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Research Article

The PPAR-γ agonist pioglitazone exerts proinflammatory effects in bronchial epithelial cells during acute *Pseudomonas aeruginosa* pneumonia

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Abstract

Pseudomonas aeruginosa is a common respiratory pathogen that causes injurious airway inflammation during acute pneumonia. Peroxisome proliferator-activated receptor (PPAR)-γ is involved in the regulation of metabolic and inflammatory responses in different cell types and synthetic agonists of PPAR-γ exert anti-inflammatory effects on myeloid cells *in vitro* and in models of inflammation *in vivo*. We sought to determine the effect of the PPAR-γ agonist pioglitazone on airway inflammation induced by acute *P. aeruginosa* pneumonia, focusing on bronchial epithelial cells. Mice pretreated with pioglitazone or vehicle (24 and 1 h) were infected with *P. aeruginosa* via the airways. Pioglitazone treatment was associated with increased expression of chemokine (*Cxcl1, Cxcl2,* and *Ccl20*) and cytokine genes (*Tnfa, Il6,* and *Cfs3*) in bronchial brushes obtained 6 h after infection. This pro-inflammatory effect was accompanied by increased expression of *Hk2* and *Pfkfb3* genes encoding rate-limiting enzymes of glycolysis; concurrently, the expression of *Sdha*, important for maintaining metabolite flux in the tricarboxylic acid cycle, was reduced in bronchial epithelial cells of pioglitazone treated-mice. Pioglitazone inhibited bronchoalveolar inflammatory responses measured in lavage fluid. These results suggest that pioglitazone exerts a selective proinflammatory effect on bronchial epithelial cells during acute *P. aeruginosa* pneumonia, possibly by enhancing intracellular glycolysis.

Keywords: pioglitazone, **Pseudomonas aeruginosa**, innate immunity, inflammation

Abbreviations: BALF, bronchoalveolar lavage fluid; CFU, colony-forming units

Introduction

Pseudomonas (P.) aeruginosa is a Gram-negative bacterium that causes severe infections of the lower respiratory tract in patients with a compromised immune system or impaired airway clearance mechanisms [1, 2]. These infections are associated with high mortality rates worldwide [1, 3]. Antibiotics, the first-line treatment for lower respiratory tract infections, exclusively target the bacterium, and the emergence of multidrug resistant *P. aeruginosa* strains has become a major burden [1, 2, 4]. Hence, the development of new therapeutic strategies able to modulate the host immune response during *P. aeruginosa* pneumonia is of great interest. *P. aeruginosa* lung infections have been reported to induce a hyperinflammatory state that can injure the airways [5, 6]. Therefore, targeting exacerbated inflammatory responses during *Pseudomonas* pneumonia represents a potential novel therapeutic option that might improve the clinical outcome of patients suffering from this infection.

The peroxisome proliferator-activated receptor (PPAR)-γ is a major regulator of cell metabolism and inflammatory responses in different cell types [7, 8]. Thiazolidinediones are synthetic agonists of PPAR-γ that were originally designed as anti-diabetics [7]. However, due to their anti-inflammatory effects PPAR-γ agonists have received much attention as potential drugs capable of modulating airway inflammatory responses in the context of bacterial infections [8–11]. Treatment with the PPAR-γ agonist pioglitazone of mice with *P. aeruginosa* pneumonia reduced tumor necrosis factor (TNF)-α and interleukin (IL)-1β release in bronchoalveolar lavage fluid (BALF) [12]. Moreover, PPAR-γ activation augmented phagocytosis and killing of *P. aeruginosa* by macrophages, and pretreatment with the PPAR-γ agonist pioglitazone resulted in reduced bacterial burdens in the lungs of mice infected with this bacterium via the airways [13].

The respiratory epithelium plays a major role in innate host defense during *P. aeruginosa* pneumonia, at least in part

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through recognition of flagellin, the principal component of the flagellum of *Pseudomonas*, by epithelial toll-like receptor 5 [14–18]. Knowledge of the effect of PPAR-γ agonists on the respiratory epithelium during bacterial infection of the airways is limited. Therefore, we here sought to determine the *in vivo* effect of pioglitazone on airway epithelial cells during murine *P. aeruginosa*-induced lung infection and inflammation.

Materials and methods

Ethical statement

All experiments were reviewed and approved by the Central Authority for Scientific Procedures on Animals (CCD) and the Animal Welfare Body (IvD) of the Amsterdam-UMC, University of Amsterdam. The animal care and use protocol adhered to the Dutch Experiments on Animals Act (WOD) and European Directive of 22 September 2010 (Directive 2010/63/EU) in addition to the Directive of 6 May 2009 (Directive 2009/41/EC).

Animals

C57BL/6NCrl mice were purchased from Charles River (Maastricht, The Netherlands). Mice were kept in the Animal Research Institute Amsterdam facility in Amsterdam UMC under standard care. All experiments were carried out with 8–10-week-old female mice.

P. aeruginosa infection

P. aeruginosa (PAK) was kindly provided by Mustapha Si-Tahar (INSERM, Tours, France). *P. aeruginosa* was cultured in Luria-Bertani (LB) medium at 37°C to log-phase; 5×10^6 colony-forming units (CFU) in 50 µl of normal saline was administered intranasally as described previously [18, 20, 22]. Twenty four and one hour prior to the infection, mice received 20 mg/kg body weight of pioglitazone (or vehicle; 10% DMSO in PBS) by intraperitoneal injection, a dose previously shown to exert an anti-inflammatory effect in a model of *Klebsiella*-induced pneumonia and sepsis [9]. Mice were terminated 6 h after induction of pneumonia.

BALF and epithelial brushes

BALF was collected by flushing the lungs via the trachea twice with 500 µl of 0.2 mM EDTA/PBS. Thereafter, epithelial brushes were performed as described by Chen K. *et al.* [34]. Briefly, PE50 tubing was sanded with sandpaper (P240) and treated with RNaseZap (Thermo Fisher Scientific, Waltham, MA, USA) before being inserted into the main bronchus via the trachea to collect epithelial tissue for RNA extraction. BALF was spun down at 1250 RPM for 10 min at 4°C, supernatants were stored for cytokine determination and cells were used to determine total cell counts (Beckman Coulter, Fullerton, CA, USA) and FACs analysis as described below.

Chemokine and cytokine measurements

All chemokines and cytokines were measured by mouse-specific ELSA's according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Flow cytometry

Cells were stained and analyzed on a FACS Canto II cytometer (BD Biosciences). The following antibodies were purchased from BD Biosciences unless otherwise listed: CD45 (30-F11), CD11c (HL3), CD11b (M1/70), Siglec-F (E50-2440), Ly-6C (AL-21), and Ly-6G (1A8; Biolegend, San Diego, CA, USA). Fixable Viability Dye kit (BD Biosciences) was used to exclude dead events. After gating for live $CD45^+$ cells, alveolar macrophages were defined as $CD11c^+SiglecF^+CD11b^-$ cells; neutrophils as $CD11c^-CD11b^+Ly6G^+$ cells; and monocytes as $CD11c^-CD11b^+Ly6G^-$ cells. Ly6C expression was used to differentiate patrolling ($Ly6C^-$) from inflammatory ($Ly6C^+$) monocytes. Data were analyzed using FlowJo software (Treestaar Inc, Ashland, OR, USA).

mRNA extraction and RT-PCR

Total RNA from epithelial brushes was isolated using a Nucleospin RNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. cDNA was synthetized using the M-MLV Reverse Transcriptase kit (Promega, Madison, WI, USA) in the presence of RNase

Table 1: Primer sequences for the quantitative RT-PCR.

Gene	Forward sequence	Reverse sequence
Actb	CTCTGGCTCCTAGCACCATGAAGA	GTAAAACGCAGCTCAGTAACAGTCCG
Pparg	CCTGCGGAAGCCCTTTGGTGACT	CCTCGATGGGCTTCACGTTCAGCA
Cxcl1	CCACTGCACCCAAACCGAAG	TCCGTTACTTGGGGACACCT
Cxcl2	CACTCTCAAGGGCGGTCAA	TCTTTGGTTCTTCCGTTGAGG
Ccl20	AGACAGATGGCCGATGAAGC	CTGCTTTGGATCAGCGCACA
Tnfa	CGAGTGACAAGCCTGTAGCC	CCTTGAAGAGAACCTGGGAGT
Il6	CTTCCTACCCCAATTTCCAATGCT	TCTTGGTCCTTAGCCACTCCTT
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Csf3	CCTGGAGCAAGTGAGGAAGA	CAGCTTGTAGGTGGCACACA
Hk2	GTGTGCTCCGAGTAAGGGTG	CAGGCATTCGGCAATGTGG
Pfkfb3	CAACTCCCCAACCGTGATTGT	TGAGGTAGCGAGTCAGCTTCT
Sdha	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
Cd36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
Acsl1	TGCCAGAGCTGATTGACATTC	GGCATACCAGAAGGTGGTGAG
Cpt1	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT

inhibitor (Thermo Fisher Scientific) with 300 ng of DNase I (Roche) treated total RNA. qPCR was performed on LightCycler 480 (Roche) using the SensiFAST SYBR No-ROX Kit (Bioline, London, UK). Data were normalized to *Actb* as a housekeeping gene. Primers used in this study are listed in Table 1.

Statistical analysis

All the analyses were done using GraphPad Prism 7.03. The number of replicates and the statistical tests used for each data is described in the figure legends. In most cases, Welch's *t*-test was used. A P value < 0.05 was considered statistically significant.

Results

Infection with *P. aeruginosa* reduces Pparg expression in bronchial epithelial cells in vivo

Previous studies reported decreased expression of *PPARG* in BALF cells from patients infected with *P. aeruginosa* [19] and in *in vitro* infected macrophages [13]. To determine the effect of *P. aeruginosa* infection on *Pparg* expression in airway epithelial cells *in vivo*, we inoculated mice intranasally with this bacterium and collected bronchial brushes 6 thereafter. Our group previously showed that bronchial brushes are highly enriched for bronchial epithelial cells as determined by the expression of the pan-epithelial cell marker *Epcam* and the club cell marker *Scgb1a1* [20–22]. Bronchial brushes had reduced

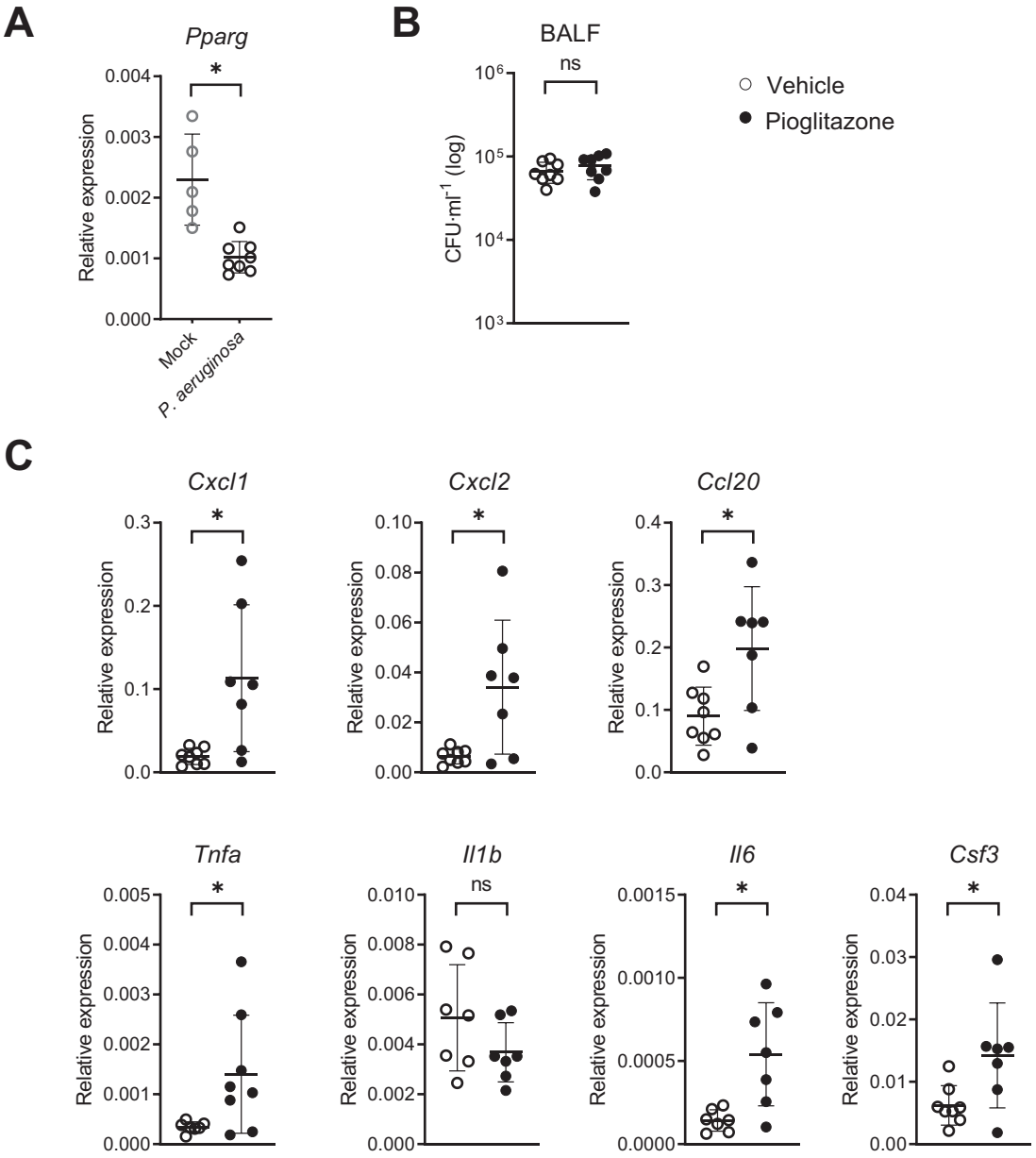

Figure 1: (A) Relative mRNA expression for *Pparg* analyzed by RT-PCR in lung epithelial brushes 6 h after *P. aeruginosa* infection or PBS administration (mock) via the airways. (B) Bacterial loads in BALF in vehicle and pioglitazone treated mice 6 h following induction of pneumonia. (C) Relative mRNA expression for the indicated immune-mediators analyzed by RT-PCR in lung epithelial brushes of mice 6 h after *P. aeruginosa* infection pre-treated with pioglitazone or vehicle. Graphs show mean and SD, and every dot represents one individual mouse. P values were calculated using Welch's *t*-test. *P < 0.05, ns (not significant).

Pparg expression after infection with *P. aeruginosa* relative to uninfected mice (Fig. 1A).

Pioglitazone enhances the expression of chemokine and cytokine genes in bronchial epithelial cells during acute P. aeruginosa pneumonia

To obtain insight into the effect of PPAR-γ stimulation in the respiratory epithelium during acute *Pseudomonas* pneumonia we treated mice with the PPAR-γ agonist pioglitazone or vehicle 24 and 1 h prior to *P. aeruginosa* infection via the airways and collected bronchial brushes and BALF 6 h thereafter. Pioglitazone did not affect bacterial loads in BALF (Fig. 1B), allowing an unbiased comparison of inflammatory responses between treatment groups (i.e. not confounded by different proinflammatory environments secondary to differences in bacterial numbers). Our group recently reported that acute *Pseudomonas* pneumonia is associated with a strong induction of genes encoding mediators of mucosal immunity in bronchial epithelial cells relative to bronchial brushes from uninfected control mice [20, 21]. Relative to vehicle control treatment, pioglitazone administration enhanced the expression of chemokine genes *Cxcl1*, *Cxcl2*, and *Ccl20* and cytokine genes *Tnfa*, *Il6*, and *Csf3* in bronchial epithelial cells, while not affecting *Il1b* expression (Fig. 1C).

Effect of pioglitazone on bronchoalveolar inflammatory responses during acute *Pseudomonas* pneumonia

Our finding of an immune-enhancing effect of pioglitazone on bronchial epithelial cells was remarkable, considering the widely reported anti-inflammatory effects of PPAR-γ agonists in models of lung inflammation [8, 10, 11, 23], including murine *Pseudomonas* pneumonia [12, 13]. We, therefore, investigated the effect of pioglitazone regarding inflammation in the bronchoalveolar compartment in our model. To this end, we harvested BALF 6 h after infection with *P. aeruginosa* and analyzed chemokine/cytokine release (Fig. 2) and cell influx (Fig. 3). Pioglitazone reduced CXCL1, TNF-α, and IL-1β protein levels in BALF, while not affecting CXCL2, CCL20, and IL-6 concentrations. BALF levels of granulocyte colony-stimulating factor (G-CSF, encoded by *Csf3*) were increased in pioglitazone-treated mice when compared to mice that received vehicle control. Whilst pioglitazone did not impact total cell counts in BALF after *P. aeruginosa* infection, it reduced neutrophil numbers. Although total monocyte numbers in BALF were low in both treatment groups, pioglitazone treatment was associated with increased monocyte numbers, which was caused by the rises in both $Ly6C^-$ (patrolling) monocytes and $Ly6C^+$ (inflammatory) monocytes. The number of alveolar macrophages was not altered by pioglitazone. These results are in agreement with previous studies [12, 13] and suggest a pro-inflammatory effect of pioglitazone specifically on respiratory epithelial cells.

Pioglitazone increases the expression of genes involved in glycolysis

We recently reported an important role for glycolysis in the induction of inflammatory responses, including CXCL1, CCL20, and G-CSF release, by human bronchial epithelial cells stimulated with flagellin, an important immune-enhancing component of *P. aeruginosa* [24]. In accordance, inhibition of glycolysis by rapamycin abrogated *Cxcl1*, *Cxl2*, and *Csf3* expression in bronchial brushes of mice administered

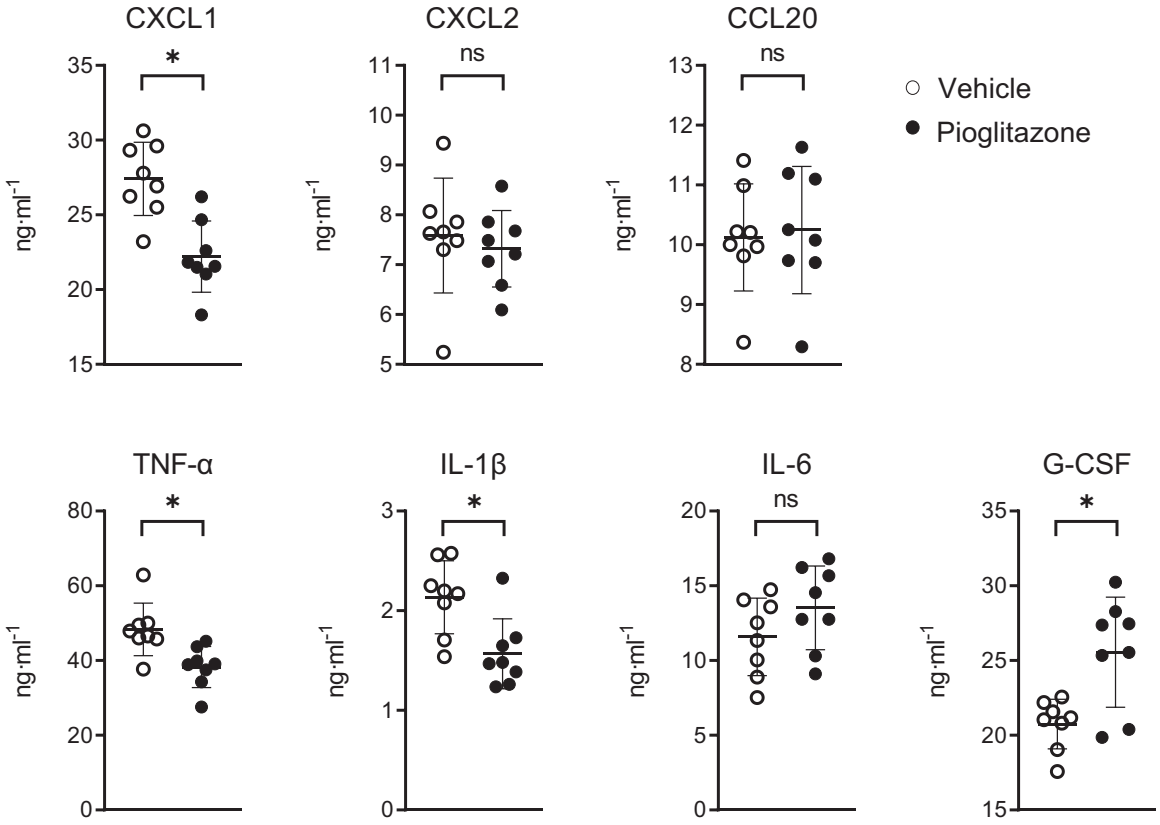

Figure 2: Chemokines and cytokines concentration in BALF after 6 h inoculation with *P. aeruginosa* to mice pre-treated with pioglitazone or vehicle. Graphs show mean and SD, and every dot represents one individual mouse. *P* values were calculated using Welch's *t*-test. $*P < 0.05$, ns (not significant).

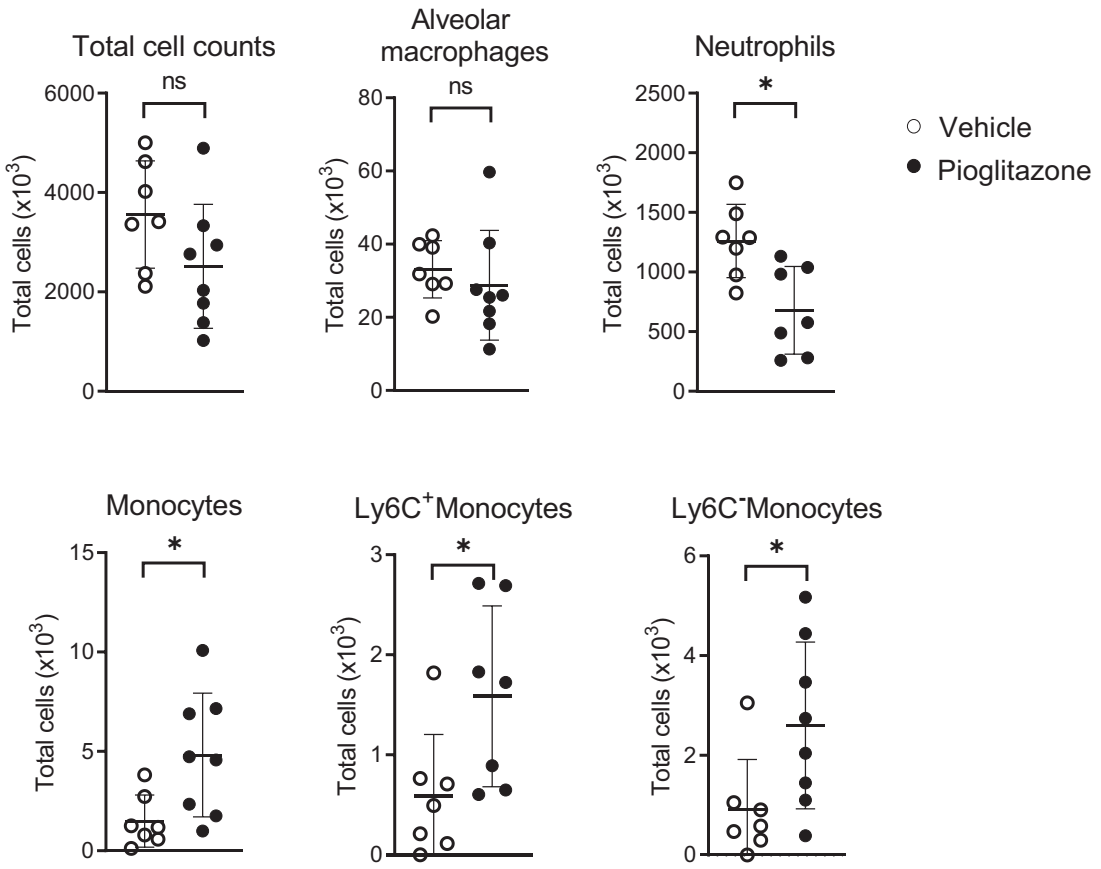

Figure 3: Total cell count, alveolar macrophage, neutrophil and monocyte numbers, as well as Ly6C$^+$ (inflammatory) and Ly6C$^-$ (patrolling) monocytes, were evaluated by flow cytometry in BALF in vehicle and pioglitazone treated mice 6 h following induction of pneumonia.Graphs show mean and SD, and every dot represents one individual mouse. *P* values were calculated using Welch's *t*-test. $*P < 0.05$, ns (not significant).

with flagellin via the airways [24]. These data prompted us to study the impact of pioglitazone on the epithelial cell expression of genes involved in glycolysis. Of interest, pioglitazone treatment was associated with enhanced expression of *Hk2* in bronchial brushes, the gene encoding hexokinase-2, the rate-limiting enzyme that mediates the first step of glycolysis by catalyzing the phosphorylation of D-glucose to D-glucose 6-phosphate (Fig. 4A). In accordance, pioglitazone also increased the expression of epithelial *Pfkfb3*, the gene encoding 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, another rate-limiting enzyme in glycolysis (Fig. 4A). In contrast, the epithelial expression of *Sdha*, encoding succinate dehydrogenase complex – subunit A, tended to be decreased in pioglitazone treated mice ($P = 0.075$ versus vehicle, Fig. 4A); SDHA participates in the tricarboxylic acid cycle by converting succinate to fumarate. Together these data suggest that pioglitazone administration resulted in a metabolic shift to glycolysis in bronchial epithelial cells during acute *P. aeruginosa* pneumonia. Pioglitazone tended to reduce the expression of genes involved in fatty acid uptake (*Cd36*) and fatty acid metabolism (*Acsl1*); differences with vehicle-treated mice did not reach statistical significance (Fig. 4B).

Discussion

PPAR-γ has been implicated as an important regulator of lung immunity by controlling the function of multiple cell types in the respiratory tract. The capacity of PPAR-γ to inhibit pro-inflammatory responses by monocytes and macrophages was reported more than 30 years ago [25, 26]. Since then many studies documented the anti-inflammatory effects of PPAR-γ agonists in models of lung inflammation [8–11, 23]. We here report remarkable pro-inflammatory effects of pioglitazone treatment in bronchial epithelial cells in mice infected with viable *P. aeruginosa* via the airways, as reflected by increased expression of chemokine and cytokine genes. Pioglitazone enhanced the epithelial expression of genes involved in glycolysis, providing a possible mechanistic link with its effect on proinflammatory gene expression.

In contrast with the abundant literature on the anti-inflammatory effects of PPAR-γ agonists on monocytes and macrophages, knowledge of the role of PPAR-γ in respiratory epithelial cells is more limited. PPAR-γ agonists inhibited IL-8 secretion by airway epithelial cell lines stimulated with a mixture of proinflammatory cytokines [12, 27], whilst the effect on *Pseudomonas* induced cytokine production was more variable [12]. Several studies reported on the function of endogenous PPAR-γ in the respiratory epithelium. Mice with targeted deletion of *Pparg* in bronchial epithelial cells presented exacerbated lung inflammation during allergen-induced airway disease, with increased cytokine concentration in BALF and increased cytokine expression in airway epithelial cells [28]. In intestinal epithelial cells, pioglitazone enhanced barrier function after infection with *Pseudomonas* [29]. In accordance with an anti-inflammatory role of endogenous PPAR-γ in colon epithelium, mice with an epithelial-specific

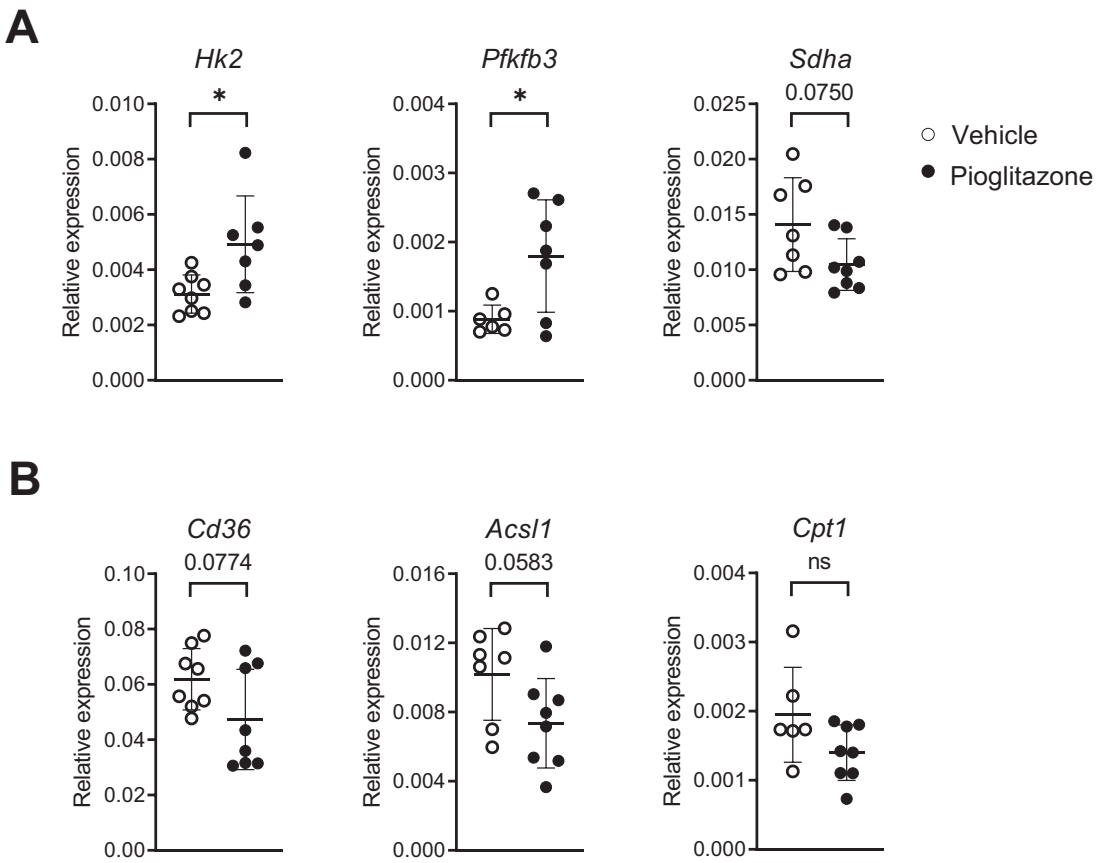

Figure 4: (A) Relative mRNA expression for the indicated glycolytic enzymes analyzed by RT-PCR in lung epithelial brushes from vehicle or pioglitazone treated mice 6 h after infection with *P. aeruginosa*. (B) Relative mRNA expression for the indicated fatty-acids metabolism enzymes analyzed by RT-PCR in lung epithelial brushes of mice from (A). Graphs show mean and SD, and every dot represents one individual mouse. *P* values were calculated using Welch's *t*-test. $*P < 0.05$.

deletion of *Pparg* showed an enhanced susceptibility to chemically induced colitis [30]. Moreover, PPAR-γ ligands reduced cytokine gene expression in colon cancer cell lines and attenuated colonic inflammation in a mouse colitis model [31]. As such, existing literature suggests an anti-inflammatory role for PPAR-γ activation in epithelial cells. We found enhanced gene expression of multiple chemokines and cytokines in bronchial epithelial cells in pioglitazone-treated mice with acute *P. aeruginosa* pneumonia. Although a definite explanation of these distinct findings is lacking, differences in the context of PPAR-γ stimulation (e.g. analyses of epithelial cell lines stimulated *in vitro* versus primary bronchial epithelial cells harvested from mice after airway infection *in vivo*) might play a role.

Pioglitazone enhanced the gene expression of rate-limiting enzymes of glycolysis hexokinase-2 and PFKFB3, whilst expression of the gene encoding SDHA (essential for an adequate metabolite flux in the tricarboxylic acid cycle) tended to be reduced. In agreement, *Hk2* has been described as a transcriptional target for PPAR-γ and pioglitazone increased hepatic HK2 levels in mice with fatty liver disease [32]. We recently showed that the primary human bronchial epithelial cells require glycolysis to sustain the production of pro-inflammatory mediators upon stimulation with flagellin, including CXCL1, CCL20, and G-CSF [24], of which the expression was enhanced in bronchial epithelial cells of pioglitazone treated mice with *P. aeruginosa* pneumonia. Moreover, inhibition of the mTOR (mechanistic target of rapamycin) pathway prevented the induction of glycolysis and limited the secretory capacity of bronchial epithelial cells in response to flagellin *in vitro* and abolished expression of *Hk2*, *Cxcl1*, *Cxcl2,* and *Csf3* in bronchial epithelial cells of mice challenged with flagellin *in vivo* [24]. Together these data suggest a functional link between pioglitazone-induced metabolic rewiring toward glycolysis and its stimulatory effect on chemokine/cytokine gene expression in bronchial epithelial cells. Further studies, in which glycolysis is inhibited specifically in epithelial cells, are needed to obtain further support for this potential mechanism of action underlying the effect of pioglitazone.

Pioglitazone exerted several anti-inflammatory effects in mice infected with *P. aeruginosa* via the airways, including inhibition of CXCL1, TNF-α, and IL-1β release and neutrophil recruitment in BALF. These data are consistent with earlier reports demonstrating the anti-inflammatory effects of PPAR-γ agonists in mouse models of *P. aeruginosa* pneumonia [12, 13] and other lung inflammation models [10, 11]. The discrepancy between pioglitazone effects expression of genes in bronchial epithelial cells and protein release in BALF likely can be explained by additional cellular sources for chemokines and cytokines besides the respiratory epithelium, particularly macrophages, which are expected to be inhibited in function by pioglitazone [10, 33]. These results further indicate that the selective pro-inflammatory effects of pioglitazone on the bronchial epithelium do not have a major

impact on typical inflammatory responses measured in BALF, which result from an interplay of different activated – predominantly myeloid – cell types.

Concluding remarks

PPAR-γ agonists exert broad anti-inflammatory effects on myeloid cells *in vitro* and models of inflammation *in vivo*. While we here confirm anti-inflammatory effects of pioglitazone in the bronchoalveolar compartment of mice during acute *P. aeruginosa* pneumonia, we found a selective pro-inflammatory effect on bronchial epithelial cells, as indicated by enhanced gene expression of several chemokines and cytokines, which was associated with increased expression of genes encoding rate-limiting enzymes of glycolysis. These results may have relevance for the administration of PPAR-γ agonists to patients with inflammatory lung disorders that affect the respiratory epithelium.

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Author contributions

Conceptualization: I.R-M., B.L.F. and T.v.d.P. Methodology: I.R-M., B.L.F. and N.A.O. Validation: I.R-M. and B.L.F. Formal analysis: I.R-M. and B.L.F. Investigation: I.R-M, B.L.F. and N.A.O. Resources: T.v.d.P. Writing - Original draft: I.R-M., B.L.F. and T.v.d.P. Writing - review and editing: I.R-M, B.L.F., N.A.O., A.F.d.V., R.S. and T.v.d.P. Visualization: I.R-M. and B.L.F. Funding acquisition: T.v.d.P.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Data availability statement

The data underlying this study are available in the article. Any further information is available from the corresponding author on request.

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