Detrimental effects of an inhaled phosphodiesterase-4-inhibitor on lung inflammation in ventilated preterm lambs exposed to chorioamnionitis are dose-dependent

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Running title:

PDE4 inhibition in ventilated preterm lambs

Abstract

Background: Treatment of bronchopulmonary dysplasia (BPD) in preterm infants is challenging due to its multifactorial origin. In rodent models of neonatal lung injury, selective inhibition of PDE4 has been shown to exert anti-inflammatory properties in the lung. We hypothesized that GSK256066, a highly selective, inhalable PDE4 inhibitor, would have beneficial effects on lung injury and inflammation in a triple hit lamb model of Ureaplasma parvum (UP)-induced chorioamnionitis, prematurity and mechanical ventilation.

Methods: 21 preterm lambs were surgically delivered preterm at 129d after 7d intrauterine exposure to UP. 16 animals were subsequently ventilated for 24 hours and received endotracheal surfactant and intravenous caffeine citrate. 10 animals were randomized to receive twice a high (10 µg/kg) or low dose (1 µg/kg) of nebulized PDE4 inhibitor.

Results: Nebulization of high, but not low doses of PDE4 inhibitor led to a significant decrease in pulmonary PDE activity, and was associated with lung injury and vasculitis, influx of neutrophils and increased pro-inflammatory cytokine mRNA levels.

Conclusions: Contrary to our hypothesis, we found in our model a dose-dependent pro-inflammatory effect of an inhaled highly selective PDE4 inhibitor in the lung. Our findings indicate the narrow therapeutic range of inhaled PDE4 inhibitors in the preterm population.

Key words: lung injury, PDE4 inhibitors, bronchopulmonary dysplasia, mechanical ventilation of neonates, prematurity

Introduction

Bronchopulmonary dysplasia (BPD) continues to be the major morbidity in infants born prematurely.[1, 2] Amongst others, BPD has been linked to an early inflammatory reaction in the developing lung associated with chorioamnionitis.[1, 3] Exposure to chorioamnionitis has also been shown to increase the risk of BPD development derived from mechanical ventilation,[4] another yet important factor originally linked to the pathophysiology of BPD.[1] In consequence of the multifactorial origin of BPD, targeted prevention and therapies are scarce.[5] Currently, anti-inflammatory therapies are used in BPD prevention and therapy, especially postnatal glucocorticosteroids.[6] However, concerns about adverse effects such as impaired neurodevelopmental outcome after systemic use of glucocorticosteroids,[7] have raised the demand to develop new anti-inflammatory therapies.[5]

Potential candidates for targeting neonatal lung inflammation are phosphodiesterase (PDE) inhibitors, such as methylxanthines and their derivates.[8] Caffeine, a methylxanthine widely used in the treatment of apnea in preterm infants, has been shown to decrease the incidence of BPD.[9] Although the mechanism is still discussed, caffeine's property as a weak, non-specific PDE inhibitor might contribute to its beneficial effect on BPD.[10] Another methylxanthine, Pentoxifylline (PTXF), has been shown to decrease the incidence of BPD when nebulized to very low birth weight preterm infants in a clinical trial,[11] possibly due to its anti-inflammatory effect on neonatal monocytes.[12] A more specific inhibition of PDE isoenzymes might therefore increase the therapeutic benefit.

Of the reported isoforms, PDE4 is the main enzyme in lung and inflammatory cells, which reduces cleavage of cAMP.[13] Therefore, in recent years PDE4 inhibitors have been developed to target different adult lung diseases like asthma and COPD.[14] In the context of BPD, selective

inhibition of PDE4 has been tested in different rodent models. In these studies, anti-inflammatory effects of PDE inhibition were identified as a possible mechanism to protect the lung from hyperoxia-induced BPD phenotype [15-18] and from lipopolysaccharide (LPS) induced lung inflammation.[19-22]

We therefore hypothesized that a new highly selective PDE4 inhibitor optimized for inhaled delivery (GSK256066) [20, 21] would exert anti-inflammatory properties in the lungs of preterm lambs exposed to chorioamnionitis and subsequent mechanical ventilation.

Materials and methods

Preparation of iPDE4

A stock solution of GSK256066 (Selleckchem, Munich, Germany) was made according to the manufacturer's guidelines by dissolving 2.5 mg GSK256066 in 1 ml DMSO 20%. Two working solutions were prepared with a concentration of 50 µg/mL and 5 µg/mL GSK 256066 in DMSO 2%, and frozen at -20°C. Immediately before administration, the working solution was thawed, and an amount of 0.2mL/kg body weight was mixed with the same amount of NaCl 0.9%, before filling a vibrating membrane nebulizer (eFlow® Neonatal Nebulizer System, PARI Pharma, Munich, Germany). The lower dose of 1 µg/kg was based on ED_{50} value of 1.1 µg/kg identified in a rat model of LPS-induced lung inflammation [20] and resembles the adult dose of 87.5 µg used in clinical trials on COPD.[23] The higher dose of 10 µg/kg was based on previous rodent studies describing anti-inflammatory effects after LPS inhalation.[21]

Animal study

The study design and the experimental protocol were in line with the institutional guidelines for animal experiments and were approved by the institutional Animal Ethics Research

Committee of Maastricht University, and the Dutch Central Animal Research Commission (CCD).

Seven days before delivery, 21 date-mated ewes underwent ultrasound-guided intraamniotic injection of Ureaplasma parvum (strain HPA 5), $5x10^5$ color changing units (CCU). One day before caesarean section, ewes were injected intramuscularly with βmethasone (12 mg, Celestone®, Schering-Plough, North Ryde, NSW, Australia). Before delivery, lambs were randomly assigned to four different treatment groups: non-ventilated controls which were sacrificed immediately (NOVENT), animals ventilated for 24h without iPDE4 treatment (Control) and two groups of ventilated animals which received 1 µg/kg GSK256066 (iPDE1) or 10 µg/kg GSK256066 (iPDE10), respectively, at 30 min and 12 h postnatal age (Fig. 1).

Lambs were surgically delivered at a gestational age of 129 days (term ~150 d), equipped with umbilical artery and vein catheters and intubated orally before clamping the cord and weighing.[24] Animals in the ventilation groups were transferred to an infant radiator bed (IW930 Series CosyCot™ Infant Warmer, Fisher & Paykel, Auckland, New Zealand) and connected to an infant ventilator (Fabian HFO®, Acutronic, Hirzel, Switzerland) with the following initial settings: SIMV, PIP 30 cmH_2O, PEEP 8 cmH_2O, ventilation rate 50/min, FiO_2 0.40. Subsequently, animals received an endotracheal dose of 200 mg/kg body weight Poractant alpha (Curosurf®, Chiesi Pharmaceuticals, Parma, Italy) and a single loading dose of caffeine citrate IV (20mg/kg, Peyona®, Chiesi Pharmaceuticals). The nebulizer was prepared as described above and placed between the tube and the connection to the ventilator circuit.[25] Ventilation was adjusted to blood gas analysis to maintain pO_2 between 60 - 90 mmHg and pCO_2 between 45 - 70 mmHg (iStat device, Point of Care Inc., Abbott Park, IL). During the experimental period of 24 hours, lambs were continuously sedated with midazolam (Actavis,

Hafnarfjordur, Iceland) and ketamine (Alfasan B.V., Woerden, The Netherlands), and parenterally fed with a 1:1 mix of glucose 20% and Ringer's solution (B. Braun Medical B.V. Oss, The Netherlands).

Necropsy

At the end of the experiment, lambs were euthanized by an intravenous injection of 10 mL pentobarbital. The thorax was opened and the lungs were removed, divided into lobes and weighed. The right upper lobe (RUL) was inflation-fixed in 10% buffered paraformaldehyde for 24 h. Lung tissue from the right middle lobe (RML) was snap frozen. Paraffin-embedded RUL sections (4 µm) were stained with hematoxylin and eosin prior to semi-quantitative scoring of lung injury, based on the composite score published by Hillman *et al.* .[26]

Immunohistochemistry

Paraffin-embedded RUL lung sections (4 µm) were stained for CD3 (DAKO A0452, Dakocytomation, Glostrup, Denmark) and MPO (DAKO A039829, Dakocytomation). Briefly, the sections were deparaffinized in an ethanol series. Endogenous peroxidase-activity was blocked by incubation with 0.3% H_2O_2 in 1 × phosphate buffered saline (PBS, pH 7.4). Antigen retrieval was performed by heating the sections in heated citrate buffer (10 mM, pH 6.0) for 10 min. To block nonspecific binding, the slides were incubated with 5% bovine serum albumin in PBS (for CD3) or 20% normal goat serum (NGS) in PBS (for MPO). For CD3, sections were incubated overnight at 4°C with the diluted primary antibody (1:200, DAKO A0452, Dakocytomation). After incubation with a swine-anti-rabbit biotin-labeled secondary antibody (DAKO E0353, Dakocytomation), immunostaining was enhanced with Vectastain ABC Peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, CA) and stained with nickel sulfate-diaminobenzidine (NiDAB). Subsequently, the sections were rinsed in Tris/Saline and

incubated with Tris/Cobalt. Counterstaining was performed with 0.1% Nuclear Fast Red. For MPO, sections were incubated for 1 hour with the 1:500 diluted primary antibody (Myeloperoxidase, Dako A0398). After incubation with the 1:200 diluted secondary antibody (Peroxidase Goat Anti-Rabbit IgG, Jackson ImmunoResearch, 111-035-045), slides were incubated with 0.02% 3-amino-9-Ethylcarbazole (Sigma A5754) dissolved in Sodium Acetate $C_2H_3NaO_2$ (0.05M, pH4.9) and a total of 0.01% H_2O_2 (Sigma H1009). After washing, background staining was performed with hematoxylin. For analysis, slides were scanned (Ventana iScan HT, Roche Diagnostics, Basel, Switzerland) and pictures were taken at 200x magnification with the Ventana Imageviewer (Roche Diagnostics). MPO- and CD3-positive cells were counted in five representative high-power fields by a blinded observer and averaged per animal.

PDE activity

PDE activity was calculated from cAMP concentration as described before.[22] Frozen lung tissue was homogenized in a buffer consisting of 30 mM HEPES and 0,1% Triton X-100 (a total volume of 4 µl per mg lung). After 10 min centrifugation at 13.000 xg, 10µl lung homogenate was mixed with 190µl PDE-assay buffer (137 mM NaCl; 2,7 mM KCL; 8,8 mM Na_2HPO_4; 1,5 mM KH_2PO_4 1mM $CaCl_2$; and 1 mM $MgCl_2$), and adding 1 µM cAMP started the reaction (incubated at 10 min at 37°C) and reaction stopped by boiling for 3 min. After centrifugation at 12.000 xg for 30 min, the cAMP concentrations in the supernatants were measured using an ELISA according to the manufacture's protocol (Enzo Life Sciences, USA). PDE activity was calculated reciprocally in all but one animal where cAMP was outside the threshold of detection.

RNA extraction and real time PCR

Total RNA was isolated from the RML using NucleoSpin® RNA Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. For quantification of total RNA, a Qubit®

2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) was used as recommended by the manufacturer. Total RNA was eluted in 60 µL nuclease-free H_2O (Sigma-Aldrich) and stored at -80 °C until reverse transcription. For RT-PCR, 1 µg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. First strand cDNA was diluted 1:10 with deionized, nuclease-free H_2O (Sigma-Aldrich) and stored at -20 °C until required for further analysis.

Quantitative real time RT-PCR (qPCR)

For quantitative detection of mRNA, 10 µL of diluted first strand cDNA were analyzed in duplicates of 25 µL reactions using 12.5 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 µL deionized H_2O, and 1 µL of a 10 µM solution of forward and reverse primers (Sigma-Aldrich). Levels of mRNA were measured for inflammatory cytokines interleukin (IL)-1, IL-6, and IL-8, for tumor necrosis factor (TNF) alpha, and for tissue inhibitor of metalloproteinase 1 (TIMP1). TIMP1 has been described as a predictive biomarker for mesenteric vasculopathy induced by PDE4 inhibition.[27] Primers were ovIL1Bfwd 5'-CCTGTCATCTTCGAAACATCC-3', ovIL1Brev 5'-GCAGAACACCACTTCTCGG-3', ovIL-6fwd 5'-CTCTCATTAAGCACATCGT-3', ovIL-6rev 5'-GATCAAGCAAATCGCCTG-3', ovIL-8fwd 5'-AAACACATTCCACACCTTTCC-3', ovIL-8rev 5'-GGATCTTGCTTCTCAGCTCTC-3', ovTNFafwd 5'-ACACTCAGGTCATCTTCTC-3', ovTNFarev 5'-GGTTGTCTTTCAGCTCCA-3', ovTIMP1fwd 5'-ACTCCGAAGTCGTCATCAG-3', ovTIMP1rev 5'-GAAGTATCCGCAGACGCTC-3', ovACTBfwd 5'-ATCTGTCGTCAGCAGGTC-3', ovACTBrev 5'-CCAACGGTACTGAGAGGA-3'. PCRs were performed on an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) using a 2-step PCR protocol after an initial denaturation at 95 °C for 10 min with 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve analysis was performed at the end of every run to verify

single PCR products. Levels of mRNAs were normalized to those of β Actin. Mean fold changes in mRNA expression were calculated by the $\Delta\Delta C_T$ method by Livak and Schmittgen.[28]

Statistics

Data were expressed as mean and standard error of means (SEM), and statistical analysis was performed using One-way ANOVA with Bonferroni post-hoc testing with IBM® SPSS version 20. Graphs were drawn with GraphPad Prism® v5.0. Significance was accepted at $p<0.05$.

Results

Baseline characteristics

Animals in different groups did not differ significantly in sex, birth weight and relative weight loss during the experiment (table 1). Blood gas analysis during ventilation showed stable results during the experimental period, with a mean pCO_2 slightly above the target range at 24h in the iPDE1 group (Fig. 2). One animal died at 12 hours due to tension pneumothorax.

PDE Activity

At sacrifice, PDE activity was highest in ventilated control animals. In the high dose group, but not in the low dose group, PDE activity was significantly decreased by approximately 80% compared to unventilated and ventilated controls 12 hours after the second dose (Fig. 3).

MPO and CD3

The number of MPO positive cells per high power field indicating neutrophils was low in unventilated and ventilated controls (Fig. 4 a), and significantly increased in the iPDE10, but not the iPDE1 group. CD3 positive cells indicating lymphocytes (Fig 4 b) were found in the lungs of all animals irrespective of ventilation or PDE4 inhibitor treatment.

Cytokines RNA

Levels of pro-inflammatory cytokines' RNA for IL-1β, IL-6, IL-8 and of TNF alpha were lowest in unventilated controls. Higher levels were found in all ventilated groups, however only animals receiving a high dose of PDE inhibitor showed a significant increase of IL-1β, IL-8 and TNF alpha levels ($p<0.05$ compared to NOVENT, Control, iPDE1, Fig 5 a,c,d). Cytokine mRNA levels in the low-dose group were comparable to or lower than levels in ventilated controls, but this effect was not statistically significant when comparing all groups. For TIMP-1, increase in mRNA levels in ventilated animals was significant only in the iPDE10 group ($p<0.05$ compared to NOVENT, $p=0.054$ vs. Control, Fig 5 f).

Lung injury score

The composite injury score in unventilated control animals was low, and ventilation alone did not lead to a significant increase of the score (Fig. 6 a-c). Animals in the iPDE10 group showed a significantly higher score for lung injury compared to unventilated and ventilated control animals, with thickened airway walls and parenchymal haemorrhage (Fig. 6 e). In this group, infiltration of inflammatory cells could additionally be found in perivascular tissue, indicating vasculitis (Fig. 6 f). Lung injury scores in the iPDE1 group were not significantly increased ($p=0.091$ vs. NOVENT, Fig. 6 d).

Discussion

Contrary to our hypothesis, we found pro-inflammatory effects and increased injury scores in the lungs of preterm lambs nebulized with 10 µg/kg per dose of a selective PDE4 inhibitor. The pro-inflammatory phenotype in our study was associated with histological signs of vasculitis and an increase in pulmonary mRNA levels of TIMP-1, a potential biomarker of intestinal vasculopathy induced by PDE4 inhibitors.[27] Mesenteric and intestinal vasculitis after oral

application of PDE4 inhibitors have been described in various animal studies and have been identified as dose-limiting adverse effect.[29, 30]

In our study, both PDE inhibition and associated pro-inflammatory effects were dose-dependent. PDE was not significantly inhibited at sacrifice in animals receiving the lower dose of PDE4 inhibitor. Of note, this was 12 hours after the second dose, so we cannot rule out a time-dependent PDE inhibition also after the low dose. However, we found no significant anti-inflammatory effect in the iPDE1 group with levels of pro-inflammatory cytokine mRNA comparable to levels in ventilated controls.

In the iPDE10 group, we observed a strong PDE inhibition 12 hours after the second dose, indicating a total blocking of PDE after administration, possibly resulting from a supra-therapeutic dose. This dose was however chosen based on previous works in rodents, where doses of 10 µg/kg GSK256066 were administered intratracheally to rats challenged with LPS inhalations. In that study, this dose had anti-inflammatory effects in the lungs and was well tolerated.[21] Our contrary findings might therefore arise from differences in the experimental setup, including choice of pro-inflammatory stimuli, route of administration, and species.

Studies showing anti-inflammatory effects of PDE4 inhibition often used rodent models and in vitro testing with lipopolysaccharide (LPS) as very strong pro-inflammatory stimulus,[19-22] and found augmented inflammation after exposure to both LPS and PDE4 inhibitor via different routes. Of the above mentioned, only one study reported higher neutrophil counts in bronchoalveolar lavage and high levels of lung keratinocyte-derived chemokine (KC, a mouse homologue of the IL-8 family) when PDE4 inhibitor treatment was administered subcutaneously without prior LPS exposure.[19] In our study, we chose a different yet clinically relevant approach to induce preterm lung injury and inflammation. Intrauterine UP exposure

has been shown to induce chorioamnionitis and to generate fetal pulmonary and systemic inflammation.[31, 32] Although pulmonary inflammation by UP is described as mild, it has been associated with BPD in several studies.[33] Acute UP infection of fetal baboons increased ventilation-associated lung injury and postnatal lung inflammation.[34] However, our data indicated that the phenotype of a more subacute pulmonary inflammation might react differently on PDE4 inhibition than on acute LPS exposure.

Previous studies also explored different routes of PDE4 inhibitor administration. In the hyperoxia-rodent model of neonatal lung-injury and BPD,[15-18] where lung inflammation and injury is induced by ongoing oxidative stress postnatally, anti-inflammatory effects of PDE4 inhibition have been reported after subcutaneous or intraperitoneal application of different PDE4 inhibitors. Systemic PDE4 inhibitor treatment has however been associated with adverse treatment effects, e.g. reduced weight gain.[15, 17] In rats treated orally for two weeks with supra-therapeutic doses of the PDE4 inhibitor rolipram, histological signs of organ damage where found in the heart, vasculature, stomach and salivary glands, but interestingly not in the lungs of treated animals.[35] In order to decrease systemic side effects, we chose for nebulization to target the lung directly, using GSK256066 as PDE4 inhibitor which is suitable for inhalation.[20] Choosing this approach, we observed inflammatory changes in the lung, indicating that the primary site of PDE4 inhibitor exposure implicates the site of major adverse effects.

Our study is limited by the fact that we used Dimethyl sulfoxide (DMSO) as a solvent for the PDE4 inhibitor. DMSO has been used in various preclinical models due to its high solubilization capacity and a very low toxicity.[36] For endotracheal use, data is conflicting: in mice, repeated endotracheal exposure against 2% DMSO for five days resulted an increase in

myeloperoxidase activity in bronchoalveolar lavage fluid, while only small number of neutrophils were recruited to the lung.[36] Endotracheal DMSO further ameliorated neutrophil influx in a hamster model of acute lung injury.[37] Based on these findings and in accordance with good animal experimental practice, we decided that the low risk of significant effects of inhalation with 1% DMSO in our model did not justify a separate control group receiving DMSO only.

Finally, translation of findings to other species and the human situation is difficult. Rats are thought to be very susceptible in terms of PDE4-inhibitor induced toxicity.[38] To our knowledge, no data from ovine models exists. The preterm lamb model has been chosen due to its similarity to human lung development, and we therefore regard our model to be suitable for preclinical testing of pulmonary drug delivery in the context of preterm lung injury and inflammation. However, it becomes clear that doses from adult humans and rodents cannot be easily translated into preterm settings. Our findings show that dose dependent toxicity and pulmonary inflammation narrows the therapeutic drug concentration of PDE4 inhibitors dramatically. These findings require thorough investigation of this group of substances before clinical use in preterm infants.

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Author Disclosure Statement

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The authors declare that no competing financial interests exist.

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Tables

Table 1: Animal characteristics

	NOVENT	Control	iPDE1	iPDE10
N	5	6	5	5
male : female	1:4	2:4	2:3	1:4
Birth weight (kg)	2.8±0.2	3.1±0.2	2.8±0.2	3.4±0.3
Relative weight loss during experiment	n.a.	-0,5%	-2,4%	-4,8%

Figure legends

Experimental groups (Fig. 1)

After initiation of ventilation, animals received 1 dose of Curosurf 200 mg/kg intratracheally and 20 mg/kg caffeine intravenously (arrow). Animals in the treatment groups received 1µg/kg (thin dotted arrows) or 10 µg/kg PDE4 inhibitor (thick dotted arrows) via nebulization at 0.5 h and 12 h.

Blood gas analysis (Fig. 2).

During ventilation, animals in the control (square), iPDE1 (diamond) and iPDE10 group (triangle) showed stable results for A) pH, B) pO_2 and C) pCO_2, with a pCO_2 slightly above the target range (thin dotted lines) at 24 h in the iPDE1 group (data as mean ± SEM, *$p<0.05$ vs. control).

PDE Activity (Fig. 3)

In lung tissue, PDE activity calculated from cAMP concentration was significantly decreased in the iPDE10 group compared to unventilated and ventilated controls ($p<0.05$ * vs. NOVENT, # vs. control).

MPO and CD3 (Fig. 4)

a) MPO positive cells indicating neutrophils, but not b) CD3 positive cells indicating lymphocytes were significantly increased in the iPDE10 group compared to NOVENT and control animals ($p<0.05$ * vs. NOVENT, # vs. control).

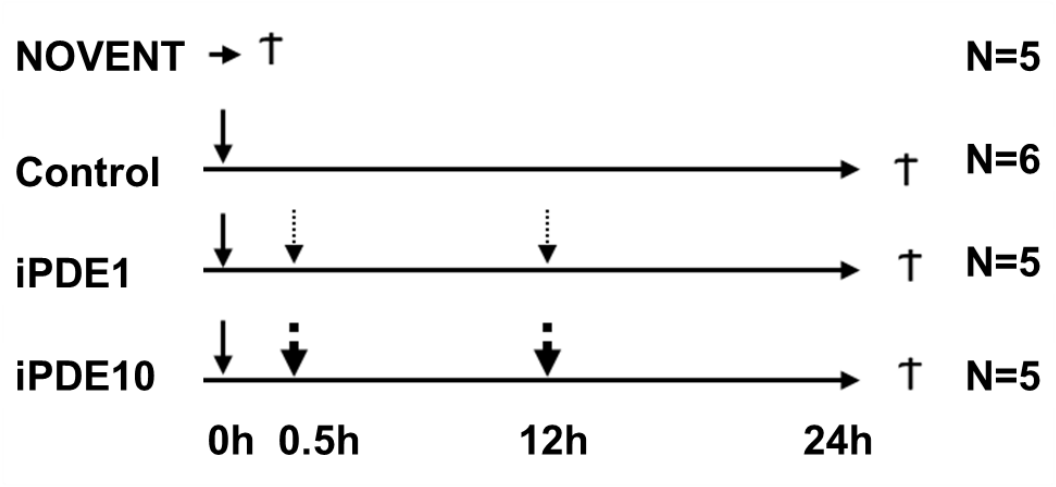
Cytokines mRNA (Fig. 5)

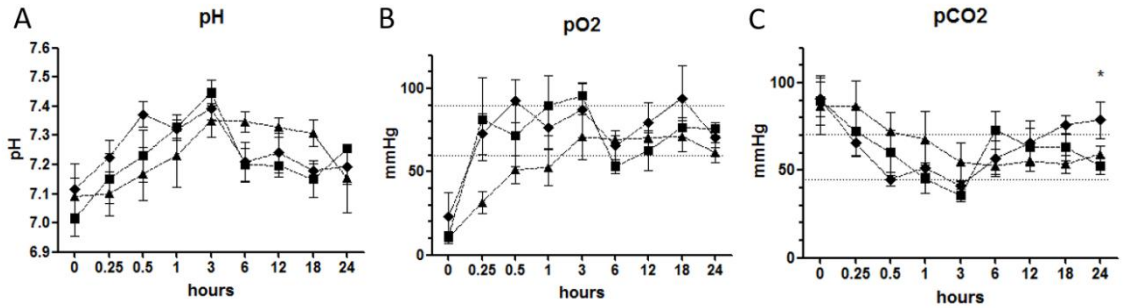
Pro-inflammatory cytokines mRNA levels of IL1β, IL-6, IL-8 and TNF-alpha increased in ventilated animals and were significantly higher for IL1β, IL-8 and TNF-alpha in the iPDE10

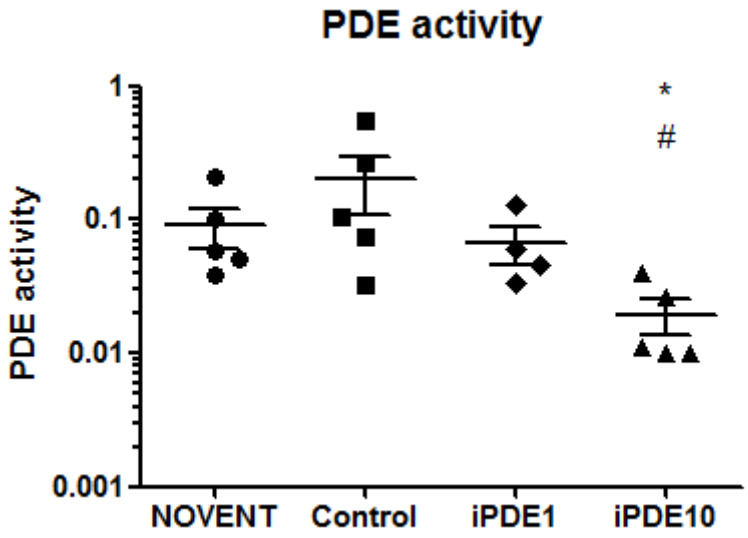
group ($p<0.05$ * vs. NOVENT, # vs. control, § vs. iPDE1, Fig. 5 A, C, D). A comparable pattern of increase was shown for TIMP-1 (E).

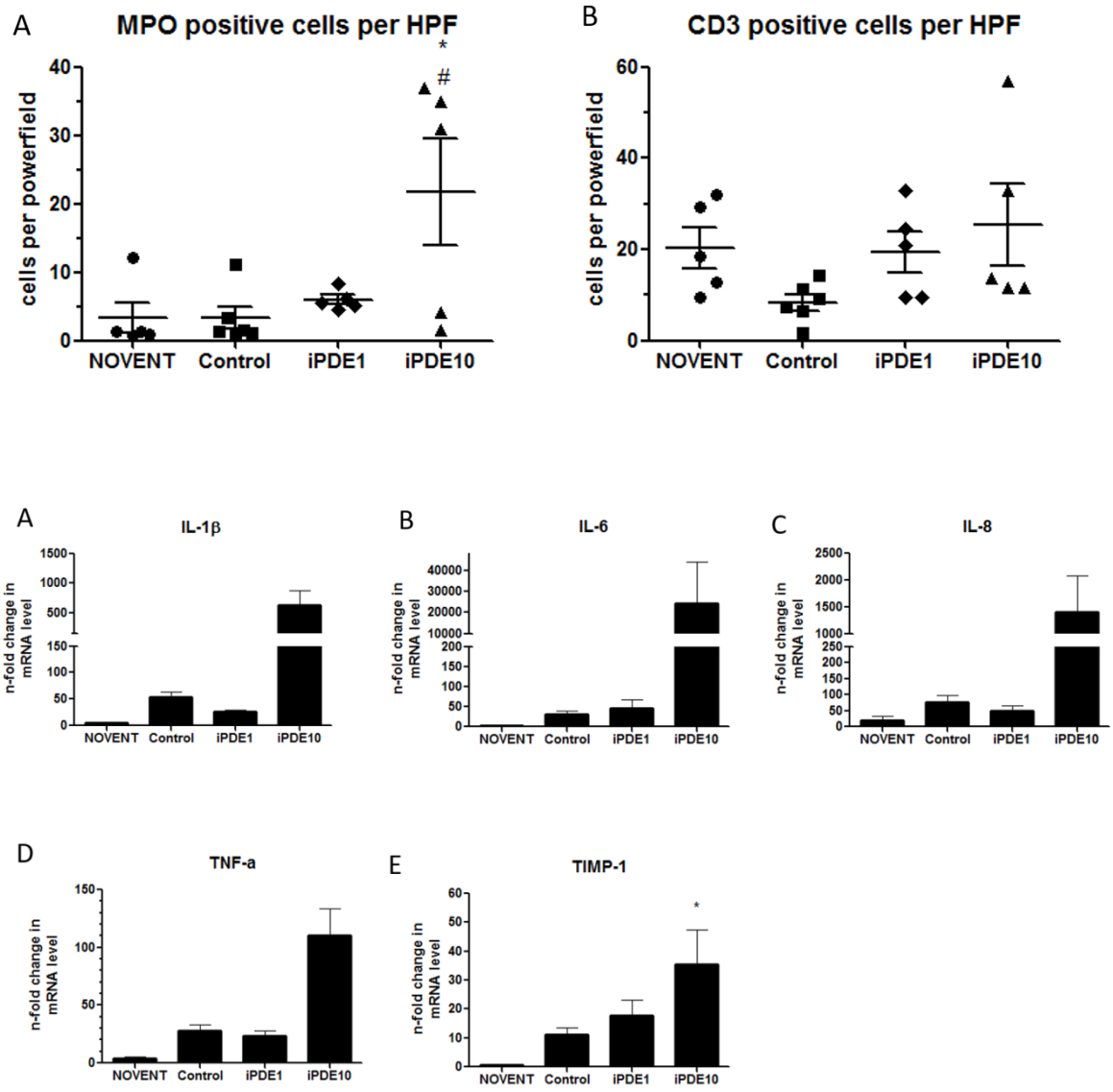
Lung injury score (Fig. 6)

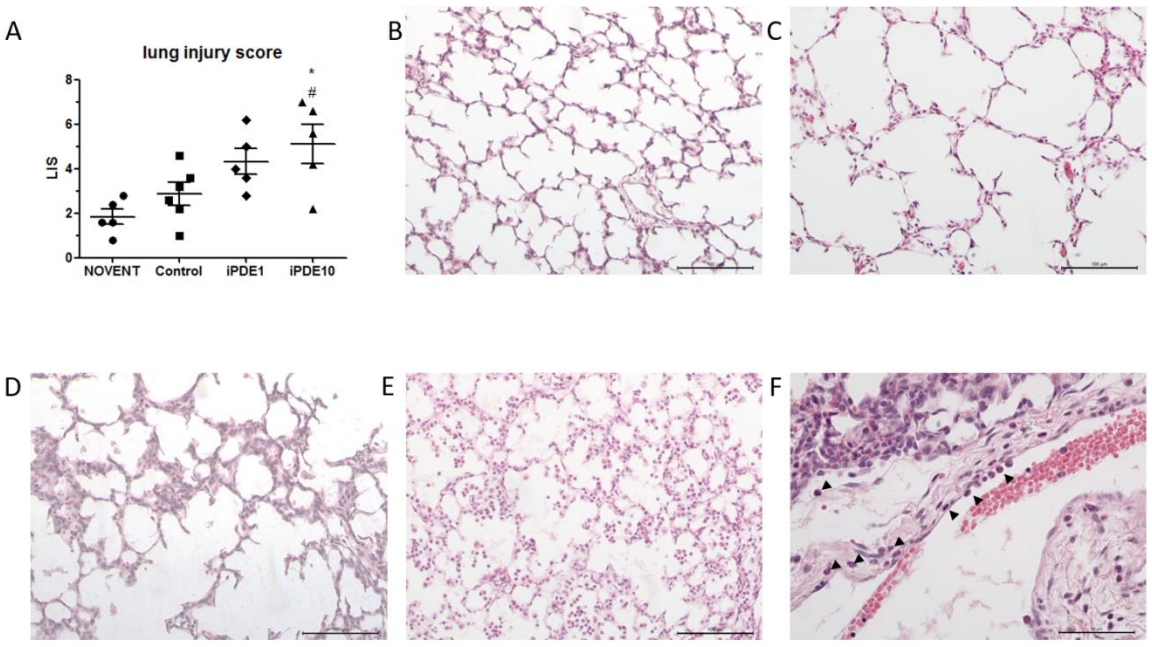
Semiquantitative lung injury score was significantly higher for iPDE10 group animals compared to not-ventilated and ventilated control animals ($p<0.05$ * vs NOVENT, # vs. control, 6 A). Representative lung slides of B) NOVENT, C) Control, D) iPDE1 and E) iPDE10 group. In the iPDE10 group, neutrophil recruitment into vessel walls indicates vasculitis (arrowheads, 6 F).


NOVENT
N=5
Control
N=6
iPDE1
N=5
iPDE10
N=5
0h
0.5h
12h
24h


A
pH
B
pO2
C
pCO2
hours
mmHg


PDE activity
PDE activity
NOVENT
Control
iPDE1
iPDE10


A
MPO positive cells per HPF
cells per powerfield
40
30
20
10
0
*
#
NOVENT
Control
iPDE1
iPDE10
B
CD3 positive cells per HPF
cells per powerfield
60
40
20
0
NOVENT
Control
iPDE1
iPDE10
A
IL-1β
n-fold change in mRNA level
1500
1000
500
150
100
50
0
NOVENT
Control
iPDE1
iPDE10
B
IL-6
n-fold change in mRNA level
40000
30000
20000
10000
200
150
100
50
0
NOVENT
Control
iPDE1
iPDE10
C
IL-8
n-fold change in mRNA level
2500
2000
1500
1000
250
200
150
100
50
0
NOVENT
Control
iPDE1
iPDE10
D
TNF-a
n-fold change in mRNA level
150
100
50
0
NOVENT
Control
iPDE1
iPDE10
E
TIMP-1
n-fold change in mRNA level
60
50
40
30
20
10
0
*
NOVENT
Control
iPDE1
iPDE10


A
lung injury score
LIS
8
6
4
2
0
NOVENT
Control
iPDE1
iPDE10
*
#
B
C
D
E
F