The effect of peroxisome proliferator-activated receptor-γ ligands on *in vitro* and *in vivo* models of COPD

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ABSTRACT Peroxisome proliferator-activated receptor (PPAR)-γ is expressed in alveolar macrophages. The anti-inflammatory potential of the PPAR-γ ligands rosiglitazone and pioglitazone were investigated using *in vitro* alveolar macrophage models and *in vivo* animal models relevant to chronic obstructive pulmonary disease (COPD).

PPAR-γ protein and gene expression in COPD alveolar macrophages was compared with control smokers and never-smokers. COPD macrophages were used to investigate the effects of PPAR-γ ligands and corticosteroids on lipopolysaccharide-induced cytokine production, alternative macrophage activation (M2) gene expression and efferocytosis. The effects of PPAR-γ ligands in a subchronic tobacco smoke model in mice were investigated.

PPAR-γ protein expression was similar in COPD patients compared to controls, although increased gene expression levels were observed in COPD patients and control smokers compared to never-smokers. PPAR-γ ligands reduced tumour necrosis factor-α and CC chemokine ligand-5, but not CXC chemokine ligand-8, in COPD alveolar macrophages; these effects were generally less than those of the corticosteroid dexamethasone. Rosiglitazone increased M2 gene expression and enhanced efferocytosis of apoptotic neutrophils. Rosiglitazone and pioglitazone attenuated airway neutrophilia in a corticosteroid-resistant mouse model of pulmonary inflammation.

We show biological actions of PPAR-γ agonists on corticosteroid-resistant disease, tobacco smoke-induced pulmonary inflammation, skewing of macrophage phenotype and clearance of apoptotic neutrophils.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors involved in cellular differentiation, metabolism and development. There are three PPAR subtypes (α, β and γ), which show distinct tissue distribution [1]. PPAR-γ is activated by endogenous agonists including the prostaglandin D_2 metabolite 15-deoxy-δ-prostaglandin J_2 [2]. The synthetic PPAR-γ ligands rosiglitazone and pioglitazone are used as insulin sensitisers for the treatment of diabetes; short- and long-term use of these drugs in patients with diabetes also reduces systemic inflammation [3].

PPAR-γ heterodimerises with the retinoid X receptor to form a complex which binds to peroxisome proliferator response elements in target genes, resulting in transcriptional upregulation (transactivation). PPAR-γ can also inhibit gene transcription through transrepression mechanisms that involve interactions with other transcription factors and their coactivators to prevent effective DNA binding [4].

PPAR-γ is expressed in human monocytes and macrophages [5]. PPAR-γ ligands inhibit the transcription of a subset of pro-inflammatory classically activated macrophage M1 genes [6], and also increase expression levels of alternative activation M2 genes that are involved in anti-inflammatory effects and tissue repair [7]. Mouse models have shown that PPAR-γ deletion from alveolar macrophages causes airway inflammation [8, 9]. Furthermore, rosiglitazone inhibits the development of lipopolysaccharide (LPS)-induced airway neutrophilia [10], and pioglitazone inhibits allergic pulmonary inflammation in mice to a similar degree to corticosteroids [11]. In addition to its role in the inflammatory response, PPAR-γ is also involved in the regulation of macrophage efferocytosis; PPAR-γ deletion reduces macrophage clearance of apoptotic cells [12]. This function of macrophages is important for the resolution of tissue injury.

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory disease caused by the inhalation of noxious substances, most commonly cigarette smoke [13]. The inflammatory response to cigarette smoke exposure can result in tissue injury and the development of emphysema due to parenchymal destruction. The number of alveolar macrophages is increased in COPD patients, and these cells contribute to disease pathophysiology through the release of inflammatory mediators and proteases [14, 15]. Recent findings show that COPD alveolar macrophages are skewed towards the M2 phenotype [16]. Alveolar macrophages appear to play a complex role in the lungs of COPD patients, being capable of releasing both pro- and anti-inflammatory cytokines as well as factors involved in tissue repair.

Corticosteroids are the most widely used anti-inflammatory therapy for COPD, but have limited clinical benefits, particularly in more severe disease [17]. Corticosteroids only partially suppress cytokine production from COPD alveolar macrophages [18–20]. There is an urgent need for more effective anti-inflammatory therapies for COPD that target macrophage function. Certainly, there is evidence from asthma clinical trials that the PPAR-γ agonist rosiglitazone has therapeutic benefits [21, 22]. There is concern about the clinical use of rosiglitazone due to cardiac side-effects [23]. One way to minimise these side-effects in respiratory disease would be to use inhaled delivery.

We have investigated the anti-inflammatory potential of the PPAR-γ ligands (rosiglitazone and pioglitazone) used in clinical practice at physiologically relevant concentrations/doses using *in vitro* and *in vivo* models relevant to COPD. We have used alveolar macrophages from COPD patients to assess the expression and function of PPAR-γ. The function of PPAR-γ in COPD alveolar macrophages was investigated by measuring pro-inflammatory cytokine production, M2 gene expression and efferocytosis.

TABLE 1 Subject demographics

	Never-smokers	Smokers	COPD
Subjects n	40	49	77
Sex female/male n/n	29/11	24/25	32/45
Age years	64.8±13.3	61.5±9.2	66.2±6.7
FEV_1 L	2.04±0.55	2.40±0.62	1.75±0.55
FEV_1 % pred	106.5±16.2	88.7±17.8	68.3±14.3
FVC	2.71±0.56	3.31±0.55	3.03±0.90
FEV_1/FVC ratio	79.2±12.8	74.3±8.1	58.8±8.7
Smoking history pack-years	NA	47.9±21.8	53.1±26.8

Data are presented as mean±SD, unless otherwise stated. COPD: chronic obstructive pulmonary disease; FEV1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; NA: not applicable.

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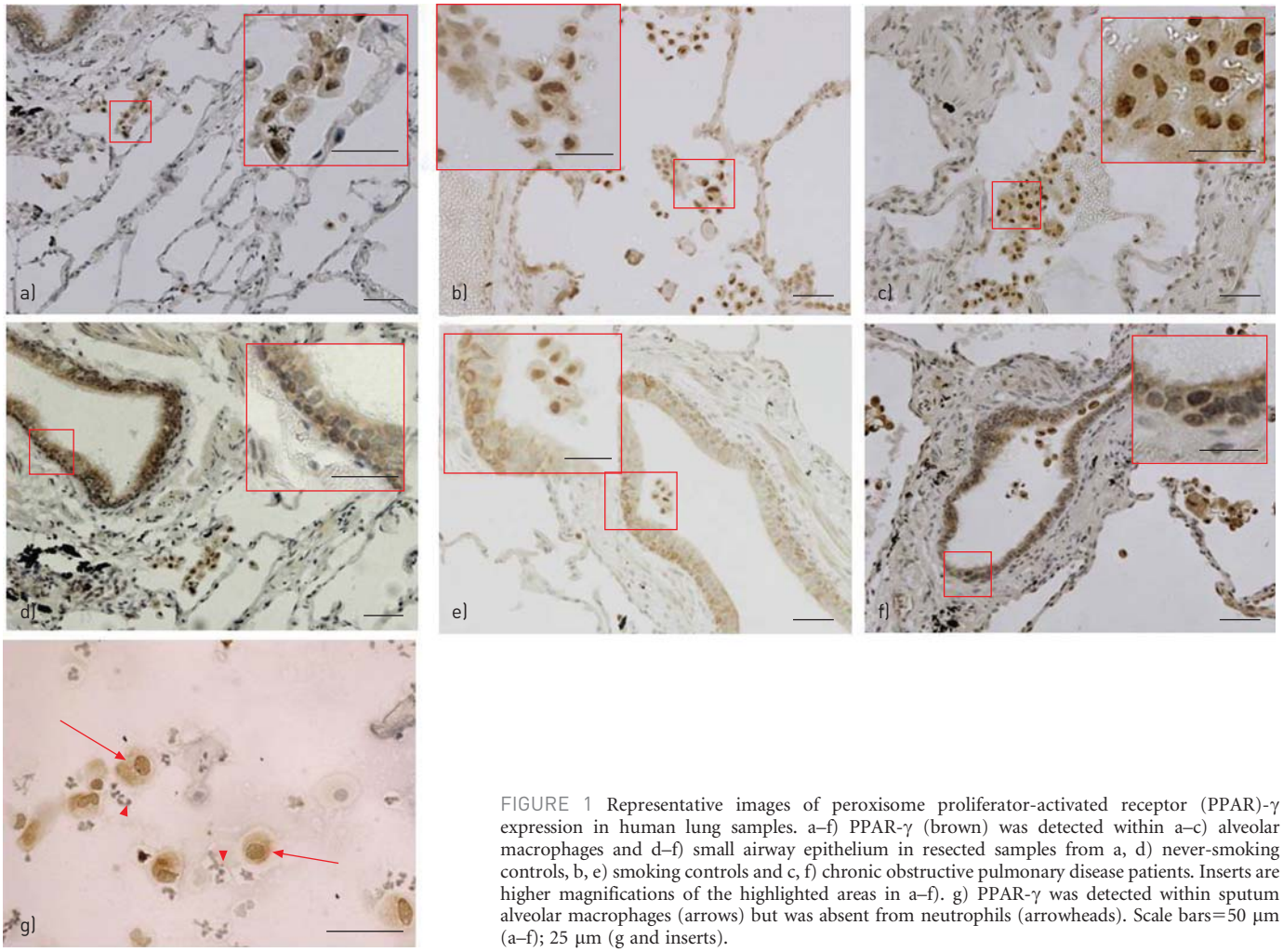

FIGURE 1 Representative images of peroxisome proliferator-activated receptor (PPAR)-γ expression in human lung samples. a–f) PPAR-γ (brown) was detected within a–c) alveolar macrophages and d–f) small airway epithelium in resected samples from a, d) never-smoking controls, b, e) smoking controls and c, f) chronic obstructive pulmonary disease patients. Inserts are higher magnifications of the highlighted areas in a–f). g) PPAR-γ was detected within sputum alveolar macrophages (arrows) but was absent from neutrophils (arrowheads). Scale bars=50 μm (a–f); 25 μm (g and inserts).

We also assessed the effects of PPAR-γ ligands in a subchronic tobacco smoke model in mice, administered both systemically and topically to the lung.

Methods

Study subjects

166 patients undergoing surgical resection for suspected or confirmed lung cancer (see table 1 for demography) were recruited. Samples from subgroups of these patients were used for individual experiments. COPD was diagnosed according to Global Initiative for Chronic Obstructive Lung Disease guidelines [24]. Smokers with normal lung function or never-smokers were recruited. All subjects gave written informed consent. This research was approved by the local research ethics committee (South Manchester Research Ethics Committee).

Macrophage culture

Alveolar macrophages were isolated from resected lung tissue as previously described [18]. To measure cytokine production, macrophages were stimulated for 24 h with LPS (1 $\mu g \cdot mL^{-1}$) after 1 h incubation with rosiglitazone, pioglitazone, dexamethasone or vehicle control containing dimethyl sulfoxide (DMSO) (0.05%); the concentration range of these PPAR-γ agonists was chosen based on published median effective concentrations between 100 nM and 1 μM [25–27]. Supernatant tumour necrosis factor (TNF)-α, CC chemokine ligand (CCL)-5 and CXC chemokine ligand (CXCL)-8 levels were determined by ELISA (online supplementary material). The effects of the drugs on cell viability were assessed by measuring lactate dehydrogenase as described in the online supplementary material.

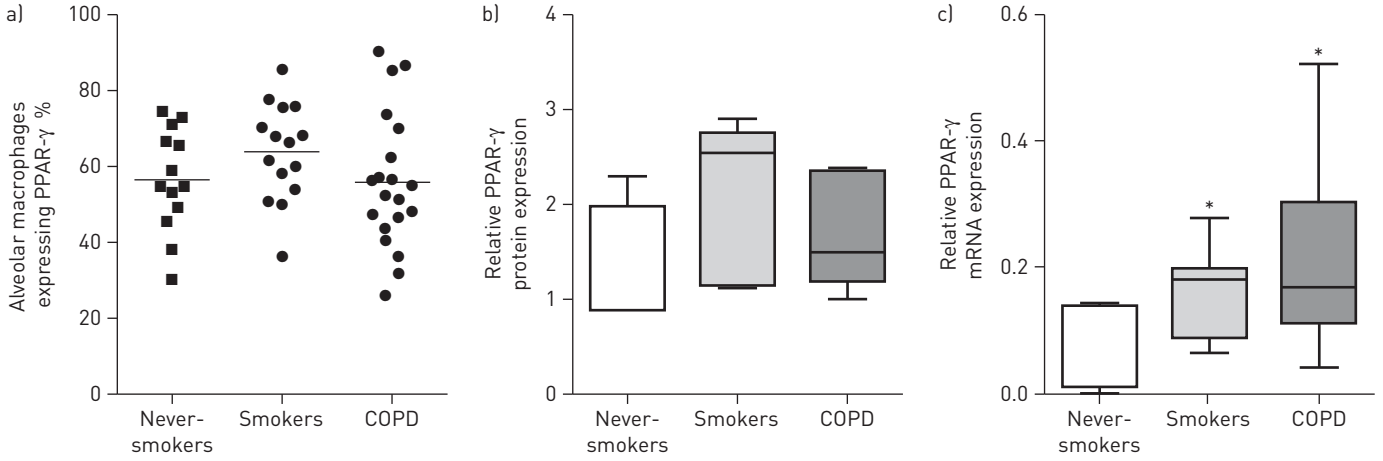

FIGURE 2 Alveolar macrophage expression of peroxisome proliferator-activated receptor (PPAR)-γ within human lung tissue. a) Percentage of alveolar macrophages that express PPAR-γ within human lung tissue. Data are presented as mean and individual scores from samples from chronic obstructive pulmonary disease (COPD) patients (n=20), smoking (n=15) and never-smoking controls (n=13). b) Relative levels of PPAR-γ protein expression in alveolar macrophages. Data are presented as median and range of relative PPAR-γ expression in alveolar macrophages from COPD patients (n=5), smokers (n=8) and never-smokers (n=4). Relative expression levels were determined normalising to β-actin. c) Relative levels of PPAR-γ mRNA expression in alveolar macrophages. Data are presented as median and range of relative PPAR-γ expression in alveolar macrophages from COPD patients (n=13), smokers (n=11) and never-smokers (n=8). Relative expression levels were determined using the ΔCt method normalising to the endogenous control (glyceraldehyde-3-phosphate dehydrogenase). *: $p<0.05$ compared to never-smokers.

To assess M2 phenotype, macrophages were incubated for 6 h and 24 h with rosiglitazone (1 μM), dexamethasone (1 μM), interleukin (IL)-4 (10 $\mu g \cdot mL^{-1}$) or media. Supernatants were removed and cell pellets lysed in Trizol (Invitrogen, Paisley, UK) for RNA extraction and PCR analysis for CD36, CD206, haemoxygenase (HO)-1 and PPAR-γ expression as described in the online supplementary material.

Immunohistochemistry

Tissue blocks were obtained from an area of the lung as far distal to the tumour as possible, and processed as described previously [28]. Blocks were labelled using anti-human PPAR-γ antibody primary antibody. Further details of methods and antibodies are described in the online supplementary material. The number of PPAR-γ-positive macrophages was calculated within the alveolar space (online supplementary material).

Western blotting

PPAR-γ protein levels in alveolar macrophages were measured by Western blotting, as described in the online supplementary material.

PPAR-γ gene expression

PPAR-γ gene expression levels were compared between macrophages from COPD patients and controls as described in the online supplementary material; the endogenous housekeeping controls glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and gene β-peptide 2-like 1 (GNB2L1) [29] were used.

Efferocytosis

Alveolar macrophages were incubated with rosiglitazone (1 μM), dexamethasone (1 μM) or media for 24 h. Treated macrophages were co-cultured with apoptotic blood neutrophils (1:5, respectively) for 90 min. Cells were stained for myeloperoxidase with O-dianisidine stain. The percentage of alveolar macrophages containing the stained neutrophils was determined. Further details are given in the online supplementary material.

Subchronic tobacco smoke mouse model of pulmonary inflammation

Female inbred C57BL/6J (or C57BL/6JAX) mice were exposed to tobacco smoke daily for four consecutive days as follows: day 1: 4 cigarettes (~32 min exposure); days 2–4: 6 cigarettes (~48 min exposure). One group of mice was exposed to air on a daily basis for equivalent lengths of time as sham controls (no tobacco smoke exposure). Bronchoalveolar lavage fluid was obtained from the mice and differential cell counts performed; further details are given in the online supplementary material.

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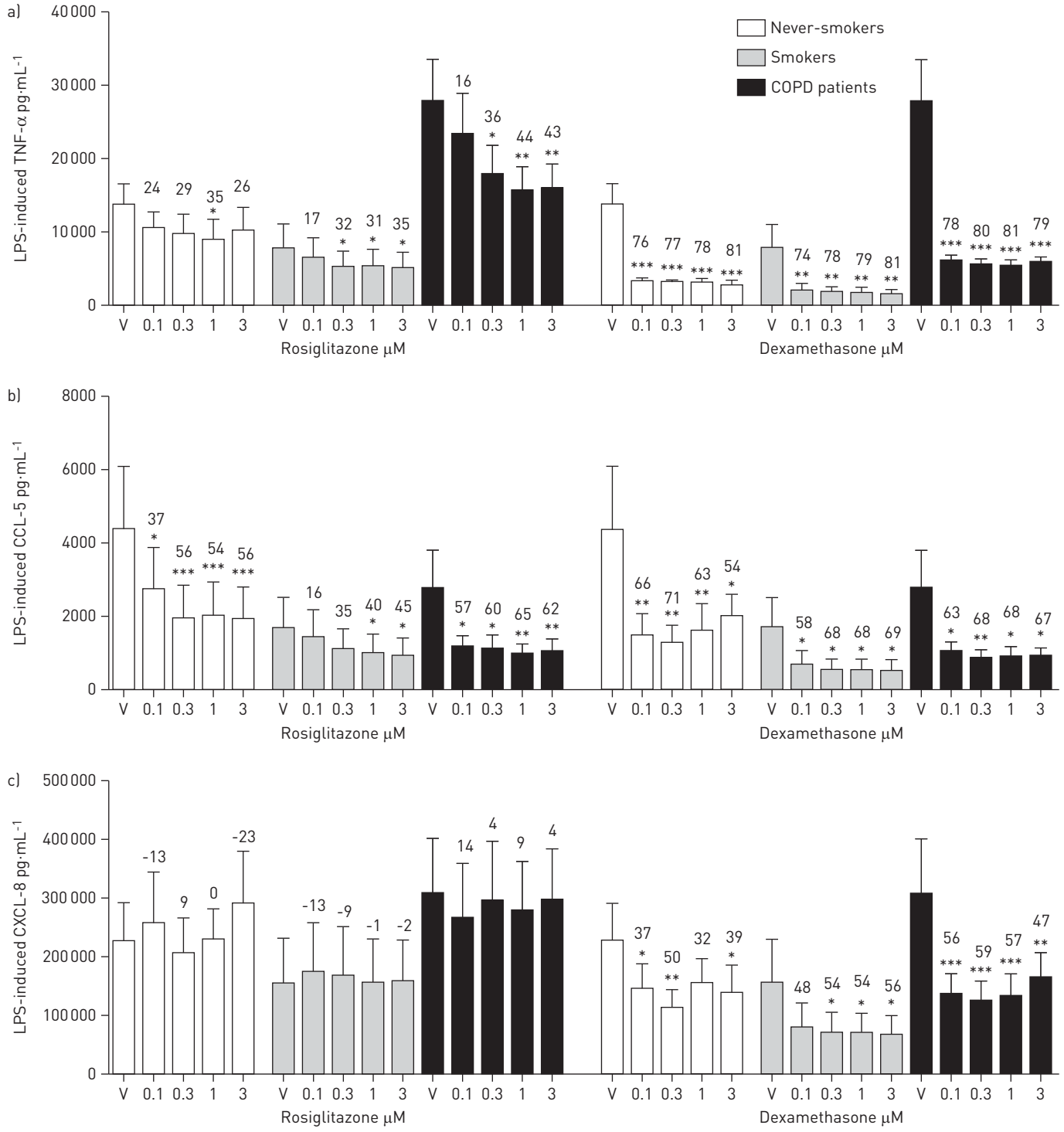

FIGURE 3 Comparative potency of dexamethasone and rosiglitazone inhibition on lipopolysaccharide (LPS)-stimulated cytokine production. LPS-induced a) tumour necrosis factor (TNF)-α, b) CC chemokine ligand (CCL)-5 or c) CXC chemokine ligand (CXCL)-8 production in alveolar macrophages from chronic obstructive pulmonary disease (COPD) patients (n=10), smoking (n=5) or never-smoking (n=7) controls with percentage inhibition shown above each bar. Data are presented as mean±SEM. V: vehicle control (0.05% dimethyl sulfoxide). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ below vehicle control.

Statistical analysis

PPAR-γ protein expression data and LPS-stimulated cytokine levels were compared between groups using unpaired t-tests. The effects of drugs on LPS-stimulated cytokine production were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. Inhibition curves for dexamethasone and rosiglitazone were compared within and between subject groups using two-way ANOVA. The effect of

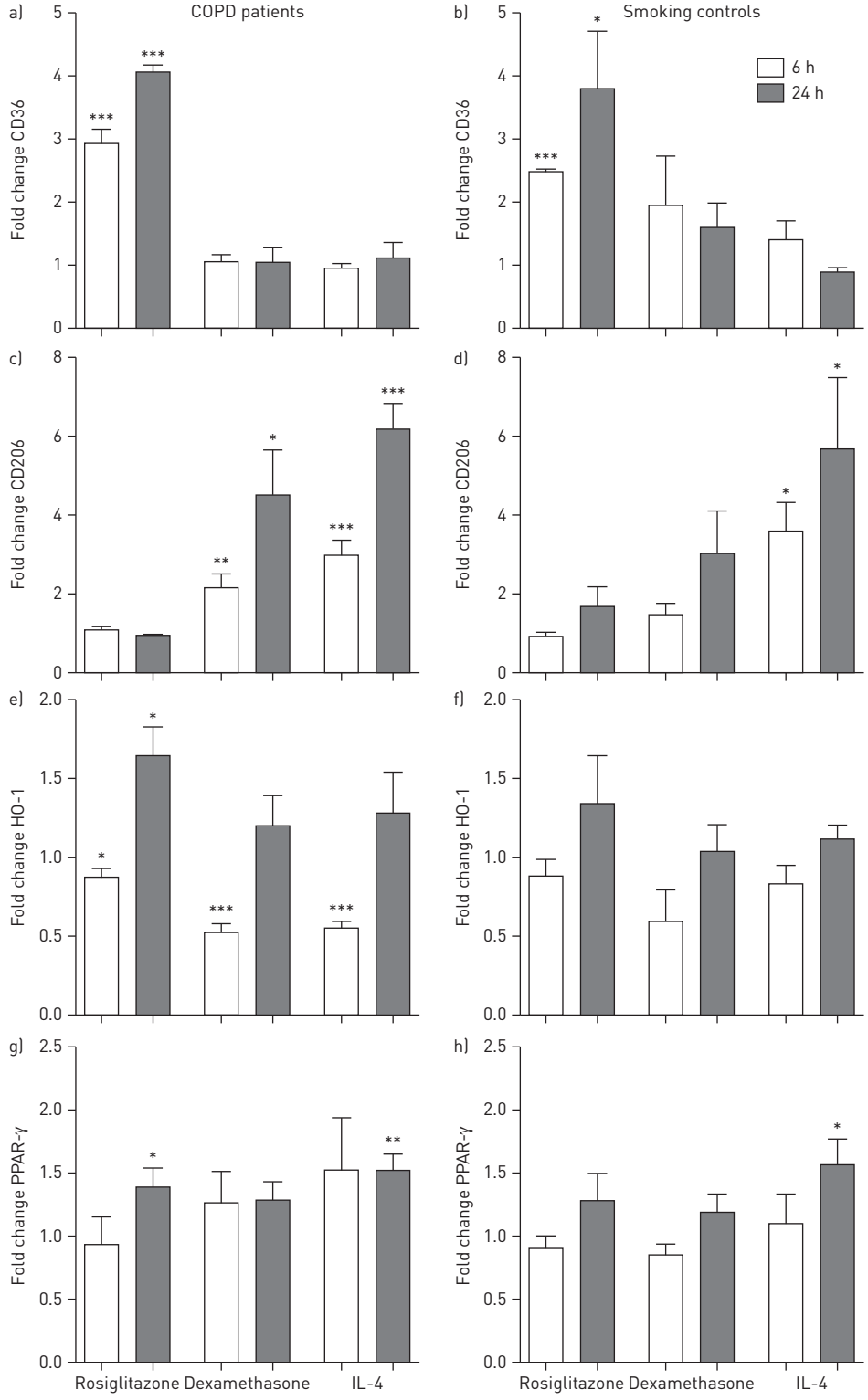

FIGURE 4 Effects of rosiglitazone, dexamethasone and interleukin (IL)-4 on CD36, CD206, haemoxygenase (HO)-1 and peroxisome proliferator-activated receptor (PPAR)-γ RNA expression in alveolar macrophages in chronic obstructive pulmonary disease (COPD) patients (n=8) and smoking controls (n=6). Alveolar macrophages were treated with rosiglitazone (1 μM), dexamethasone (1 μM), IL-4 (10 ng·mL^{-1}) or left unstimulated for 6 h and 24 h. RNA was extracted for PCR analysis of: a, b) CD36; c, d) CD206; e, f) HO-1; and g, h) PPAR-γ. Data are presented as mean±SEM of fold increase of gene expression above unstimulated controls. Relative expression levels were determined using the ΔΔCt method normalising to the endogenous control (glyceraldehyde-3-phosphate dehydrogenase). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ significantly different to time-matched unstimulated control.

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PPAR-γ agonists on efferocytosis was assessed using paired t-tests. Mann–Whitney U-tests were performed to compare relative mRNA expression levels of PPAR-γ between subject groups. Paired t-tests were performed to determine expression regulation of M2 genes compared to unstimulated controls. Subchronic tobacco smoke mouse model data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. All statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). $p<0.05$ was considered significant.

Results

PPAR-γ protein expression in alveolar macrophages

PPAR-γ was detected in alveolar macrophages and small airway epithelium using peripheral lung tissue from 20 COPD patients, 15 smokers and 13 never-smokers (fig. 1). PPAR-γ staining in macrophages was predominantly nuclear in these tissue sections; this was further demonstrated using immunofluoresence and a nuclear-specific stain (see online supplementary material and fig. S1). There were no differences between the proportion of macrophages expressing PPAR-γ in COPD patients compared to smokers and never-smokers (means 56%, 64% and 57%, respectively, $p=0.3$) (fig. 2a). Immunohistochemistry on induced sputum samples showed that PPAR-γ was expressed in macrophages but not neutrophils (fig. 1g).

Western blotting was used to analyse PPAR-γ protein levels using alveolar macrophages from five COPD patients, eight smokers and four never-smokers (fig. 2b). There were no differences between groups in the levels of PPAR-γ protein relative to β-actin.

PPAR-γ gene expression in alveolar macrophages

PPAR-γ mRNA levels were significantly increased in alveolar macrophages from 13 COPD patients and 11 smokers compared to eight never-smokers ($p=0.01$ and $p=0.03$, respectively) relative to GAPDH (fig. 2c), with similar results obtained for GNB2L1 (online supplementary material and fig. S2a). No difference in PPAR-γ mRNA levels were observed between COPD patients and smokers ($p=0.8$) (fig. 2c).

Inhibition of LPS-induced cytokine production

Alveolar macrophages from 10 COPD patients, five smokers and seven never-smokers were used to evaluate the effects of rosiglitazone (0.1–3 μM) on LPS-induced TNF-α, CCL-5 and CXCL-8 production (fig. 3a–c). The absolute levels of LPS-induced TNF-α were significantly higher in COPD patients compared to smokers ($p<0.05$). There were no other differences between groups for TNF-α, CCL-5 or CXCL-8 production.

Rosiglitazone caused concentration-dependent inhibition of TNF-α, with maximal inhibition of 44% in COPD patients, 35% in smokers and 35% in never-smokers. Rosiglitazone inhibited LPS-induced CCL-5, with maximal inhibition of 65% in COPD patients, 45% in smokers and 56% in never-smokers. A plateau of inhibition for both TNF-α and CCL-5 was reached at 1–3 μM. The effects of rosiglitazone did not differ between groups for TNF-α or CCL-5 ($p=0.9$ and $p=0.6$, respectively). Rosiglitazone did not inhibit LPS-induced CXCL-8 in any group.

Dexamethasone significantly reduced TNF-α production to a greater degree than rosiglitazone in COPD patients, smokers and never-smokers ($p=0.02$, $p=0.0001$ and $p=0.01$, respectively) (fig. 3a). There was no difference in the reduction of CCL-5 by dexamethasone compared with rosiglitazone in COPD patients, smokers or never-smokers ($p=0.3$, $p=0.08$ and $p=0.9$, respectively). Dexamethasone, but not rosiglitazone, inhibited LPS-induced CXCL-8 in all groups (fig. 3c). There was no difference in the effect of dexamethasone between groups for any of the cytokines ($p>0.05$ for all comparisons).

The effects of rosiglitazone and dexamethasone on cell viability and basal cytokine release from alveolar macrophages were assessed (online supplementary material and figs S3 and S4). Dexamethasone but not rosiglitazone significantly inhibited basal release of TNF-α, CCL-5 or CXCL-8 ($p<0.05$ for all cytokines). Neither drug caused loss of cell viability at any concentration. These experiments also showed that the concentration of DMSO used (0.05%) had no effect on macrophage cytokine production or cell viability.

The effects of pioglitazone were compared to rosiglitazone in alveolar macrophages from 11 COPD patients. The effect of these ligands on TNF-α production was similar (online supplementary material and fig. S5).

Induction of M2 genes and PPAR-γ

Alveolar macrophages from eight COPD patients and six smokers were used to compare the effects of rosiglitazone (1 μM), dexamethasone (1 μM) and IL-4 on the expression levels of the M2 genes CD36, CD206 and HO-1 and PPAR-γ (fig. 4). Rosiglitazone significantly increased CD36 mRNA expression levels in COPD patients and smokers at 6 h and 24 h, while dexamethasone and IL-4 had no effect. Rosiglitazone significantly increased HO-1 mRNA expression levels in COPD patients after 24 h ($p=0.01$). Dexamethasone significantly decreased HO-1 mRNA levels after 6 h ($p=0.0002$) with levels returning to

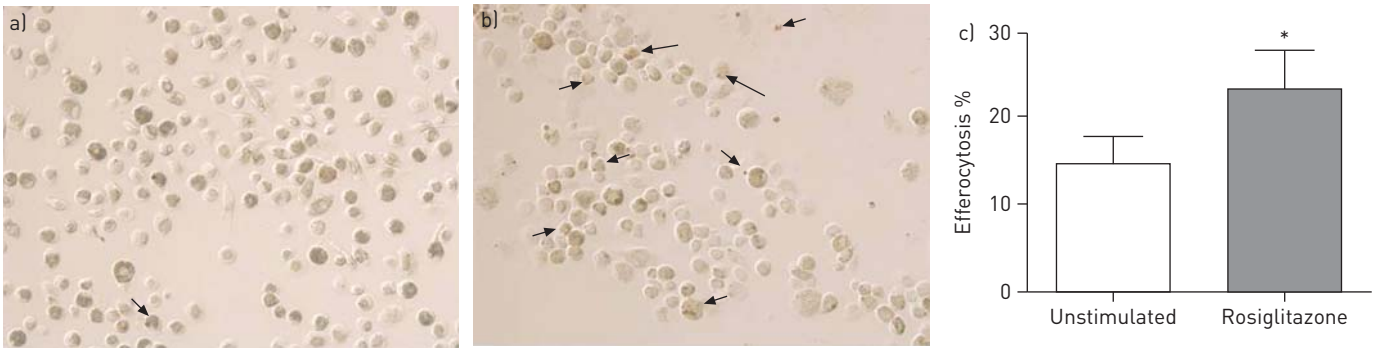

FIGURE 5 Effects of rosiglitazone on the ability of alveolar macrophages to efferocytose apoptotic neutrophils. Alveolar macrophages from chronic obstructive pulmonary disease patients (n=8) were a) left untreated or b) treated with rosiglitazone (1 μM) for 24 h. Apoptotic neutrophils were co-cultured with treated macrophages (5:1, respectively) for 90 min. Cells were fixed and stained with O-dianisidine (arrows indicate positively stained macrophages). c) Percentage of positively stained macrophages (% efferocytosis). Data are presented as mean±SEM. *: $p<0.05$.

baseline at 24 h. Rosiglitazone had no significant effect on CD206 mRNA expression levels, while dexamethasone and IL-4 significantly increased CD206 mRNA expression levels after 24 h in COPD patients ($p=0.02$ and $p=0.0001$, respectively).

IL-4 significantly increased PPAR-γ mRNA expression levels in COPD patients and smokers after 24 h, while rosiglitazone also increased PPAR-γ mRNA expression levels in COPD patients after 24 h. Dexamethasone had no significant effects on PPAR-γ mRNA expression levels.

Efferocytosis assay

Alveolar macrophages from eight COPD patients were used to investigate the effects of rosiglitazone (1 μM) on the efferocytosis of apoptotic blood neutrophils (fig. 5). The percentage of alveolar macrophages containing the stained neutrophils (fig. 5a and b) was significantly increased following 24 h treatment with

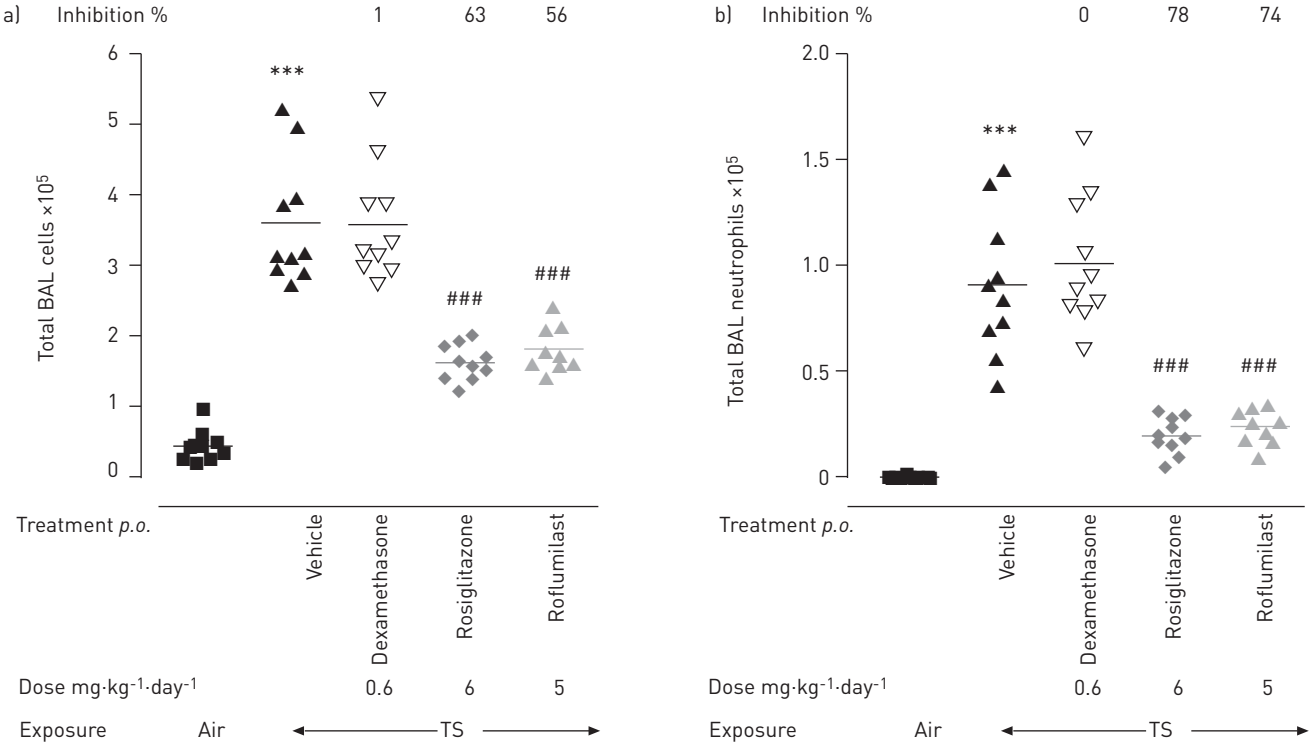

FIGURE 6 Effects of orally (*p.o.*) administered rosiglitazone on the subchronic tobacco smoke (TS) mouse model of pulmonary inflammation. Dexamethasone (0.3 mg·kg^{-1} twice daily), rosiglitazone (3 mg·kg^{-1} twice daily) and the phosphodiesterase-4 inhibitor roflumilast (5 mg·kg^{-1} once daily) were administered orally for 4 days on the subchronic TS mouse model of pulmonary inflammation. Total a) bronchoalveolar lavage (BAL) cells and b) neutrophils were counted. Data are presented as mean and individual points. Percentage inhibitions of vehicle control are shown above each condition. ***: $p<0.001$ above air control; ###: $p<0.001$ below vehicle control.

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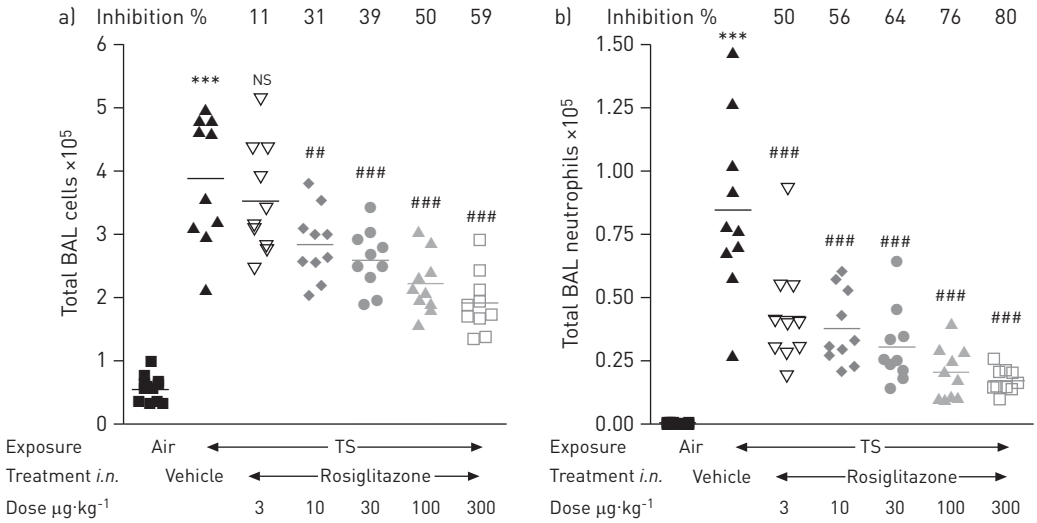

FIGURE 7 Effects of intranasally (*i.n.*) administered rosiglitazone on the subchronic tobacco smoke (TS) mouse model of pulmonary inflammation. Rosiglitazone (3–300 µg·kg^{-1} twice daily) was administered *i.n.* for 4 days. Total a) bronchoalveolar lavage (BAL) cells and b) neutrophils were counted. Data are presented as mean and individual points. Percentage inhibitions of vehicle control are shown above each condition. NS: nonsignificant. ***: $p<0.001$ above air control; ##: $p<0.01$ and ###: $p<0.001$ below vehicle control.

rosiglitazone (24%) above untreated cells (15%) ($p=0.02$). There was no effect of dexamethasone (1 µM) on efferocytosis (n=3 COPD patients) (online supplementary fig. S6).

Subchronic tobacco smoke mouse model of pulmonary inflammation

The effects of dexamethasone (0.3 mg·kg^{-1} twice daily), rosiglitazone (3 mg·kg^{-1} twice daily) and the phosphodiesterase-4 inhibitor roflumilast (5 mg·kg^{-1} once daily) administered orally for 4 days on the subchronic tobacco smoke mouse model of pulmonary inflammation was investigated (fig. 6). Rosiglitazone and roflumilast significantly inhibited the total number of bronchoalveolar lavage inflammatory cells and the number of neutrophils (mean inhibition 78%, $p<0.001$ and 74%, $p<0.001$, respectively), while dexamethasone had no effect.

Intranasal rosiglitazone at doses of 3–300 µg·kg^{-1} once daily for 4 days caused dose-dependent inhibition of tobacco smoke-induced pulmonary inflammation (fig. 7). Significant inhibition of total cells were observed at doses of 10 µg·kg^{-1} and above, while there was significant inhibition of neutrophil numbers at all doses (fig. 7). Consistent with the results observed for rosiglitazone, orally and intranasally administered pioglitazone (10 mg·kg^{-1} twice daily and 100 µg·kg^{-1} twice daily, respectively) also inhibited pulmonary inflammation in this tobacco smoke model (online supplementary figs 7 and 8).

Discussion

This is the first study using COPD alveolar macrophages to show that PPAR-γ agonists reduce pro-inflammatory cytokine production. Rosiglitazone suppressed both TNF-α and CCL-5 production from LPS-stimulated macrophages, although to a lower extent than dexamethasone. In the subchronic tobacco smoke mouse model, rosiglitazone and pioglitazone both attenuated airway neutrophilia, while corticosteroids had no effect. This differentiation between PPAR-γ agonists and corticosteroids for anti-inflammatory effects was also observed for macrophage phenotype and efferocytosis. Rosiglitazone increased the gene expression levels of CD36 and HO-1, which are involved in the efferocytosis of apoptotic cells and oxidative stress defence, respectively. These increases in M2 activity may be beneficial in tissue repair and resilience against cigarette smoke-induced tissue damage. We also showed that increased CD36 gene expression by rosiglitazone was associated with enhanced efferocytosis of apoptotic neutrophils.

It has been shown in mice that PPAR-γ deletion from alveolar macrophages is associated with mild pulmonary inflammation and failure of inflammation resolution [9]. In COPD alveolar macrophages, we observed that PPAR-γ exerts a level of control on certain elements of the acute inflammatory response (TNF-α and CCL-5 production) and also alters macrophage phenotype in a manner that aids the clearance of apoptotic neutrophils. These mouse and human data suggest that PPAR-γ plays a complex role in the control of inflammation, through specific actions that control acute inflammation and inflammation resolution. We were not able to dissect which of these two mechanisms was principally responsible for the effects of the

PPAR-γ ligands on airway neutrophilia caused by chronic cigarette smoke exposure. This is an important question, and could be addressed in the future by studying the time-course of the effects reported here.

Neutrophils are key effector cells in COPD, producing pro-inflammatory cytokines and proteases that mediate tissue destruction [30]. Rosiglitazone inhibits LPS-induced airway neutrophilia in an animal model [10], and here we show that rosiglitazone inhibits cigarette smoke-induced pulmonary neutrophilia in an animal model. H1N1 infection after 4 days of cigarette smoke exposure causes corticosteroid-resistant pulmonary inflammation in mice that is attenuated by pioglitazone [31]. This model of infection coupled with cigarette smoke-induced inflammation can be argued to resemble exacerbations of COPD. Inflammation in COPD is driven by a variety of stimuli, including oxidative stress, cytokines and toll-like receptor (TLR) stimulation by bacteria and viruses. Previous animal model data [10, 31] and the current results therefore show evidence of efficacy for PPAR-γ agonists in the prevention of inflammation caused by oxidative stress and/or TLR stimulation.

The activity of rosiglitazone in our subchronic tobacco smoke model was similar to roflumilast when administered orally. We used pioglitazone and rosiglitazone at the doses that are effective in animal models of diabetes [26, 27]. These PPAR-γ ligands have systemic side-effects when administered orally, such as cardiac failure [32]. COPD patients often suffer with cardiovascular comorbidities [33], making the use of oral PPAR-γ agonists in such patients difficult. The therapeutic index for the treatment of pulmonary diseases may be optimised by topical delivery, and is probably the only way that PPAR-γ agonists can be developed as potential COPD therapies. We observed that the anti-inflammatory effects of pioglitazone and rosiglitazone were maintained during intranasal administration, using lower doses. This supports the development of inhaled anti-inflammatory PPAR-γ agonists for the treatment of COPD that would minimise the potential for side-effects.

Human monocytes and monocyte-derived macrophages (MDMs) from smokers have higher PPAR-γ expression levels compared to never-smokers [5]. Maturation of monocytes towards macrophages increases PPAR-γ expression [34], and PPAR-γ expression in alveolar macrophages is 10-fold higher compared to monocytes [35]. We therefore focused on alveolar macrophages to measure PPAR-γ expression and function, rather than using monocytes or MDMs.

PPAR-γ gene expression levels were higher in alveolar macrophages from both COPD patients and smokers compared to never-smokers, but the levels of PPAR-γ protein expressed by macrophages and proportion of macrophages staining for PPAR-γ protein in tissue was similar in COPD patients compared to controls. This suggests that the upregulation of PPAR-γ mRNA expression in smokers and COPD patients is not translated into protein expression. Nevertheless, we show that PPAR-γ protein is expressed in a high proportion of alveolar macrophages. It is well known that the absolute number of alveolar macrophages is increased in patients with COPD [15], and our results indicate that PPAR-γ expression will be widespread in this cell population.

It has been reported that the PPAR-γ ligands 15d-PGJ_2 and troglitazone inhibited LPS-induced TNF-α production in alveolar macrophages and MDMs from healthy subjects [5, 35]. We now confirm this finding in COPD alveolar macrophages, with a similar effect size. The lack of effect of rosiglitazone on CXCL-8 has been reported previously using the human monocytic cell line THP-1 [36]. Nuclear hormone receptors such as PPAR-γ and glucocorticoid receptor suppress the upregulation of only a subset of LPS-induced genes in macrophages [37], and the lack of effect of rosiglitazone on CXCL-8 indicates that this gene is not regulated by PPAR-γ.

PPAR-γ expression is known to be increased by IL-4 in murine macrophages and human monocytes [38, 39] and we now confirm this using COPD alveolar macrophages. IL-4 drives macrophages towards an M2 phenotype, and it has been shown that airway levels of IL-4 are increased in patients with chronic bronchitis [40]. This cytokine may, therefore, play a role in the upregulation of PPAR-γ mRNA expression in COPD macrophages. PPAR-γ ligands upregulate PPAR-γ expression in human monocytes and MDMs [5], and we confirm this positive feedback mechanism in COPD alveolar macrophages. The effect of IL-4 and rosiglitazone on PPAR-γ mRNA expression did not occur with dexamethasone, further differentiating the effects of PPAR-γ ligands and corticosteroids on COPD alveolar macrophages.

We observed that PPAR-γ activation upregulated CD36 and HO-1 mRNA expression, but had no effect on CD206. Our findings for CD36 have also previously been observed in healthy alveolar macrophages [35]. PPAR-γ activation stabilises HO-1 mRNA expression in mouse peritoneal macrophages [7]. Cigarette smoke extract upregulates macrophage HO-1 mRNA levels [41] and HO-1 levels are decreased in the lungs of COPD patients compared to controls [42]. The effects of PPAR-γ activation on CD36 mRNA did not occur with dexamethasone, and represent potential molecular mechanisms that may be therapeutically important in defence against oxidative stress.

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Macrophage-specific deletion of PPAR-γ in mice decreases the efferocytosis of apoptotic cells, while PPAR-γ activation enhances efferocytosis by MDMs [12] and alveolar macrophages from healthy donors [35]. Here we observe that rosiglitazone treatment of COPD alveolar macrophages increases efferocytosis of apoptotic neutrophils. PPAR-γ upregulation of efferocytosis may be mediated through the upregulation of CD36, which is essential for phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages [43, 44]. Anti-CD36 antibodies block PPAR-γ-mediated efferocytosis by healthy alveolar macrophages [35]. This may be a therapeutically advantageous function of PPAR-γ agonists in promoting inflammation resolution.

We obtained macrophages from patients undergoing cancer surgery. Only a minority of these patients are never-smokers, which limited our scope to perform experiments in this patient group. Consequently, some of our experiments did not contain never-smoker controls.

We have shown biological actions of a PPAR-γ agonist on COPD-relevant models including cigarette smoke-induced pulmonary inflammation and skewing of macrophage phenotype to aid the clearance of apoptotic neutrophils. The development of inhaled PPAR-γ agonists may have important effects on associated COPD clinical outcomes such as exacerbation rates and the rate of disease progression, while limiting the side-effect profile. The translation into COPD clinical trials of the *in vitro* and *in vivo* findings reported here is now required.

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