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Phosphodiesterase type 4 inhibitors cause pro-inflammatory effects *in vivo*

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Abbreviations: BAL, Bronchoalveolar lavage; LPS, lipopolysaccharide; PDE, phosphodiesterase; KC, keratinocyte-derived chemokine; MIP-2, macrophage inhibitory protein-2, HUVEC, human umbilical vein endothelial cell; cAMP, cyclic adenosine monophosphate

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Abstract

PDE_4 inhibitors are currently being evaluated as potential therapies for inflammatory airway diseases. However, this class of compounds has been shown to cause an arteritis/vasculitis of unknown etiology in rats and cynomolgus monkeys. Studies in rodents have demonstrated the anti-inflammatory effects of PDE_4 inhibitors on LPS-induced airway inflammation. The aim of this work was to assess the direct effects of PDE_4 inhibitors on inflammatory cells and cytokine levels in the lung in relation to therapeutic effects. The effects of the PDE_4 inhibitors, roflumilast (3-cyclo-propylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide) and piclamilast (3-(cyclopentyloxy)-N-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide)), were assessed *in vivo*, using Balb/c mice, and *in vitro*, in un-stimulated human endothelial and epithelial cell lines. In Balb/c mice, LPS challenge caused an increase in neutrophils in BAL and lung tissue and BAL TNF-α levels, which were inhibited by treatment with either roflumilast or piclamilast (30-100 mg/kg, s.c.). However, roflumilast and piclamilast alone (100 mg/kg) caused a significant increase in plasma and lung tissue keratinocyte-derived chemokine (KC) levels, and lung tissue neutrophils. *In vitro*, both piclamilast and roflumilast caused an increase in IL-8 release from HUVEC but not BEAS-2B cells, suggesting that one source of the increased KC may be endothelial cells. At doses that antagonized an LPS-induced inflammatory response, the PDE_4 inhibitors possessed pro-inflammatory activities in the lung that may limit their therapeutic potential. The pro-inflammatory cytokines KC and IL-8 therefore may provide surrogate biomarkers, both in pre-clinical animal models and in the clinic, to assess potential pro-inflammatory effects of this class of compounds.

Introduction

Phosphodiesterase type 4 (PDE_4) inhibitors are currently being evaluated as potential therapies for inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma. Indeed, roflumilast and cilomilast are currently in phase III clinical trials for one or both diseases. However, this class of compounds has been associated with some toxicity issues (a topic reviewed in some detail recently (Giembycz, 2005)). PDE_4 inhibitors have been shown to cause arteritis and vasculitis (inflammation of the arteries and vessels), in rats (Langle *et al.*, 1994; Larson *et al.*, 1996; http://www.fda.gov/ohrms/dockets/ac/03/transcripts/3976T1.doc; Mecklenburg *et al.*, 2006) and minipigs (Vogel *et al.*, 1999). This finding was demonstrated more recently in cynomolgus monkeys which was the first observation using this class of compounds in non-human primates (Losco *et al.*, 2004). Recent data provided further evidence for inflammatory implications of PDE_4 inhibitors. Evaluations of the ryanodine-receptor complex in knockout mice and in human cells suggest that reducing PDE_{4D} activity causes defective RyR2-channel function that is associated with heart failure and arrhythmias (Lehnart *et al.*, 2005). To date, there does not appear to be published data reporting any kind of pro-inflammatory effect in the lung in response to treatment with single or multiple doses of this class of compounds.

The effects of PDE_4 inhibitors in animal models of LPS – induced airway inflammation have been described in the literature. In mice, PDE_4 inhibitors have been shown to inhibit neutrophil numbers, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) levels and matrix metalloproteinase (MMP)-9 activity in the bronchoalveolar lavage (BAL; Corbel *et al.*, 2002). In other experiments PDE_4 inhibitors

demonstrated suppression of lung myeloperoxidase (MPO) activity, serum TNF-α levels (Miotla *et al.*, 1998), BAL neutrophilia and lymphocyte numbers (Trifilieff *et al.*, 2002). Several PDE_4 inhibitors have also been demonstrated to be effective in rat models. Studies have shown inhibition of neutrophil numbers (Trifilieff *et al.*, 2002; Billah *et al.*, 2002; Spond *et al.*, 2001; Kuss *et al.*, 2003) and neutrophil activation in BAL (Trifilieff *et al.*, 2002). In guinea pigs, rolipram treatment was shown to completely reverse the bronchoconstriction produced by chronic treatment with LPS (Toward and Broadley, 2002).

However, the effects of these inhibitors on both BAL and lung tissue inflammation have not been extensively investigated and assessment of biomarker levels appears to have been limited mainly, but not exclusively, to measurement of TNF-α. More importantly, the direct effects of this class of compounds on inflammatory cells and biomarkers in the lung have not yet been elucidated.

The objective of the experiments described here was to determine the effects of two PDE_4 inhibitors, roflumilast (3-cyclo-propylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide) and piclamilast (3-(cyclopentyloxy)-N-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide)), either alone or against LPS induced airway inflammation in Balb/c mice, in order to determine if there were inflammatory effects additional to those already extensively described in the literature. Cellular inflammation, TNF-α levels and the levels of the neutrophilic chemokines keratinocyte-derived chemokine (KC) and macrophage inhibitory protein-2 (MIP-2), shown to be established markers of inflammation in response to LPS (Schwartz *et al.*, 1994; Korsgren *et al.*, 2000; Birrell *et al.*, 2004; Haddad *et al.*, 2001; Ulich *et al.*, 1995), were measured in the

airway lumen as well as in lung tissue. The effects on these parameters in the circulation were also determined. Additionally, in order to assess the direct effects of PDE_4 inhibitors on structural cells, concentration response experiments were performed using primary human umbilical vein endothelial cells (HUVEC) and a human bronchial epithelial cell line (BEAS-2B).

Methods

Supplemental Methods

Materials and Methods

Male Balb/c mice (20 – 25g) were purchased from Harlan (HSD; Indianapolis, IN, USA) and housed for five days before initiating experiments. Food and water were supplied *ad libitum*. All experiments were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines, in a program approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Effect of roflumilast, piclamilast and dexamethasone on LPS induced airway and plasma mediator release in the Balb/c mouse

Balb/c mice were challenged with an aerosol of endotoxin free sterile water or LPS in a perspex pie chamber (0.3 mg/ml for 10 minutes). Vehicle (polysorbate 80 suspension, 5 ml/kg), roflumilast or piclamilast (0.3 – 100 mg/kg) were administered subcutaneously 30 minutes prior to challenge. The positive standard, dexamethasone (1 mg/kg), was dosed subcutaneously 1 hour before LPS challenge. Three hours after challenge mediator release was determined. The dose of LPS and sampling time point after challenge used were determined from previous experiments carried out in house (data not shown). In a separate experiment both compounds were dosed at 30 and 100 mg/kg and lung tissue protein expression was assessed. The effect of PDE4 inhibitor or steroid alone was measured at a single dose (100 mg/kg and 1 mg/kg, respectively).

Effect of roflumilast, piclamilast and dexamethasone on LPS induced cellular inflammation in the Balb/c mouse

Balb/c mice were challenged with an aerosol of endotoxin free sterile water or LPS (0.3 mg/ml for 10 minutes). Vehicle (polysorbate 80 suspension, 5 ml/kg), roflumilast or piclamilast (3, 10, 30 and 100 mg/kg) were administered subcutaneously 30 minutes prior to challenge. The positive standard, dexamethasone (1 mg/kg), was dosed subcutaneously 1 hour before LPS challenge. Three hours after challenge cellular inflammation in the airway lumen and lung tissue was determined. As described above the effect of PDE4 inhibitor or steroid alone was determined at a single dose.

Effect of roflumilast, piclamilast and dexamethasone on basal cellular inflammation in the circulation

Balb/c mice were challenged with an aerosol of endotoxin free sterile water (10 minutes). Vehicle (polysorbate 80 suspension, 5 ml/kg), roflumilast or piclamilast (100 mg/kg) were administered subcutaneously 30 minutes prior to challenge. Dexamethasone (1 mg/kg, s.c.) was included as a control and dosed 1 hour prior to water challenge. One vehicle group was challenged with LPS (0.3 mg/ml for 10 minutes). Three hours after challenge white cell numbers in the circulation were determined. As described above the effect of PDE4 inhibitor or steroid alone was determined at a single dose.

Effect of roflumilast and piclamilast on basal IL-8 release from primary HUVEC and transformed BEAS-2B cells

Primary human umbilical vein endothelial cells (HUVEC) and the transformed human bronchial epithelial cell line, BEAS-2B, were obtained from Cambrex Bio Science

Walkersville (MD, USA) and the American Type Culture Collection (ATCC; Manassas, VA, USA), respectively. HUVEC were grown to confluence in tissue culture flasks (75 cm^2) containing 15 ml of EGM-2 complete medium then seeded onto 96 well plates at an initial density of 3200 cells per well. Cells were treated upon reaching sub-confluence and were used at passages 3 to 4. BEAS-2B cells were grown to near confluence in 75 cm^2 tissue culture flasks containing 15 ml of LHC9 medium, which was changed to LHC8 medium 18 hours before assay. Cells were seeded into 96 well plates at a density of 50,000 cells per well and allowed to adhere for approximately 2 hours before treatments.

In order to determine if the PDE4 inhibitors caused an increase in basal levels of IL-8, cells were treated with roflumilast, piclamilast (10^{-10} – 10^{-4}M) or 0.5% DMSO vehicle. IL-1β (10 ng/ml) and LPS (100 ng/ml) treatments were included as stimulus controls in order to compare compound effects. Cells were incubated at 37°C in a thermostatically controlled incubator at a 5% CO_2 atmosphere. After 24 hours the amount of IL-8 released into the cell culture supernatant was measured by ELISA, according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Quantification of airway inflammation following LPS challenge

Three hours after water or LPS challenge, animals were bled from the tail vein, then euthanized with sodium pentobarbital (50 mg/kg) and the trachea cannulated. Bronchoalveolar lavage (BAL) cells were recovered from the airway lumen by flushing the airways with RPMI 1640 (three 0.3 ml washes, pooled (Invitrogen, Carlsbad, California, USA)) delivered through the tracheal cannula and removed after a 30 second

interval. The lungs were then perfused with RPMI 1640 to remove the blood pool of cells, removed and finely chopped. The inflammatory cells were extracted from the lung tissue by collagenase digest as described by Underwood *et al* (1997) using 6ml of digest fluid (RPMI 1640/10% fetal bovine serum (FBS) containing collagenase (1 mg/ml) and DNase (25 µg/ml)).

Total white cell counts were determined on the EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) using an FITC-conjugated antibody to CD45. Briefly, in 100mm flow tubes 100µl of sample was stained with 10µl CD45 FITC and incubated in the dark at room temperature for 10 minutes. Samples were then lysed/fixed with 500µl of OptilyseC and incubated for a further 10 minutes under the same conditions. 500µl of phosphate buffered saline (PBS) was added to the samples and vortexed, then, immediately before reading, 100µl of flow count beads were added to each sample to function as a calibrator. Samples were read for 30 seconds using Expo 32 software and the amount of total white blood cells was counted.

Cytospins of the BAL and lung tissue digest samples were prepared by centrifugation of 100 µl aliquots in a cytospin (Shandon Scientific, Pittsburgh, PA, USA) at 700 rpm for 5 minutes, low acceleration at room temperature. Slides were fixed and stained on a Hema-tek 2000 (Spectron Corp., Kirkland, WA, USA) with modified Wright's-Giemsa stain. Differential counts on 200 cells per slide were performed following standard morphological criteria, and the percentage of neutrophils was determined.

Assessment of cytokine protein expression

BAL and blood samples were centrifuged at 1900 rpm and 3500 rpm, respectively, for 10 minutes at 4°C and the resultant supernatant and plasma removed and used to measure cytokine levels. For the determination of cytokine protein levels in the lung tissue, the lungs were homogenized in 2ml of ice-cold saline using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). The samples were then centrifuged at 5000 rpm for 15 minutes and the resultant supernatants were used for cytokine protein quantification.

In the BAL and lung tissue protein levels for TNF-α, KC and MIP-2 were determined by enzyme-linked immunosorbent assay (ELISA) using mouse Duosets according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). For lung tissue, cytokine levels were further corrected for total protein content, which was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Blood samples were assessed for KC only as previous in-house experiments had determined that there was no increase in TNF-α or MIP-2 levels following LPS challenge (data not shown).

Assessment of circulating cellular inflammation

Total white cell counts were determined in whole blood samples as described above. Blood smears were prepared on microscope slides using 10 µl of sample, then slides were stained and fixed on the Hema-tek 2000 as described above. Differential counts on 100 cells per slide were performed following standard morphological criteria, and the percentage of neutrophils was determined.

Materials

Sodium pentobarbital (50 mg/kg) was obtained from Abbott Laboratories (North Chicago, IL, USA). RPMI 1640 and fetal bovine serum were from Invitrogen (Carlsbad, California, USA). Roche Diagnostics (Pleasanton, CA, USA) supplied the collagenase, DNase and penicillin streptomycin solution. Roflumilast (3-cyclo-propylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide) and piclamilast (3-(cyclopentyloxy)-N-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide) were synthesized by the Medicinal Chemistry Department of Theravance Inc. (South San Francisco, CA, USA). Polysorbate 80 suspension was prepared by the pharmacology department of Theravance Inc. LPS, dexamethasone, DMSO and Wright's-Giemsa stain were purchased from Sigma-Aldrich (St. Louis, MO, USA). CD45 FITC and reagents for the flow cytometer were purchased from Beckman Coulter (Fullerton, CA, USA). LHC8 and LHC9 media were purchased from BioSource International (Camarillo California USA). EGM-2 complete medium was obtained from Cambrex Bio Science Walkersville (MD, USA). All ELISA kits and Duoset kits were obtained from R & D Systems (Minneapolis, MN, USA).

Analysis

Values are expressed as mean ± S.E.M. of n independent observations. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. A p value of less than 0.05 was considered to be statistically significant.

Results

Effect of roflumilast and piclamilast on LPS-induced cytokine release in the BAL

Challenge with an aerosol of LPS resulted in a significant increase in BAL TNF-α, MIP-2 and KC levels (figures 1 and 2 A, B and C). Treatment with roflumilast or piclamilast caused a dose dependent inhibition of TNF-α which was significant at 30 and 100 mg/kg (figures 1 and 2 A). However there was no inhibition of LPS-induced elevations in MIP-2 or KC levels following treatment with either compound (figures 1B and C, 2B and C). The synthetic glucocorticoid, dexamethasone, caused a significant inhibition of all three biomarkers.

Neither roflumilast, piclamilast nor dexamethasone had any effect on basal BAL levels of TNF-α, MIP-2 or KC (figures 1 and 2).

Effect of roflumilast and piclamilast on cytokine release in the plasma and lung tissue

Treatment with roflumilast and piclamilast had no inhibitory effect on the increase in plasma KC levels caused by challenge with inhaled LPS (figures 3A and B). However, both compounds caused an increase in basal levels of KC, compared to vehicle control. These stimulatory effects were greater than those observed following LPS challenge. Additionally, treatment with roflumilast at 100 mg/kg also caused a significant increase in KC levels in the LPS challenged group. This may reflect the sum of the LPS response plus the increase in basal KC described for roflumilast. Dexamethasone significantly attenuated the LPS-induced elevation in plasma KC levels. In a separate experiment, both inhibitors were also shown to caused a significant increase in basal

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levels of KC when tested at a lower dose of 30mg/kg, compared to vehicle control (from 187.5 ± 17.9 to 1410 ± 382.1 and 1982 ± 629.5 pg/ml for roflumilast and piclamilast, respectively; $p < 0.05$). Although this was a separate experiment, the increase in KC observed was lower than that produced by the PDE_4 inhibitors tested at 100 mg/kg in the present study.

Neither roflumilast nor piclamilast treatment caused any increase in the levels of either TNF-α or MIP-2 protein levels in the plasma. As with vehicle control, levels remained below the limit of detection of the assay (data not shown).

Neither PDE_4 inhibitor caused any inhibition of the LPS-induced increase in lung tissue KC levels (figure 3C), however treatment with both roflumilast and piclamilast alone caused a significant increase in levels of this chemokine compared to vehicle control. Dexamethasone treatment caused a significant inhibition of KC levels in the lung tissue. Both TNF-α and MIP-2 levels were increased in the lung in response to LPS challenge (895 ± 68.9 to 1872.7 ± 164.8 and 1656 ± 76.9 to 2272.3 ± 166.4 pg/mg of tissue, respectively). Treatment with roflumilast caused a significant inhibition of TNF-α at 100mg/kg but no inhibition of MIP-2 protein levels (838.8 ± 54.3 and 2167.4 ± 124.6 pg/mg of tissue). Treatment with piclamilast resulted in significant inhibition of TNF-α at 30 mg/kg and MIP-2 at 100 mg/kg (892.4 ± 45.6 and 1715.4 ± 96.9 mg/kg of tissue). Dexamethasone caused significant inhibition of both biomarkers. Neither PDE_4 inhibitor caused any increase in levels of these mediators (data not shown).

Effect of roflumilast and piclamilast on LPS-induced cellular inflammation

LPS challenge caused a significant increase in neutrophils in the BAL, which was inhibited in a dose dependent manner by roflumilast, reaching significance at 30 and 100 mg/kg of the inhibitor (figure 4A). Although not statistically significant the lowest dose of roflumilast used (3 mg/kg) produced 62% inhibition (data assessed using non-parametric one-way ANOVA with appropriate post-test). LPS challenge also resulted in a significant increase in lung tissue neutrophils that was unaffected at any dose of roflumilast tested (figure 4B). Treatment with dexamethasone resulted in a significant inhibition of neutrophils in both compartments. Interestingly, roflumilast appeared to have a pro-inflammatory effect in the lung tissue, where basal neutrophil numbers were increased (figure 4B). Indeed, this apparent pro-inflammatory effect of roflumilast in the lung tissue may account for the lack of inhibition of inflammation produced upon LPS challenge. Even at the highest dose tested (100 mg/kg), cell numbers were similar to the levels produced by treatment with roflumilast alone.

LPS challenge caused a significant increase in neutrophils in the BAL and lung tissue, which was inhibited in a dose dependent manner by piclamilast, reaching significance at 100 mg/kg and 30 mg/kg respectively (figures 5A and B). Treatment with dexamethasone caused a significant inhibition of neutrophil number in both compartments. Piclamilast also appeared to have a pro-inflammatory effect in the lung tissue (figure 5B). Basal neutrophil numbers were increased following treatment with piclamilast, although this did not reach statistical significance ($p = 0.06$).

Effect of roflumilast and piclamilast on basal cell burden in the blood

Treatment with piclamilast caused a significant increase in neutrophil numbers in the circulation (from 433.7 ± 52 to 1199.3 ± 167.3 cells x 10^3/ml; $p < 0.05$). Indeed, this was of a similar magnitude to the increase in neutrophils produced by LPS challenge (1254.7 ± 119.7 cells x 10^3/ml). This effect is very similar to that observed in the lung tissue, in that there was no increase in total white cell numbers but an increase in neutrophil numbers. Treatment with dexamethasone had no effect on either neutrophils or eosinophils. In contrast treatment with roflumilast had no effect on neutrophil numbers (234.7 ± 43.9 to 371.9 ± 94.9 cells x 10^3/ml).

Effect of roflumilast and piclamilast on basal IL-8 release *in vitro*

In HUVEC roflumilast caused a significant increase in basal IL-8 levels at both 10^{-5} and 10^{-4}M (from 244.4 ± 26.9 to 898.2 ± 36.2 pg/ml, 10^{-4}M; figure 6A). Piclamilast caused a significant increase in basal IL-8 levels at 10^{-4}M (from 214.9 ± 15.6 to 472.3 ± 83.7 pg/ml; figure 6B). The increases in response to either compound were appreciable and for roflumilast, was comparable to that elicited by LPS treatment (1250.7 ± 32.5 pg/ml). In the bronchial epithelial cell line, BEAS-2B, neither PDE_4 inhibitor caused any significant increase in release of IL-8 compared to vehicle control, even at 10^{-4}M (roflumilast: 36.6 ± 2.8 to 47.7 ± 10 pg/ml; piclamilast: 36.6 ± 2.8 to 51.6 ± 13.4 pg/ml). For comparison, the positive control, IL-1β, caused an increase in IL-8 levels up to 6701 ± 258.8 pg/ml. This suggests that, *in vivo*, the source of the KC may be endothelial and not epithelial cells.

Discussion

The objective of the experiments described here was to determine the direct effects of two PDE_4 inhibitors, roflumilast and piclamilast, in Balb/c mice. The data demonstrates that the PDE_4 inhibitors possess both pro and anti-inflammatory properties.

LPS challenge, administered as an aerosol or via the intratracheal route, has been shown to induce airway inflammation in mice (Schwartz *et al.*, 1994; Korsgren *et al.*, 2000; Birrell *et al.*, 2004) and rats (Haddad *et al.*, 2001; Ulich *et al.*, 1995). Treatment with roflumilast and piclamilast caused a reduction of neutrophil numbers in the BAL, a finding that agrees with published data. In mice (Corbel *et al.*, 2002), treatment with piclamilast (assessed from 1 to 30 mg/kg) or NVP-ABE171 (Trifilieff *et al.*, 2002) reduced BAL neutrophil numbers. Meanwhile in rats, inhibition of BAL neutrophilia was observed using cilomilast, SCH 351591, NVP-ABE171, rolipram and AWD 12-281 (Trifilieff *et al.*, 2002; Billah *et al.*, 2002; Spond *et al.*, 2001; Kuss *et al.*, 2003; Escofier *et al.*, 1999). The effect on BAL neutrophils observed here appears to be associated with a dose dependent inhibition of BAL TNF-α levels. Experiments using PDE_{4B} knockout mice, also highlighted a role for TNF-α (Jin and Conti, 2002; Jin *et al.*, 2005). However, contradictory data has been described where the inhibitory effects of rolipram, against LPS induced MPO activity, were not affected by a TNF-α specific antibody (Miotla *et al.*, 1998). In a rat model of LPS induced lung inflammation the effects of rolipram and cilomilast were shown to be independent of TNF-α production (Spond *et al.*, 2001). In the experiments described here, the PDE_4 inhibitors were shown to inhibit lung tissue TNF-α levels and treatment with piclamilast led to a significant reduction of lung tissue neutrophils at a dose of 30 mg/kg. This provides further evidence that the effects of PDE_4

inhibitors may be mediated via TNF-α. However, it should also be considered that the effects of PDE_4 inhibitors may be mediated by cytokines which were not measured here. It is also noteworthy that there was no significant inhibition of lung tissue neutrophilia following treatment with roflumilast. The neutrophilic chemokines, KC and MIP-2 are mouse homologues of the IL-8 family, whose members have been shown to be increased in response to LPS challenge in rodents (Birrell *et al.*, 2004; Yamasawa *et al.*, 1999; Holmes *et al.*, 2002; McCluskie *et al.*, 2004). Our data show that levels of both mediators were increased, in response to challenge with LPS, in BAL and lung tissue. However, there was no reduction in the levels of either chemokine following treatment with the PDE_4 inhibitors, suggesting that these compounds only partially inhibit the LPS-mediated inflammatory response. Although there does not seem to be any published data that addresses the effects of PDE_4 inhibition on these chemokines in aerosolized LPS models, the effect of rolipram on the increased neutrophilia and chemokine release in response to Klebsiella pneumoniae infection has been investigated (Soares *et al.*, 2003). Although neutrophil numbers were decreased, this was not due to inhibition of KC, but rather of TNF-α, a finding which is in agreement with our data. However, in human peripheral blood monocytes stimulated with LPS, treatment with PDE_4 inhibitors was found to have no effect on IL-8 but effectively inhibited TNF-α levels (Yoshimura *et al.*, 1997).

Our data show that levels of KC were increased in the plasma in response to aerosolized LPS challenge; however, there were no increases in the levels of either TNF-α or MIP-2. This is possibly an indication that KC is very sensitive to LPS stimulation. Alternatively, KC release could be secondary to the release of other pro-inflammatory mediators.

Both PDE_4 inhibitors demonstrated pro-inflammatory properties *in vivo* in non-LPS stimulated mice at the single dose tested. KC levels were significantly increased in both the lung tissue and plasma and appear to be associated with an increase in lung tissue and plasma neutrophil numbers. This may account for the attenuated inhibition of LPS-induced lung tissue neutrophil numbers by these compounds. BAL neutrophil numbers were substantially reduced by PDE_4 inhibitors (100 mg/kg). This effect was greater than that observed after treatment with the positive control dexamethasone. However, in the lung tissue, the inhibition produced by roflumilast and piclamilast at the highest dose (100 mg/kg) was similar to the pro-inflammatory effect produced by the compounds at the same dose. Indeed, roflumilast did not produce a significant inhibition of lung tissue neutrophils at any dose tested. It is noteworthy that the current results do not preclude the possibility that residual blood neutrophils may affect the observations made in lung tissue. The increase in basal levels of KC in the lung tissue may also explain the lack of inhibition by these compounds on LPS-induced KC levels. Additionally, there was no increase in basal KC levels in the BAL. Since KC is generated in the vasculature, the time course of the experiment may allow for extravasation into the interstitial fluid, but not into the BAL. It should be considered that using the current markers the first indications of a pulmonary and systemic pro-inflammatory effect were seen at 100 mg/kg. It is conceivable that other metabolic mechanisms that result in an underlying pro-inflammatory effect could be present at lower doses.

The mechanisms by which PDE_4 inhibitors increase levels of KC are not known. Further experiments are required to assess compound effects on transcription factors and biomarker mRNA levels in order to fully understand our findings. Recently, *in vitro* data

suggested that the mechanism of action by which roflumilast inhibits inflammatory mediators was through inhibition of NF-κB, p38 MAP kinase and JNK activation in macrophages (Kwak *et al.*, 2005). Investigation of these transcription factors *in vivo* may elucidate the mechanism of action of these compounds.

To determine the cellular source of KC, experiments were conducted in human endothelial and epithelial cells, which are known to release IL-8 (Mul *et al.*, 2000; Khair *et al.*, 1996; Striz *et al.*, 1998). Roflumilast and piclamilast treatment led to a significant increase in IL-8 release from HUVEC, but not from BEAS-2B cells. This data would suggest that the source of KC observed in our *in vivo* experiments may be endothelial rather than epithelial cells. However, the possibility of other cellular sources cannot be excluded. Since IL-8 was released from only one of the two cell types tested *in vitro*, it also suggests that this effect is specific, rather than simply a general cytotoxic effect due to a high concentration (10^{-4}M) of compound used. The airway diseases to which these inhibitors are being targeted require chronic therapy; therefore, any observed pro-inflammatory effect of these compounds could be a cause for concern.

Agents that elevate cyclic adenosine monophosphate **(**cAMP) levels have shown an increase in IL-8 release from different cell types. This has been demonstrated in granulosa cells using dibutyryl cAMP (Zeineh *et al*., 2003) and in Hela cells using forskolin (Iourgenko *et al.*, 2003). However the effects of these agents on the release of IL-8 from epithelial cells are controversial. β_2-agonists have been shown to increase IL-8 levels released from human bronchial epithelial cells (Linden, 1996; Korn *et al.*, 2001); an effect not shared by either forskolin, or the specific PDE4 inhibitor, rolipram

(Fuhrmann *et al*., 1999). It appears that the effects of elevating cAMP levels on IL-8 release in this particular cell type remain unclear.

The toxicity issues of this class of compounds is associated with arteritis in several species (Langle *et al.*, 1994; Larson *et al.*, 1996; Mecklenburg *et al.*, 2006; Vogel *et al.*, 1999), including cynomolgus monkeys (Losco *et al.*, 2004). Recent data also highlighted a role in heart failure and arrhythmias (Lehnart *et al.*, 2005). The data presented here now demonstrate further pro-inflammatory activities of selective PDE_4 inhibitors. This is the first time such properties have been observed in the lung. Although the doses at which pro-inflammatory effects were observed in this animal model are higher than those used in clinical studies, they are within the range of efficacious doses published previously for similar animal models (Corbel *et al.*, 2002). It should be considered that animal models may be less sensitive to the anti-inflammatory effects of PDE_4 inhibition and that the clinical doses may be sub-maximal for efficacy in order to maintain a therapeutic window, yet side effects remained a problem even at those lower doses (Sturton and Fitzgerald, 2002).

In summary, the data presented here show for the first time that the PDE_4 inhibitors exhibit pro-inflammatory activities in the lung that may limit their therapeutic potential in the chronic treatment of inflammatory respiratory diseases. Additionally, these data suggest that KC and IL-8 may provide surrogate biomarkers, in both pre-clinical animal models and in the clinic, with which to measure potential pro-inflammatory effects of this class of compounds. This may in turn aid in the discovery of safer PDE_4 inhibitors which can be used in the treatment of inflammatory airway diseases such as asthma and COPD.

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Legends for Figures

Figure 1: Effect of roflumilast on LPS-induced inflammatory mediator levels in the airway lumen. Mice were treated with vehicle or roflumilast (0.3 – 100 mg/kg, s.c.) 30 minutes before challenge with an aerosol of water or LPS (0.3mg/ml, 10 minutes). Dexamethasone (1 mg/kg, s.c.) was dosed 1 hour before water or LPS challenge. 3 hours after challenge BAL samples were collected and TNF-α (A), MIP-2 (B) and KC (C) levels were measured by ELISA. Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated control group; * = $p < 0.05$ compared to stimulated control group, treatment groups, n = 6-12; vehicle groups n = 18).

Figure 2: Effect of piclamilast on LPS-induced inflammatory mediator levels in the airway lumen. Mice were treated with vehicle or piclamilast (0.3 – 100 mg/kg, s.c.) 30 minutes before challenge with an aerosol of water or LPS (0.3mg/ml, 10 minutes). Dexamethasone (1 mg/kg, s.c.) was dosed 1 hour before water or LPS challenge. 3 hours after challenge BAL samples were collected and TNF-α (A), MIP-2 (B) and KC (C) levels were measured by ELISA. Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated control group; * = $p < 0.05$ compared to stimulated control group, treatment groups, n = 6-12; vehicle groups n = 18).

Figure 3: Effect of roflumilast and piclamilast on LPS-induced inflammatory mediator levels in the plasma and lung tissue. Mice were treated with vehicle, roflumilast or piclamilast (0.3 – 100 mg/kg, s.c. (30 and 100 mg/kg for lung tissue)) 30 minutes before challenge with an aerosol of water or LPS (0.3mg/ml, 10 minutes). Dexamethasone (1 mg/kg, s.c.) was dosed 1 hour before water or LPS challenge. 3 hours after challenge samples were collected and KC levels were measured by ELISA (A, roflumilast - plasma KC; B, piclamilast - plasma KC; C, lung tissue KC). Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated control group; * = $p < 0.05$ compared to stimulated control group, n = 6-12; A and B vehicle groups, n = 18).

Figure 4: Effect of roflumilast on LPS-induced cellular burden in the airway lumen and lung tissue. Mice were treated with vehicle or roflumilast (3 – 100 mg/kg, s.c.) 30 minutes before challenge with an aerosol of water or LPS (0.3mg/ml, 10 minutes). Dexamethasone (1 mg/kg, s.c.) was dosed 1 hour before water or LPS challenge. 3 hours after challenge BAL and lung tissue samples were collected and cell numbers were determined (A, BAL neutrophils; B, lung tissue neutrophils). Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated control group; * = $p < 0.05$ compared to stimulated control group, n = 6).

Figure 5: Effect of piclamilast on LPS-induced cellular burden in the airway lumen and lung tissue. Mice were treated with vehicle or piclamilast (3 – 100 mg/kg, s.c.) 30 minutes before challenge with an aerosol of water or LPS (0.3mg/ml, 10 minutes). Dexamethasone (1 mg/kg, s.c.) was dosed 1 hour before water or LPS challenge. 3 hours after challenge BAL and lung tissue samples were collected and cell numbers were determined (A, BAL neutrophils; B, lung tissue neutrophils). Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated control group; * = $p < 0.05$ compared to stimulated control group, n = 9).

Figure 6: Effect of roflumilast and piclamilast on basal IL-8 release from primary HUVEC. Cells were treated with roflumilast, piclamilast (10^{-10} – 10^{-4}M) or 0.5% DMSO vehicle for 24 hours. LPS (100 ng/ml) and IL-1β (10 ng/ml) were included as stimulus controls. After 24 hours cell culture supernatants were removed and assessed for IL-8 release by ELISA. Values are expressed as mean ± SEM. Statistical analysis was made using a Mann-Whitney U test for unpaired data, or, for multiple comparisons, a Kruskal-Wallis test followed by a Dunn's post-test. († = $p < 0.05$ compared to non-stimulated vehicle; n = 9 from three separate experiments).

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Figure 1

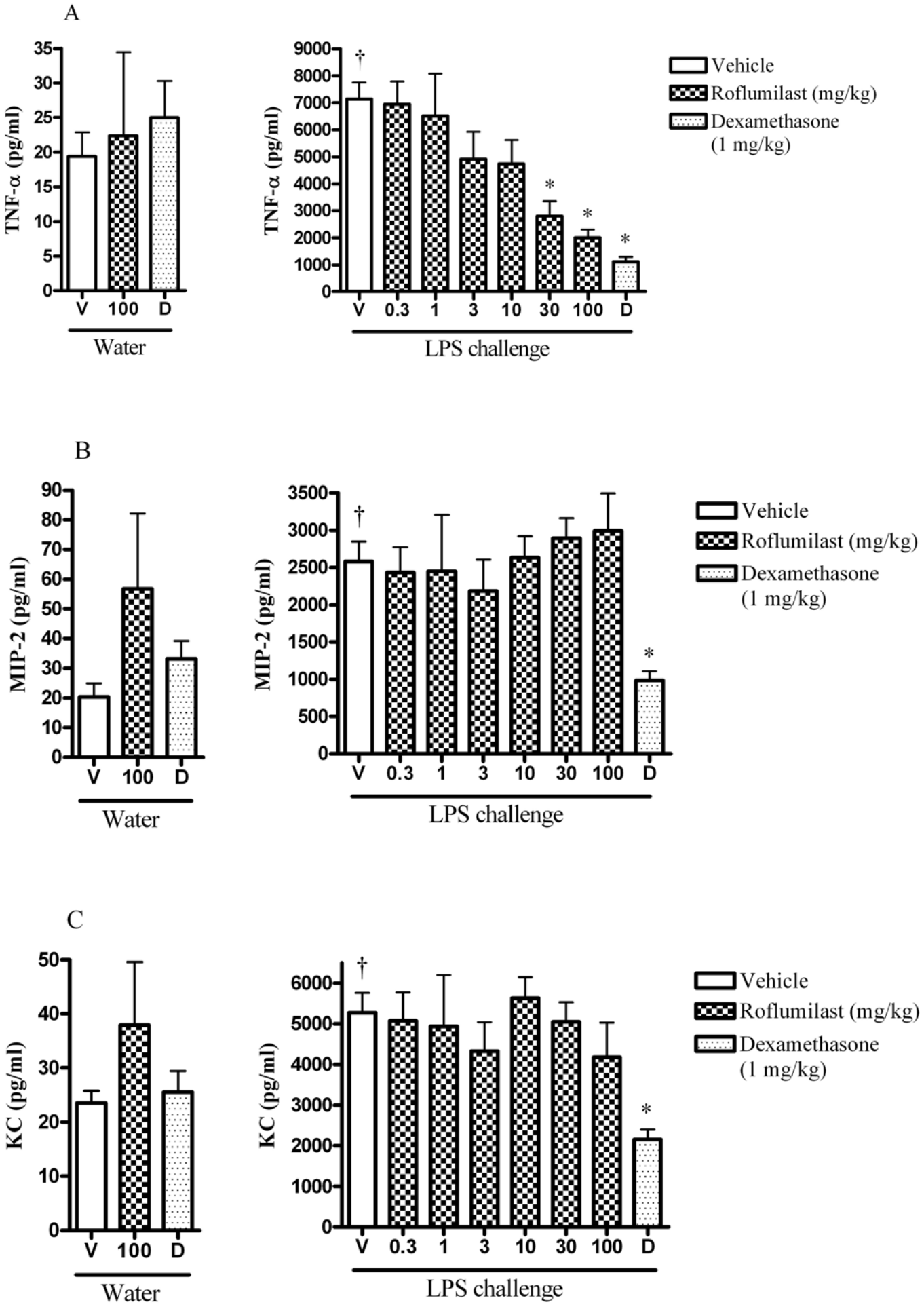

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Figure 2

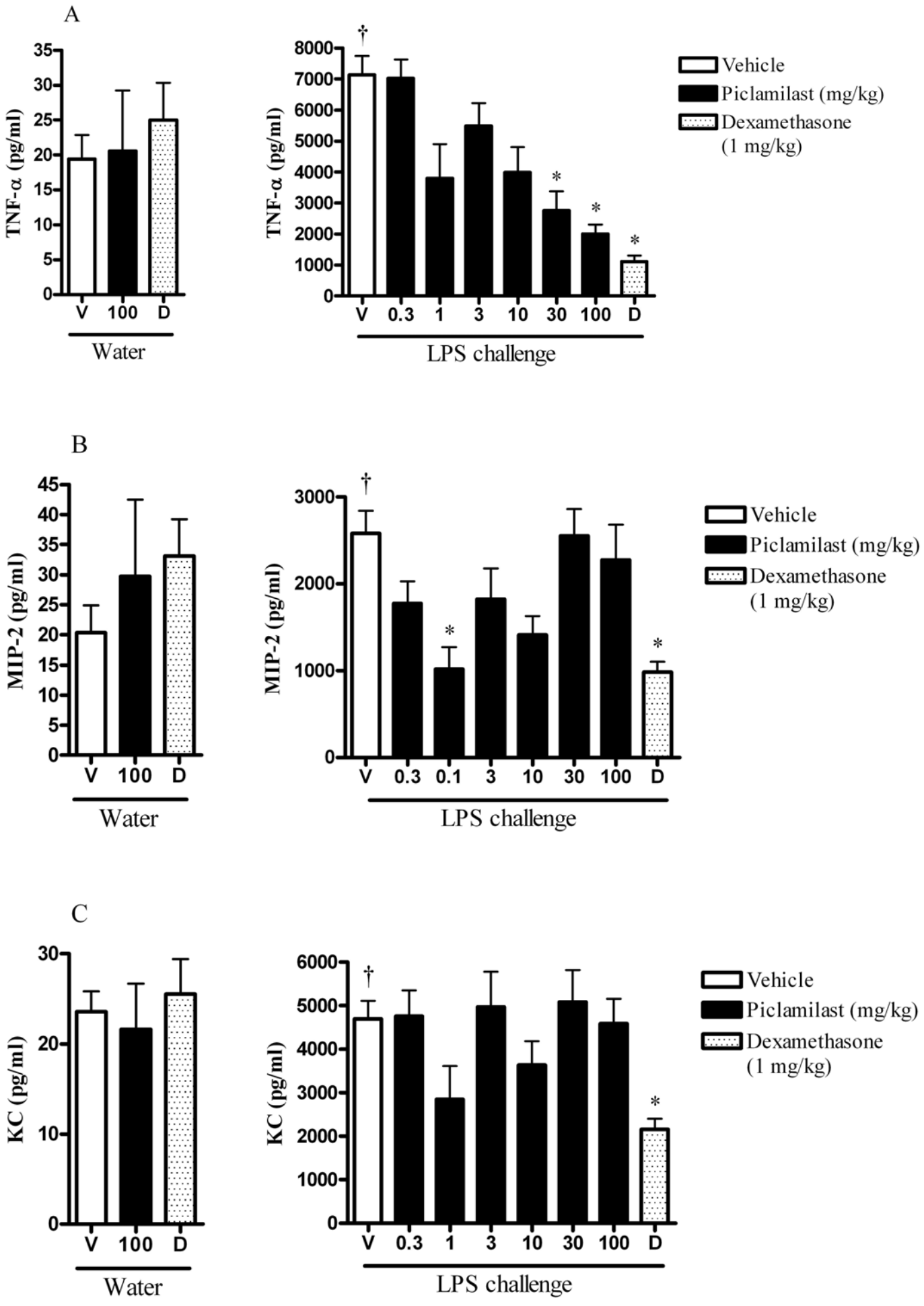

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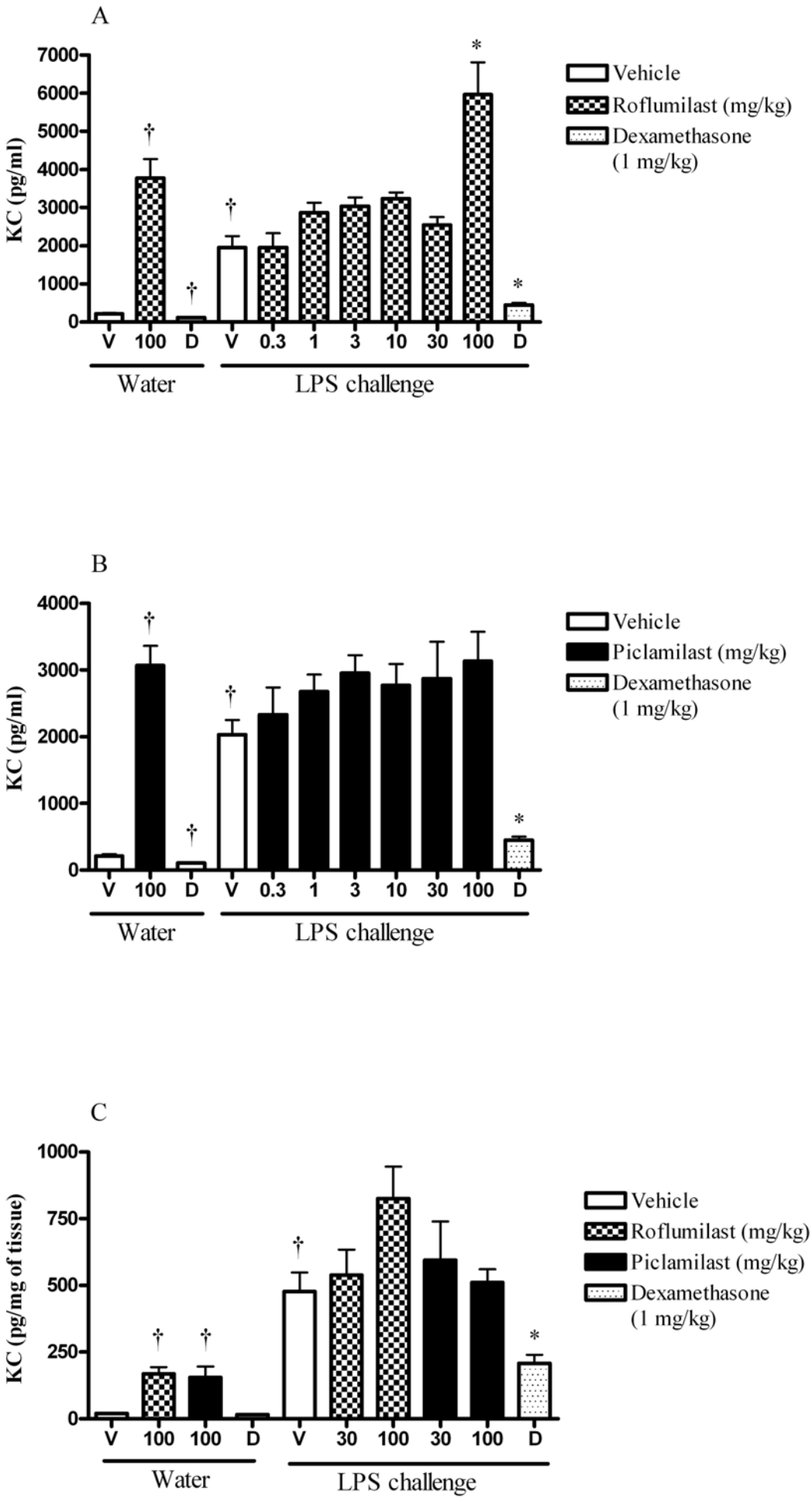
Figure 3



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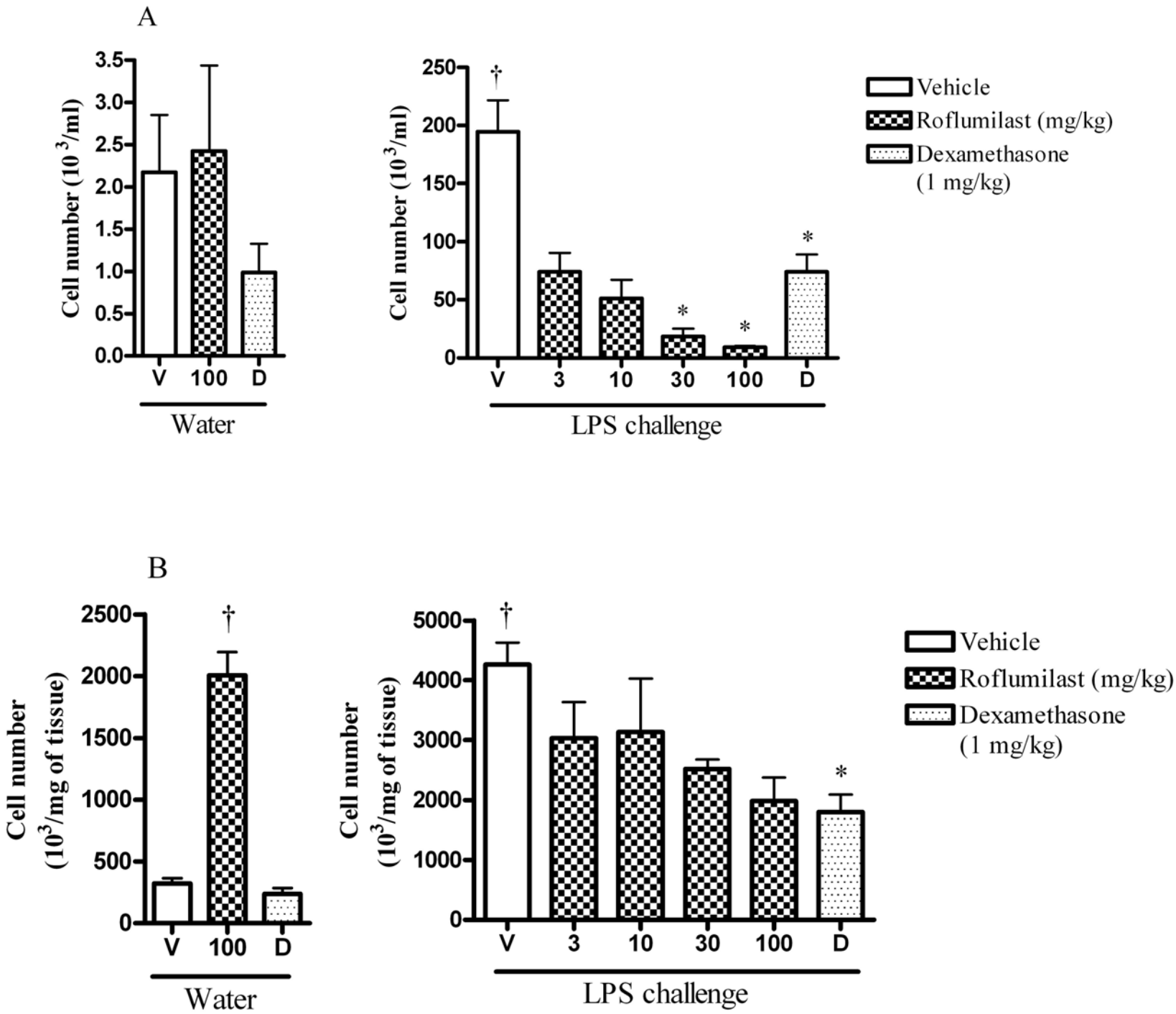

Figure 5

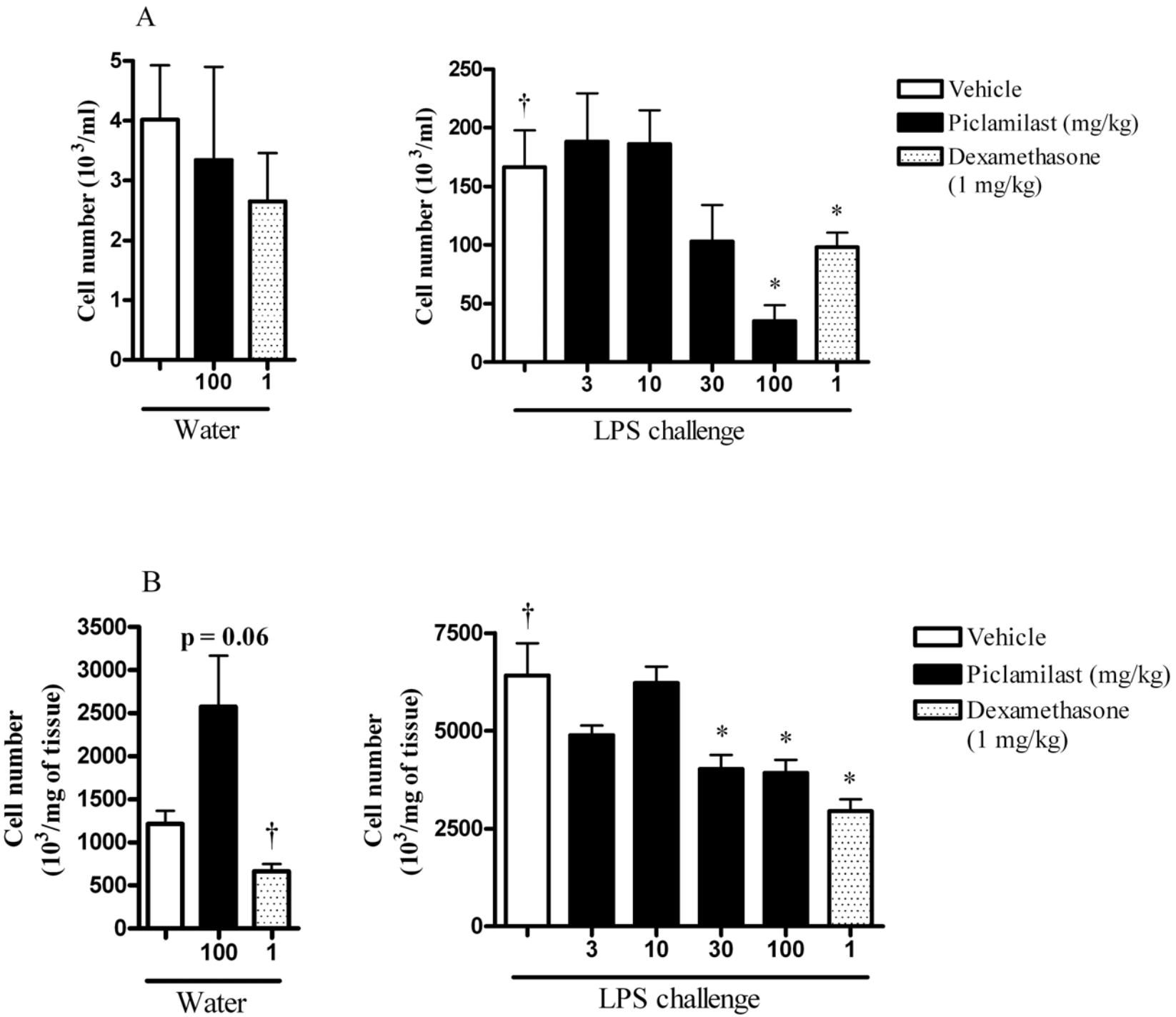

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Figure 6