose of terminal anesthetic (sodium pentobarbitone 200 mg i.p.) followed by exsanguination 24 hours after the last exposure. There were sham, time-matched controls for each time point.

Assessing compound efficacy in models of acute CSE-induced inflammation

For the acute CSE model, the CSE regimen was performed as described above once a day and for 3 consecutive days. For studies with budesonide, the mice were dosed with either budesonide (1 mg kg⁻¹) or vehicle (saline with 2% NMP) 1 hour before each air or smoke exposure by intranasal (i.n.) administration under short-acting anaesthetic as described previously [10]. For studies with roflumilast and fluvastatin, the mice were dosed with either roflumilast (3 mg kg⁻¹) or fluvastatin (2 mg kg⁻¹) or vehicle (0.5% CMC) *per os* (p.o.) 1 hour before and (for fluvastatin-treated and vehicle control mice) 5 hours after each air or smoke exposure. The doses and dosing schedule for each compound were based on those that we and others have previously shown to be effective in other preclinical mouse models [9,13,16,17]. Twenty-four hours after the last exposure, animals were sacrificed with an overdose of terminal anesthetic (sodium pentobarbitone 200 mg i.p.) followed by exsanguination.

Assessing compound efficacy in models of chronic CSE-induced inflammation

For the chronic CSE model, the CSE regimen was performed as described above once a day, 5 days a week, for 14 weeks. During weeks thirteen and fourteen, mice were dosed with compounds or vehicles (as described above) concurrent with CSE. As before, animals were sacrificed with an overdose of terminal anaesthetic (sodium pentobarbitone 20 mg i.p.) followed by exsanguination 24 hours after the last exposure.

Preparation of bronchoalveolar lavage fluid (BALF)

After animals were sacrificed, BALF was collected, processed, and BALF inflammatory cell numbers determined as described previously [10].

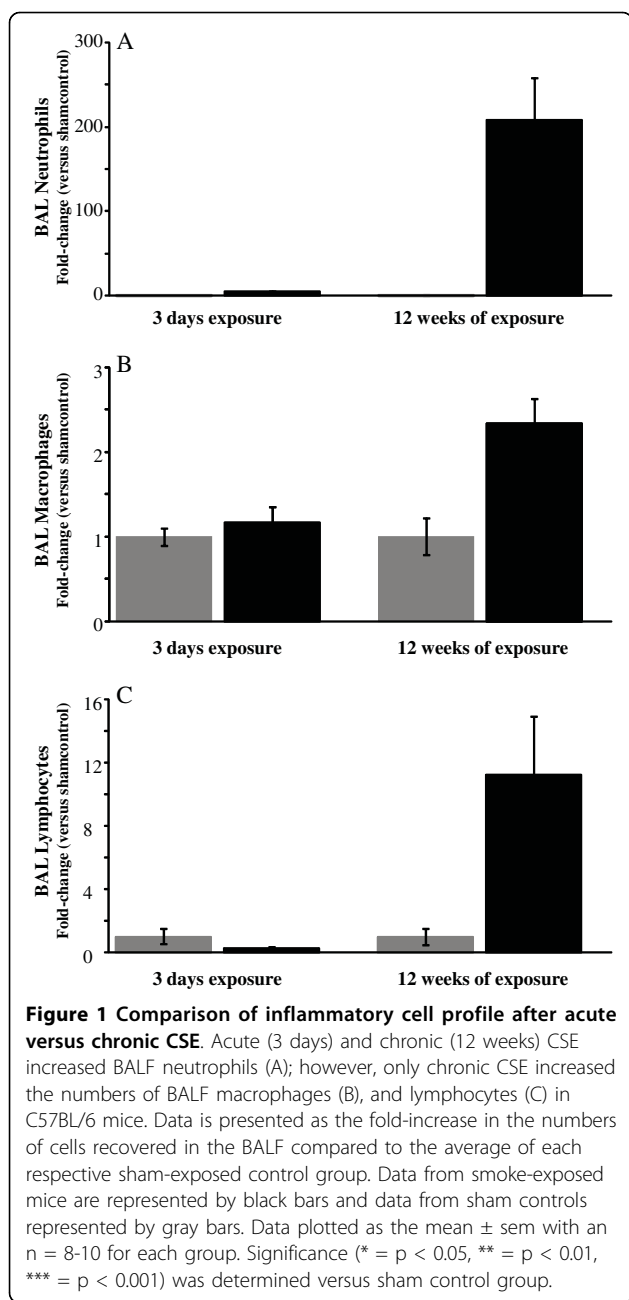
Statistical Analysis

All data are presented as Mean ± Standard Error of Mean (SEM). For time course studies, a Student's t-test was used comparing all smoke-exposed animals to their corresponding time-matched sham-exposed controls. For the compound studies, a one-way ANOVA with Dunnett correction for multiple comparisons was used. A P value of less than 0.05 was considered significant. Power calculations were based on t-tests, assuming unequal variances (Satterthwaite approximation), and were based on group means and standard deviations derived from historical data. All sample sizes were based on 80% power with a two-sided alpha = 0.05. Calculations were performed using the software package NQUERY ADVISOR.

Results

Time-dependent changes in BALF inflammatory cell numbers over 3 months of CSE

In a previous study we confirmed the bi-phasic nature of the inflammatory response to CSE over a 26 week period (data not shown). The data in figure 1, was from a separate study comparing the inflammatory phenotypes that are observed after an acute (3 days) or chronic (12 weeks) exposure period. Both acute and chronic CSE increased the numbers of BALF neutrophils recovered (Figure 1A), although it's clear chronic exposure led to a greater increase relative to each groups' respective sham controls. The numbers of neutrophils increased more than 5-fold over the $2.2 \pm 0.4 \times 10^3$ cells mL⁻¹ recovered in the sham-exposed controls ($p > 0.01$) after 3 days of CSE; however, there was more than a 200-fold increase over the $1.7 \pm 0.9 \times 10^2$ cells mL⁻¹ recovered in the sham-exposed mice after 12 weeks of CSE ($p > 0.001$). Increases in BALF macrophages (Figures 1B), and lymphocytes (Figures 1C) were only observed after chronic CSE. After 3 days of CSE, there were no significant increases over the numbers of macrophages ($9.7 \pm 1.0 \times 10^4$ cells mL⁻¹) or lymphocytes ($1.6 \pm 0.8 \times 10^3$ cells mL⁻¹)



recovered in the BALF of sham-exposed mice. After 12 weeks of CSE, however, the numbers of macrophages increased more than 2-fold over the $4.2 \pm 0.9 \times 10^4$ cells mL^{-1} recovered in the sham-exposed mice ($p > 0.01$). Similarly, BALF lymphocyte numbers increased more than 10-fold over the $3.0 \pm 1.1 \times 10^3$ cells mL^{-1} recovered in the sham-exposed mice ($p > 0.01$).

Effect of prophylactically administered anti-inflammatory compounds on CSE-induced acute inflammation

After 3 days of CSE, there was an increase in BALF neutrophil numbers in vehicle-treated mice compared to

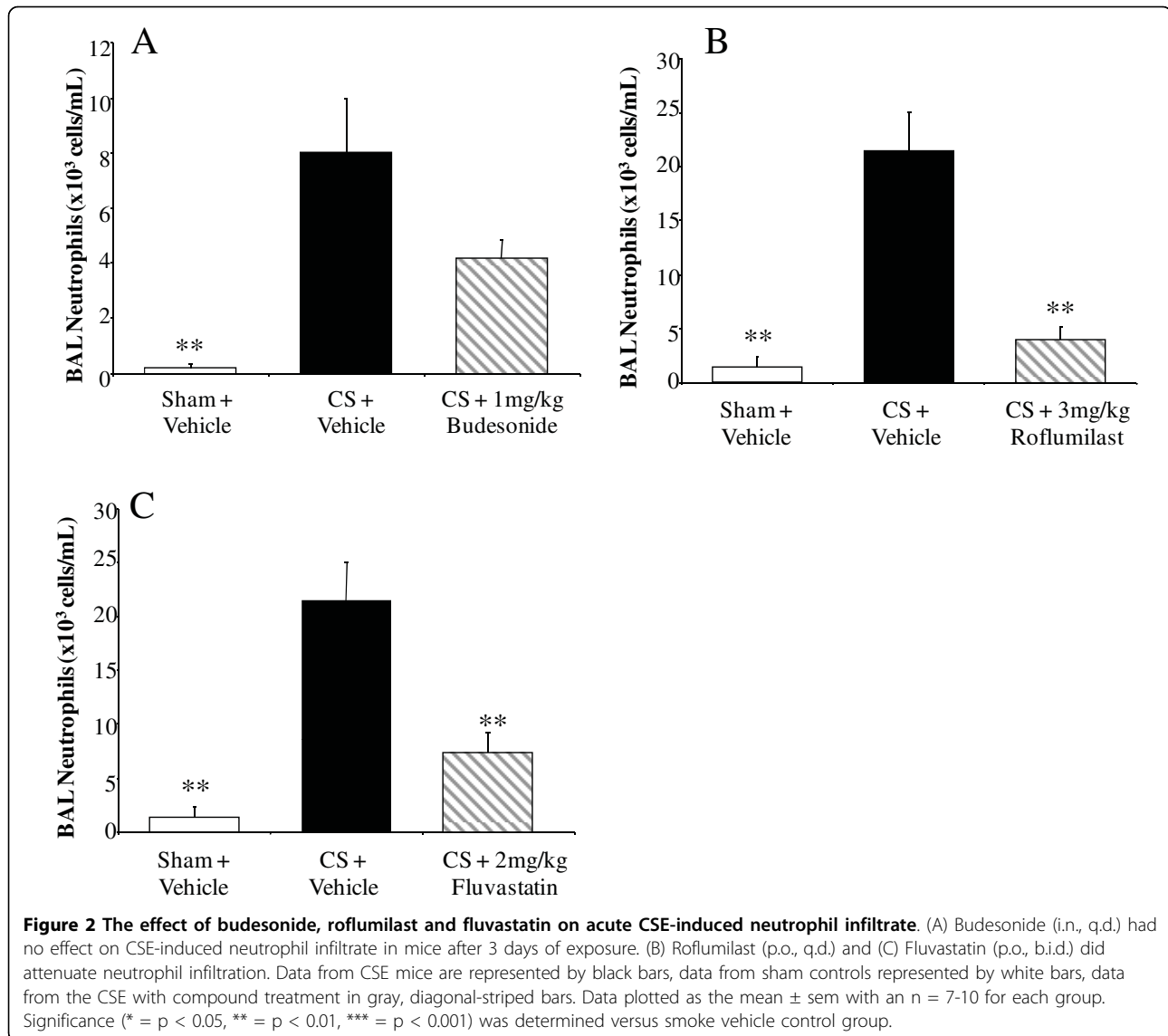
sham-exposed, vehicle-treated controls ($p < 0.01$) (figure 2A-C). Budesonide, administered i.n., had no effect on neutrophil numbers (Figure 2A). Conversely, roflumilast (Figure 2B) and fluvastatin (Figure 2C) administered p.o. significantly reduced the numbers of BALF neutrophils by $87 \pm 5\%$ and $71 \pm 9\%$, respectively ($p < 0.01$).

Effect of therapeutically administered anti-inflammatory compounds on CSE-induced chronic inflammation

Chronic CSE increased the numbers of BALF neutrophils, macrophages and lymphocytes in the all vehicle-treated groups compared to sham-exposed, vehicle-treated controls. Budesonide (1 mg kg^{-1} , i.n., q.d.) had no effect on BALF neutrophil or macrophage numbers (Figure 3A and 3B). Budesonide did, however, reduce the number of lymphocytes recovered by $91 \pm 4\%$ ($p < 0.01$) (Figure 3C). Roflumilast trended towards reducing the increase in BALF neutrophils by $40 \pm 10\%$ (Figure 4A), macrophages by $47 \pm 13\%$ (Figure 4B) and lymphocytes by $56 \pm 10\%$ (Figure 4C); however these effects on BALF leukocyte numbers were not statistically significant. Fluvastatin reduced the number of neutrophils by $74 \pm 5\%$ (Figure 5A) and macrophages by $64 \pm 7\%$ (Figure 5B) in the BALF ($p < 0.05$), but the reduction of BALF lymphocytes was not statistically significant (Figure 5C).

Discussion

These data confirm that there are different inflammatory phenotypes after either an acute or chronic CSE. The most obvious difference being the greater numbers and spectrum of inflammatory cell infiltrate present in the airways after a chronic exposure compared to the predominantly low-grade neutrophilic inflammation after an acute exposure. We also demonstrated that the acute (prophylactic) CSE models can be used to identify compounds with potential anti-inflammatory efficacy, but could not be used to predict the therapeutic efficacy of the same compounds on chronic CSE-induced inflammation. This is the first time the prophylactic and therapeutic effects of these 3 broad spectrum anti-inflammatory compounds have been assessed in these models. Again, we focused our assessment of efficacy around the numbers of inflammatory cells recovered in the BALF as this is a direct preclinical correlate to endpoints used in early proof-of-concept studies in man. Additionally, infiltrating inflammatory cells (particularly macrophages and lymphocytes) have been directly linked to the subsequent development of COPD-like lung pathologies in these modeling systems [18,19]. We did not assess levels of cytokines or chemokines in the BALF or lung tissue for several reasons. First, changes in the levels of these mediators are not acceptable biomarkers at the present time for studies conducted in

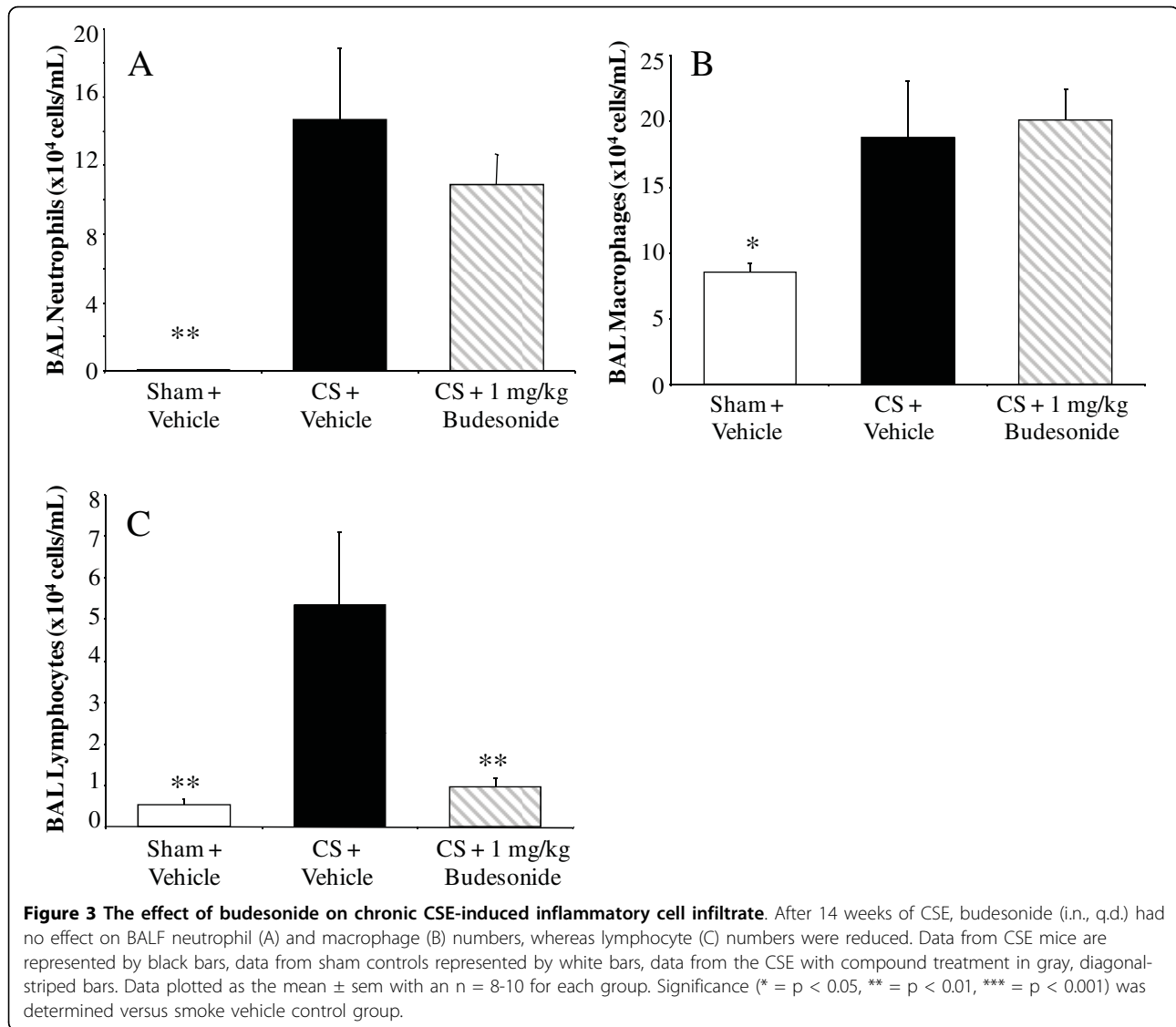


COPD patients because they do not consistently track with disease progression. Second, we and others [20,21] have shown that the effects anti-inflammatory molecules (e.g. steroids) have on chemokine levels do not necessarily align with their ability to block cell infiltrates. Finally, investigating the molecular mechanisms responsible for the effects of these 3 compounds in the models was beyond the scope of these studies and (for the reasons just described) would require more than an assessment of cytokine or chemokine production. These data will, however, be important to collect in future studies elucidating the specific mechanisms of these compounds in these models.

The response to CSE in rodents has both an acute phase consisting of neutrophil infiltrate peaking after one week of exposures and a chronic phase consisting

of neutrophils, macrophages and lymphocytes that begins after one month of exposures as previously reported by us and others [1,14]. Between weeks 1 and 4 the inflammation goes through a transition period, where neutrophil numbers decline, while macrophages and lymphocytes begin to increase, but not in a completely progressive fashion. After 1 month the inflammatory response is progressive, more pronounced, and eventually leads to airway remodeling and emphysema. We tested 3 mechanistically distinct anti-inflammatory compounds in both the 3-day and 14-week CSE models to determine whether these subtle differences in the inflammatory phenotype during each phase of the response affected compound efficacy.

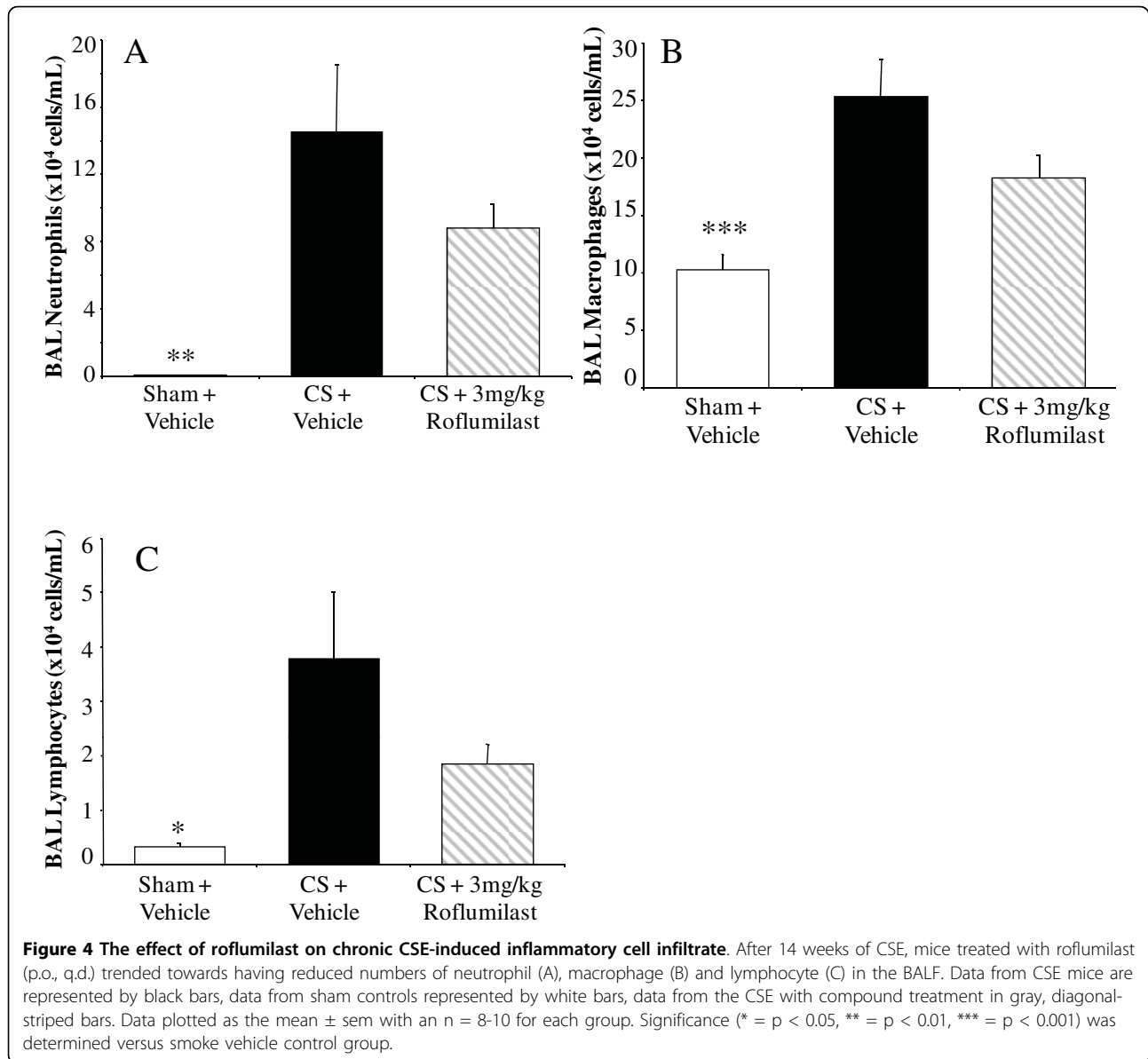
In the acute models, CSE consistently induced an increase in the number of neutrophils recovered in the



BALF and as such this remained the primary endpoint in the acute model. We and others have previously shown that glucocorticoids cannot affect the acute inflammatory changes induced by CSE at doses which can attenuate allergen-induced inflammation [2,9,13,22]. We confirmed our previous findings (conducted using BALB/C mice) here, using C57BL/6 mice as again budesonide had no effect on acute CSE-induced neutrophilia in this strain. Similarly, budesonide had no effect on chronic CSE-induced macrophage or neutrophil infiltration in the lung. There was, however, a profound effect on lymphocytic infiltrate that may be due to budesonide's effect on the thymus [23,24]; however, the mechanism for this effect on lymphocytes still requires further investigation. These findings reflect the inability of glucocorticoids to attenuate the inflammation observed in COPD patients. Additionally, the data

suggest that the CSE models can be used for investigating mechanisms related to steroid-resistant inflammation and for identifying approaches that may be able to restore steroid efficacy in COPD [2].

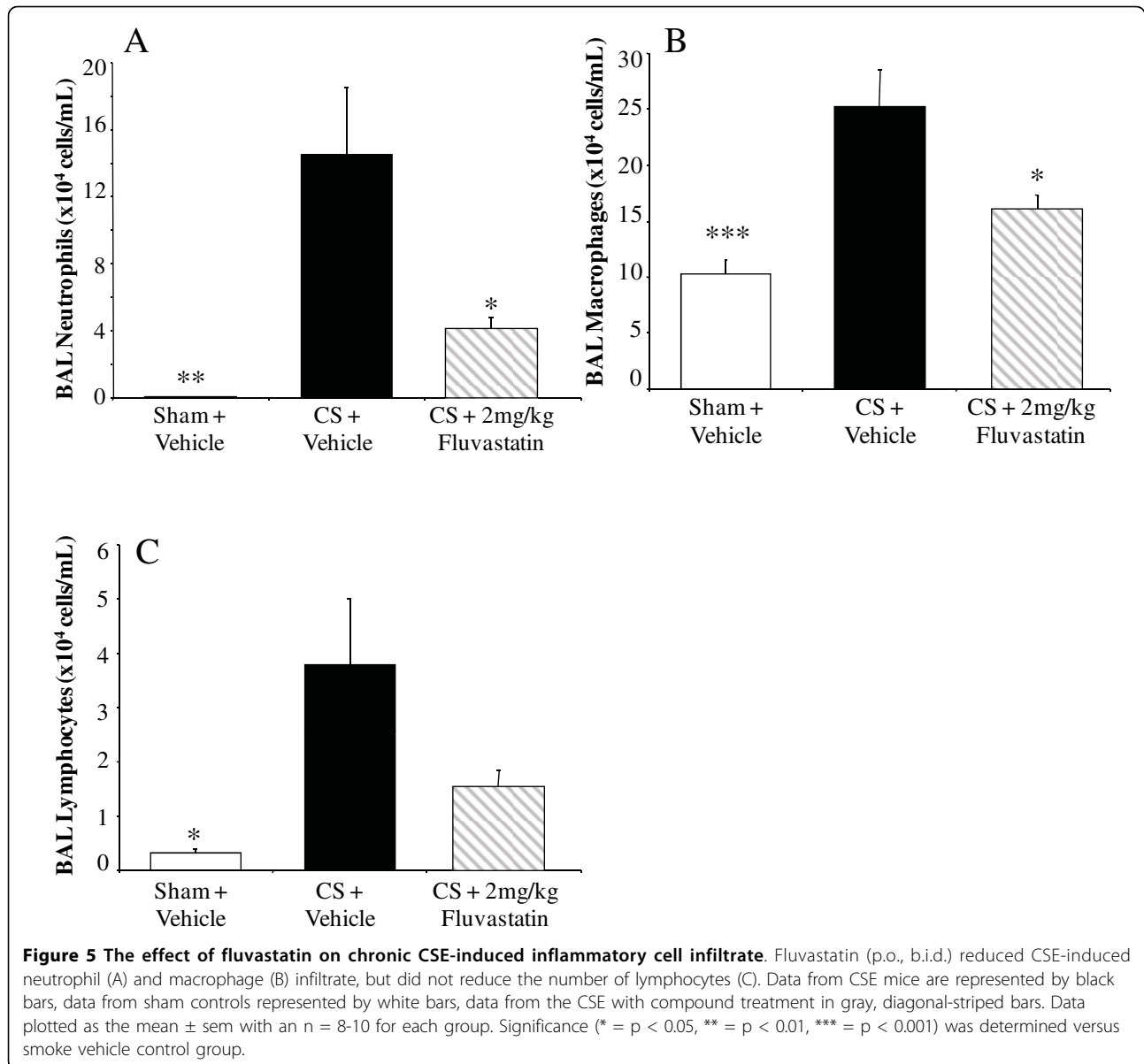
Statins, on the other hand, have been reported to slow the rate of lung function decline and reduce mortality in COPD patients [25,26]; however, no one as yet has looked at whether statins affect the inflammation associated with the disease. Prophylactic administration of a statin (i.e., simvastatin) has previously been demonstrated to inhibit inflammation, emphysema and remodeling of the lung vasculature after chronic CSE in Sprague-Dawley rats [13]. It is unclear how statins act as anti-inflammatory agents, although their ability to block adhesion molecules and preventing the prenylation of proteins involved in inflammatory signaling (e.g. GTP-binding proteins) are well documented [27-29].



In our acute (prophylactic) system, fluvastatin attenuated acute neutrophilia induced by CSE. When we tested fluvastatin in the more chronic (therapeutic) model, it reduced the numbers of neutrophil and macrophage recovered in the BALF, while there only a modest reduction in lymphocyte infiltration, but the latter was not significant. These data are encouraging and imply that statins may prove to be effective anti-inflammatory treatments for COPD.

We also assessed the effect of a PDE4 inhibitor, roflumilast, in our models as it has previously been shown to reduce both acute and chronic CSE-induced inflammation in rodents when administered prophylactically at similar doses [11,12,16]. Here, we show that while roflumilast can reduce acute CSE-induced inflammation

when given prophylactically, it failed to significantly reduce an established chronic inflammation when administered therapeutically. We propose that our results differ from those reported by Martorana and colleagues [11] due to the different dosing schedules (prophylactic versus therapeutic). Their results did, however, suggest that higher doses were needed to inhibit the chronic response. Our findings are in accordance with those reported by Le Quement and colleagues [16] who found that roflumilast reduced BALF neutrophils after 4 days of CSE, but could not attenuate the numbers of BALF macrophages after 11 days of CSE. The authors attributed these differences to PDE4 inhibitors' inability to inhibit macrophage activation and recruitment [16]. Our data from the chronic CSE system demonstrate that



roflumilast does not effectively reduce inflammatory cell recruitment in general. These data, along with that reported by Le Quement and colleagues [16], do suggest that there are different mechanisms driving the acute and chronic phases of the inflammatory response. Roflumilast has demonstrated very limited efficacy in the clinic as well, which has largely been attributed to dose-limitation associated with roflumilast's side-effect profile. It has been reported that roflumilast can reduce the number of inflammatory cells recovered from COPD patients by approximately 30-50% [30]. This level of inhibition is consistent with what we observed in the chronic CSE experiment; however, these *in vivo* models are typically powered to identify a $\geq 50\%$ inhibitory

effect. As such, these observations suggest that the chronic model is a more rigorous assessment of a compound's anti-inflammatory efficacy that may be more reflective of the clinical situation.

Conclusions

The data reported here demonstrate that overall, the prophylactic effects of compounds in the acute CSE models can identify compounds with anti-inflammatory efficacy; however, effects in acute, prophylactic systems did not reliably predict those observed in chronic models where compounds were administered therapeutically. This suggests that mechanisms that are involved in the initiation of CSE-induced inflammation may not be the

same as those involved in the progression of the chronic response. Thus, we conclude that the acute CSE model is a robust, primary modeling system that can be used to assess the potential efficacy of candidate compounds, particularly those with broad spectrum anti-inflammatory effects or that target neutrophilic inflammation. However, testing candidate compounds in a chronic system more akin to the clinical situation where a progressive chronic inflammation (with a broader spectrum of inflammatory cell infiltrate) is already established in the lungs would always be prudent to get a more complete understanding of a compound's range of effects.

List of abbreviations

COPD: Chronic obstructive pulmonary disease; CS: Cigarette smoke; CSE: Cigarette smoke exposure; BALF: Bronchoalveolar lavage fluid; p.o.: Per os (by mouth); i.n.: Intranasal; q.d.: Quaque die (once daily); b.i.d.: Bis in die (twice a day)

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Authors' contributions

W-YHW, AM, GK, WP, JM, DW, and CSS contributed to the acquisition and analysis of the data, have contributed to the drafting of the manuscript, read and approve of the final version of this manuscript. CSS designed the studies and drafted the manuscript.

Competing interests

The authors declare that they have no competing interests.

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