On-line Supplement

Methods

ELISA analysis

Supernatant levels of TNF-α, CCL5 and CXCL-8 were determined by ELISA as per the manufacturers' instructions (R&D Systems, Abingdon, UK). The lower limits for quantification were 15.623pg/ml for TNFα; 15.63pg/ml for CCL5; 31.25pg/ml for CXCL-8.

Macrophage culture

Alveolar macrophages from 5 smokers were incubated with rosiglitazone, dexamethasone or vehicle control containing DMSO (0.05%) for 24hrs. 1% Triton-X was used as a cell death positive control. Supernatant TNF-α, CCL5 and CXCL-8 levels were determined by ELISA. Alveolar macrophage membrane integrity was assessed by analysing the release of cytoplasmic lactate dehydrogenase (LDH) in the supernatant using the LDH based *in vitro* toxicology assay kit (Sigma) as follow; Briefly, 50 µl of culture supernatant was placed in 96-well plate with 100 µl lactate dehydrogenase assay mixture. Plates were incubated in the dark for 20 mins. Reactions were stopped by adding 15 µl 1M HCl to each well. Absorbance was measured using the 490 nm wavelength on the spectrophotometer.

Western blot analysis

Alveolar macrophages from 5 COPD patients, 8 S and 4 NS were lysed using RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) containing phosphatase (Sigma-Aldrich) and protease inhibitors (Calbiochem, Nottingham, UK). Cell lysates were diluted in sample buffer [62.5 mM Tris, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.01% bromphenol blue, pH 6.8] were electrophoresed on SDS-

polyacrylamide gels (10%) and transferred to Hy-bond ECL membranes (Whatman, Kent, UK). Membranes were incubated with blocking buffer [5% dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS/Tween 20) for 4 h at room temperature and then incubated with mouse anti-human PPAR-γ antibody (clone E-8, Santa Cruz Biotechnology) (diluted in blocking buffer) at 4 °C overnight. After washing in TBS/Tween 20, the membranes were incubated for 60 min with a peroxidase-conjugated rabbit anti-mouse antibody (Sigma) (diluted in wash buffer), washed again, and the antibody-labeled proteins were visualized by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Densitometric analysis was performed by normalising band density to that for β-actin using Quantity One v4.6.1 software (Bio-Rad, Hertfordshire, UK).

Immunohistochemistry

Fresh lung tissues, as far distal to tumour as possible, were fixed in formalin and paraffin embedded. Lung tissues were cut into 4μm sections and lifted onto polysine coated glass slide. Following heat induced epitope retrieval (HIER) in Tris-EDTA buffer pH9 (10mM Tris Base, 1mM EDTA, 0.5% Tween 20) microwaved for 20mins at 800W. Mouse anti-human PPAR-γ antibody (clone E-8, Santa Cruz Biotechnology) diluted in 1.5% normal serum (Vector Labs, Peterborough, UK) was applied overnight at 4°C. PPAR-γ was detected using biotinylated horse anti-mouse IgG secondary antibody (Vector) in conjunction with an avidin-biotin peroxidase complex (Vector) and diaminobenzidine (DAB) substrate. Sections were counterstained with Meyer's haematoxylin (Sigma, Poole, UK). Incubation in suitable isotype control antibody (Vector) was used as negative controls.

The number of PPAR-γ positive macrophages was calculated within the alveolar space. At least 200 macrophages, defined as mononuclear cells with well represented cytoplasm present

in the alveolar spaces and not attached to the alveolar walls, were counted and the number of PPAR-γ positive cells was presented as a percentage. Digital micrographs were obtained using a Nikon Eclipse 80i microscope equipped with a QImaging digital camera and ImagePro Plus 5.1 software (MediaCybernetics, Marlow, UK). All micrographs were stored as tiffs and were cropped, where applicable, using Adobe Photoshop 6.0.

Immunofluorescence

Fresh lung tissues, as far distal to tumour as possible, were fixed in formalin and paraffin embedded. Lung tissues were cut into 4µm sections and lifted onto polysine coated glass slide. Following heat induced epitope retrieval (HIER) in Tris-EDTA buffer pH9 (10mM Tris Base, 1mM EDTA, 0.5% Tween 20) microwaved for 20mins at 800W. Mouse anti-human PPAR-γ antibody (clone E-8, Santa Cruz Biotechnology) diluted in 1.5% normal serum (Vector Labs, Peterborough, UK) was applied overnight at 4°C. PPAR-γ was detected by incubating sections in Alexa 568 conjugated rabbit anti-mouse IgG (1:200) for 90mins at 37°C. Cell nuclei were detected using 3, 3'- diaminobenzadine (DAPI, Invitrogen, Paisley, UK).

Lung macrophages were seeded in chamber slides for 24hrs then fixed in 4% paraformaldehyde for 10mins. Following washes in PBS, macrophages were incubated in 1.5% normal serum and PPARγ detected as described above.

Digital micrographs were obtained as described above.

PCR

Total RNA was purified from cell lysates using RNeasy kits (Qiagen, Crawley, UK) according to manufactuers' instructions. DNA contamination was prevented by on-column

addition of DNase (Qiagen, Crawley, UK) according to manufactures' instructions. TaqMan reverse transcription- PCR (RT-PCR) was performed on RNA from alveolar macrophages. Using the Verso^{TM} 2-Step QRT-PCR kit (Thermo Scientific, Surry, UK) total RNA was converted to cDNA, 50ng of which was used in 25µl reactions containing specific primers and probes for PPAR-γ, premade Taqman gene expression assay for CD36, mannose receptor (MR), hemeoxygenase-1 (HO-1) or endogenous controls glyceraldehyde-3phosphate dehydrogenase (GAPDH) and gene beta-peptide 2-like 1 (GNB2L1) (Applied Biosystems). Thermal cycling was carried out on a Stratagene MX3005P (Agilent Technologies, West Lothian, UK). Relative expression levels were determined using the ΔCt method normalizing to the endogenous control or the ΔΔCt method normalizing to the endogenous control and to unstimulated samples.

Blood neutrophil isolation

Polymorphonuclear (PMN) leukocytes were isolated from peripheral blood of healthy subjects as previously described [28]. Briefly, venous blood was layered onto MonoPoly Resolving medium (MP Biomedicals, Cambridge, UK) and centrifuged at 800g for 45mins at 18ºC. PMNs were resuspended in phenol red free RPMI (Sigma Aldrich, Poole, UK).

Efferocytosis assay

Alveolar macrophages were incubated with rosiglitazone (1µM), dexamethasone (1µM) or media alone for 24hrs. Blood neutrophils were cultured for 20hrs in RPMI to cause apoptosis which was verified using TUNEL assay (Supplementary Figure 9). Treated macrophages were co-cultured with apoptotic neutrophils (1:5 respectively) for 90mins, washed in PBS and fixed with 4% paraformaldehyde for 10mins before final wash. Cells were stained for

myeloperoxidase with O-diansidine stain at 37ºC for 15mins. Digital micrographs were obtained as described previously.

TUNEL assay for detection of apoptotic PMN

DNA fragmentation was analysed using the In Situ Cell Death Detection Kit, Fluorescein kit (Roche Products Limited, UK). Briefly, cytospin slides of freshly isolated PMNs, 20h post culture PMNs and PMNs treated with 0.1% Triton X as a positive control. Cells were fixed in 4% paraformaldehyde for 30 mins before rinsed in PBS. 50µl of Label solution was added to the negative-TUNEL control and 50µl enzyme solution (1/10 in label solution) was added to each of the remaining slides. Slides were protected from light and incubated for 1hr at 37^{o}C. Cells were counterstained with DAPI (1/50,000 in PBS for 5min in the dark) and analysed under fluorescent microscope. The percentage of cells undergoing DNA fragmentation was calculated

Sub-chronic tobacco smoke mouse model of pulmonary inflammation

A.1 Animals

Female inbred C57BL/6J (or C57BL/6JAX) mice (body weights on initial day of use: ~15 - 22g) were obtained from Charles River labs, full barrier bred and certified free from specified micro-organisms on receipt. The mice were housed, up to 5 per cage, in individually ventilated, polycarbonate solid bottomed cages (IVC) with grade 8 aspen chip bedding. Environment (airflow, temperature and humidity) within the cages was controlled by the IVC system (Techniplast). Food (RM 1, Special Diet Services) and water were provided *ad libitum*. In addition they received once a weekly, a teaspoon sized amount of RM 1 forage

granules). Individual animals were identified by unique coloured “Pentel” markings on their tails, weighed and randomly assigned to treatment groups.

A.2 Exposure of animals to TS daily for 4 consecutive days

In this exposure protocol, mice were exposed in groups of 5 in individual clear polycarbonate chambers (27 cm x 16 cm x 12 cm). The TS from the cigarettes was allowed to enter the exposure chambers at a flow rate of 100 ml/min. In order to minimise any potential problems caused by repeated exposure to a high level of TS (6 cigarettes), the exposure of the mice to TS was increased initially from 4 cigarettes at the start of the exposure period (day 1) to a maximum of 6 cigarettes on day 2. The exposure schedule used in this study was therefore as follows:

Day 1: 4 cigarettes (approximately 32 min exposure)

Day 2-4: 6 cigarettes (approximately 48 min exposure)

One further group of mice was exposed to air on a daily basis for equivalent lengths of time as sham controls (no TS exposure).

A.3 Bronchoalveolar lavage and cytospin analysis

Bronchoalveolar lavage was performed as follows:

The trachea was cannulated using a Portex nylon intravenous cannula (pink luer fitting) shortened to approximately 8 mm. Phosphate buffered saline (PBS) was used as the lavage fluid. A volume of 0.4 ml was gently instilled and withdrawn 3 times using a 1ml syringe and then placed in an Eppendorf tube and kept on ice prior to subsequent determinations.

Cell counts:

Lavage fluid was separated from cells by centrifugation and the supernatant decanted and frozen for subsequent analysis. The cell pellet was re-suspended in a known volume of PBS and total cell numbers calculated by counting a stained (Turks stain) aliquot under a microscope using a haemocytometer.

Differential cell counts were performed as follows:

The residual cell pellet was diluted to approximately 10^5 cells per ml. A volume of 500 µl was placed in the funnel of a cytospin slide and centrifuged for 8 min at 800 rpm. The slide was air dried and stained using Wrights/Giemsa stain as per the proprietary instructions. When dried and cover-slipped, differential cells were counted using light microscopy. Up to 400 cells were counted for each slide. Cells were differentiated using standard morphometric techniques.

Results

PPAR-γ protein expression in alveolar macrophages

PPAR-γ was detected using immunofluorescence in alveolar macrophages within a lung tissue section (Supplementary Figure 1 A-B) and perfused alveolar macrophages cultured in chamber slides (Supplementary Figure 1 D-E). PPAR-γ staining was predominately nuclear as shown by co-localisation with the nuclear specific stain DAPI (indicated by white arrows in Supplementary Figure 1 C and F).

PPAR-γ gene expression in alveolar macrophages

PPAR-γ mRNA levels were significantly increased in alveolar macrophages from 13 COPD patients and 11 S compared to 8 NS ($p=0.01$ and $p=0.02$ respectively) relative to GNB2L1 (Supplementary Figure 2 A). No difference in PPAR-γ mRNA levels was observed between COPD patients and S ($p=0.8$). There was no difference in GAPDH mRNA levels relative to GNB2L1 between groups ($p>0.05$ all comparisons; Supplementary Figure 2 B).

Effects of rosiglitazone and dexamethasone on basal cytokine production from alveolar macrophages

Alveolar macrophages from 5 smokers were incubation with rosiglitazone (3μM), dexamethasone (3μM) or vehicle control containing DMSO (0.05%) for 24hrs (Supplementary Figure 3). Rosiglitazone and vehicle control did not inhibit basal release of TNF-α, CCL5 or CXCL-8 from alveolar macrophages. Dexamethasone significantly inhibited basal release of TNF-α, CCL5 or CXCL-8 from alveolar macrophages ($p<0.05$ for all cytokines).

Effects of rosiglitazone and dexamethasone on alveolar macrophage cell membrane integrity

Alveolar macrophages from 5 smokers were incubated with rosiglitazone (3μM), dexamethasone (3μM) or vehicle control containing DMSO (0.05%) for 24hrs with and without LPS (1μg/ml). Cells were also treated with 1% Triton-X for 24hrs as a positive death control which significantly increased LDH activity within the culture supernatants above unstimulated control ($p<0.001$) (Supplementary Figure 4). LPS stimulation did not increase LDH activity. Neither rosiglitazone nor dexamethasone increased LDH activity in the presence or absence of LPS. These data confirm that none of the culture conditions used for experiments in this paper caused loss of alveolar macrophage membrane integrity and therefore viability.

Comparative potency of rosiglitazone and pioglitazone on LPS stimulated TNF-α production from alveolar macrophages

The effects of rosiglitazone were compared to pioglitazone in alveolar macrophages from 11 COPD patients (Supplementary Figure 5). There was no significant difference between the maximal inhibition of rosiglitazone (41%) compared to pioglitazone (33%).

Detection of DNA fragmentation in 20h PMN

20h culture of PMN caused a significant increase in DNA-fragmentation, a measure of apoptosis, compared to freshly isolated (0h) PMN (Supplementary Figure 9).

Figure Legends

Supplemtary Figure 1. Representative photomicrographs for the detection of PPARγ (Red) by immunoflurescence within (A-C) alveolar macrophages within resective tissue section and (D-E) perfused alveolar macrophages cultured in chamber slides. Cell nuclei (B&E) were

detected using DAPI (Blue). Composite images (C&F) highlight the nuclear location of PPARγ (arrows).

Supplemtary Figure 2. Alveolar macrophage expression of PPAR-γ within human lung tissue. Data shows levels of PPAR-γ (A) and GAPDH (B) mRNA expression relative to GNB2L1 in alveolar macrophages. Data shown are median ± range of relative PPAR-γ expression in alveolar macrophages from 13 COPD patients, 11 S and 8 NS. Relative expression levels were determined using the ΔCt method normalizing to the endogenous control. * $P<0.05$ compared to NS

Supplementary Figure 3. Effects of rosiglitazone and dexamethasone on basal cytokine production from alveolar macrophages. Data shown are mean ± SEM of TNF-α (A), CCL5 (B) or CXCL-8 (C) production in alveolar macrophages from S (n=5).
* = significantly below unstimulated control ($p<0.05$).

Supplementary Figure 4. Effects of rosiglitazone and dexamethasone on alveolar macrophage cell membrane integrity. Data show mean ± SEM for LDH activity.
*** = significant increase in LDH activity compared to unstimulated control ($p<0.001$)
Supplementary Figure 5. Comparative potency of rosiglitazone and pioglitazone on LPS stimulated TNF-α production from alveolar macrophages. Data shown are mean ± SEM of LPS-induced TNF-α production in alveolar macrophages from COPD patients (n=11) treated with rosiglitazone (0.1-3 μM), pioglitazone (0.1-3 μM) or vehicle control (0.05% DMSO). Percentage inhibition in text above each bar. ** = significantly below Vehicle control ($p<0.01$)

Supplementary Figure 6. Effects of dexamethasone on the ability of alveolar macrophages to efferocytose apoptotic neutrophils.

Alveolar macrophages from 3 COPD patients were left untreated or treated with dexamethasone (1μM) for 24 hrs. Apoptotic neutrophils were co-cultured with treated macrophages (5:1 respectively) for 90mins. Cells were fixed and stained with O-diansidine. Data shown are individual percentage of positively stained macrophages (% Efferocytosis).

Supplementary Figure 7. Effects of orally administered pioglitazone on the sub-chronic tobacco smoke mouse model of pulmonary inflammation. Piogiltazone (10mg / kg bid) was administered orally for 4 days on the sub-chronic tobacco smoke mouse model of pulmonary inflammation. Total BAL cells (A) and neutrophils (B) were counted. Data shown are mean and individual points. Percentage inhibitions of vehicle control are shown above each condition. One way ANOVA was performed followed by Bonferroni's Multiple Comparison Test. p.o = per os (oral administration). *** = significantly above air control ($p<0.001$).

= significantly below Vehicle control ($p<0.001$)

Supplementary Figure 8. Effects of intranasally administered pioglitazone on the sub-chronic tobacco smoke mouse model of pulmonary inflammation. Pioglitazone (100μg / kg bid) was administered intranasally for 4 days on the sub-chronic tobacco smoke mouse model of pulmonary inflammation. Total BAL cells (A) and neutrophils (B) were counted. Data shown are mean and individual points. Percentage inhibitions of vehicle control are shown above each condition. One way ANOVA was performed followed by Bonferroni's Multiple Comparison Test. i.n = intranasal administration. *** = significantly above air control ($p<0.001$).

##, ### = significantly below Vehicle control ($p<0.01$, $p<0.001$ respectively)

Supplementary Figure 9. Detection of DNA fragmentation in 20h PMN. Freshly isolated PMNs (0h PMN), 20h post culture PMNs (20h PMN) and PMNs treated with 0.1% Triton X as a positive control (0h PMN + 0.1% Triton X). Images are representative of three independent experiments. Percentage of positive stained cells written below.