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# IκB kinase-2 independent and dependent inflammation in airway disease models: relevance of IKK-2 inhibition to the clinic

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Running title: IKK-2 inhibition and inflammation

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Abstract

NF-κB is a transcription factor believed to be central in the expression of numerous inflammatory genes and the pathogenesis of many respiratory diseases. We have previously demonstrated increased NF-κB pathway activation in a steroid-sensitive animal model of LPS-driven airway inflammation. Interestingly, this phenomenon was not observed in a steroid-insensitive model of elastase-induced inflammation in the rat. The aim of this study was to gather further evidence to suggest that these similar profiles of neutrophilic inflammation can be NF-κB-dependent or -independent by determining the impact of an IκB kinase-2 (IKK-2) inhibitor, TPCA-1. In the LPS model TPCA-1 blocked the increase in NF-κB:DNA binding, a marker of NF-κB pathway activation. This inhibition was associated with a reduction in inflammatory mediator release (TNFα/IL-lβ/MMP-9) and lung inflammatory cell burden (neutrophilia/eosinophilia); data paralleled with a steroid and in human cell based assays. In the elastase-driven inflammation model, in which our group has previously failed to measure an increase in NF-κB:DNA binding, neither TPCA-1, nor the steroid, impacted on mediator release (IL-1β/MMP-9) or cellular burden (neutrophilia/lymphomononuclear cells). This is the first study examining the effect of an IKK-2 inhibitor in well validated models which mimic aspects of the inflammatory lesion evident in diseases such as COPD. In conclusion we have demonstrated that animal models with similar profiles of airway inflammation can be IKK-2 inhibitor/steroid sensitive or insensitive. If both profiles of inflammation exist in the clinic, then this finding is extremely exciting and may lead to greater understanding of disease pathology and the discovery of novel anti-inflammatory targets.

Introduction:

The nuclear factor-kappa B (NF-κB) transcription factor plays a key role in the normal "physiological" induction of pro-inflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors and enzymes (Baldwin, 2001). These NF-κB regulated mediators (eg. TNFα, IL-8, IL-6, IL-1β, MIP-1α and GROα) have been suggested to play a central role in a variety of acute and chronic inflammatory diseases (Barnes, 2001). Therefore, it has been suggested that blocking the NF-κB pathway may represent a possible disease modifying therapy (Barnes *et al*., 1997).

NF-κB is activated in response to a number of stimuli including physical and chemical stress, lipopolysaccharide (LPS), double-stranded RNA, T- and B-cell mitogens and pro-inflammatory cytokines (Rothwarf *et al*., 2000; Beyaert *et al*., 2000; Karin and Lin, 2002; Li Q, 2002). NF-κB induced gene expression is controlled by a complex series of proteins and enzymes. In resting cells, the majority of NF-κB is bound to an IκB inhibitory protein which holds the complex in the cytoplasm. Upon appropriate stimulation of the cell, the IκB protein is phosphorylated and ubiquinated, which leads to subsequent proteasome-mediated degradation. With the IκB removed the transcription factor can translocate to the nucleus and bind consensus sequences on DNA, which can then lead to gene transcription. The critical phosphorylation of the IκB protein, in the classical pathway, is performed by the IκB kinase (IKK) complex, which consists of at least three subunits, two catalytic subunits (IKK-1 and -2 also known as IKKα and IKKβ) and a regulatory subunit IKKγ (NEMO) (Scheidereit, 1998; Karin, 1999; Courtois *et al*., 2001). Of the two catalytic subunits IKK-2 is 20 fold more active than IKK-1 in the phosphorylation of IκB (Mercurio *et*

*al*., 1997) and it has been shown that IKK-2, and not IKK-1, is important in NF-κB activation *in vivo* (Hu *et al*., 1999; Li L *et al*., 1999; Li Q *et al*., 1999a; Li Q *et al*., 1999b; Takeda *et al*., 1999). In addition, IKK-2 has been shown to directly phosphorylate NF-κB, which in turn may enhance its transactivating potency (Sakurai *et al*, 2003). For this reason there has recently been a search for a small molecular weight inhibitor of IKK-2, for the potential treatment of inflammatory diseases.

Previously our group has utilised an IKK-2 inhibitor, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide) which has a $pIC_{50}$ of 7.7±0.2 on the isolated kinase and has twenty-two fold selectivity over IKK-1 and > 550 fold selectivity over other kinases and enzymes (Podolin *et al*., 2005). In cultured primary human airway smooth muscle cells (HASM) we have demonstrated that TPCA-1 inhibits IL-1β stimulated IκB phosphorylation, NF-κB:DNA binding, an NF-κB reporter assay and cytokine release. Interestingly, a steroid had no effect on NF-κB DNA binding and only a partial inhibitory effect on the reporter assay suggesting that steroids and IKK-2 inhibitors do not share a common mechanism of action. Further evidence of this was observed in the same cell based assay system where we have shown complete inhibition of cytokines such as G-CSF with an IKK-2 inhibitor with minimal or no inhibition evoked by a steroid (Birrell *et al*., 2005a). In addition, we have shown in a pre-clinical, steroid-sensitive, rodent model of asthma that TPCA-1 inhibits antigen-induced NF-κB pathway activation, by measuring a reduction in the level of NF-κB binding to DNA. Treatment with TPCA-1 in this model resulted in an inhibition of antigen induced mediator release, at the gene and protein level, and a reduction in airway eosinophilia (Birrell *et al*., 2005a).

We have recently demonstrated an increase in NF-κB pathway activation in a steroid-sensitive rat model of LPS-induced airway inflammation, a model which is

characterised by increases in neutrophilia and mediator release (e.g. TNFα, IL-1β and MMP-9) (Birrell *et al*., 2005b). In addition, we have also shown that an elastase-driven rat model, with a similar profile of airway inflammation, appears to be NF-κB-independent and resistant to steroid treatment (Birrell *et al*., 2005c). The data generated in these two models would suggest that similar profiles of airway inflammation can be NF-κB-dependent and -independent depending on the stimulus employed. This suggestion is extremely interesting and may help in understanding the pathophysiology of steroid-resistant diseases and also highlight potential novel anti-inflammatory targets.

The aim of this study was to generate additional data to aid in understanding the molecular mechanisms involved in the steroid-resistance observed in the elastase model and the steroid-sensitivity observed in the LPS model, as described above. As stated above we have previously demonstrated that an IKK-2 inhibitor appears to have a more comprehensive anti-inflammatory profile which differs to that of a steroid (Birrell *et al*., 2005a). Therefore we felt it would be extremely interesting to profile this class of anti-inflammatory in the two models by focusing on the key cells and mediators that are thought to play a role in the pathogenesis of diseases such as COPD.

Materials and Methods:

*Effect of TPCA-1 on LPS induced cytokine release from human monocytes*

The human monocytic cell line, THP-1, was purchased from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Salisbury, Wiltshire, UK). The cells were then cultured in Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen, Ltd, UK) with Glutamax I (Invitrogen Ltd, UK) supplemented with 10% FCS and 1% antibiotic and antimycotic solution (Penicillin, Streptomycin, Amphotericin B – Sigma-Aldrich Co., Poole, UK) at 37°C in a humidified atmosphere (95% air, 5% (v/v) $CO_2$). They were cultured into 75cm$^2$ flasks, and the media was replaced every 48-72 hours. For testing of the compounds, cells (400,000) were added to each well of a 24 well plate. Vehicle (DMSO, 0.1% v/v, was maintained in all treatments), TPCA-1 (30nM – 10µM) or the intra-assay positive control compound, dexamethasone (1µM) was added to wells 60 minutes prior to stimulation with vehicle or LPS (0.1µg/ml, *Escherichia coli* serotype 0111:B4 from Sigma, UK). Twenty-four hours after stimulation the culture fluid was collected and stored at -80°C until needed for cytokine assessment. Each experiment was performed in triplicate and repeated on three separate occasions. Cytokine levels in the supernatants were assessed using specific Enzyme-Linked-Immuno-Sorbent Assay (ELISA) from R&D systems (DuoSet) following the manufacturer's instructions.

In parallel studies performed in 6 well plates as above, the effect of TPCA-1 on cytokine gene expression was assessed using a method detailed in Birrell *et al* (2005a). Briefly, the cell pellet was collected 2 hours after stimulation and mRNA was extracted and converted into cDNA. Levels of gene expression were assessed using a TaqMan real-time PCR machine (ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Warrington, UK). Results were analysed using the

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Sequence Detection Software (Applied Biosystems, Warrington, UK), and the relative amount of target gene transcript was normalised to the amount of 18S internal control transcript in the same cDNA sample. The difference between 18S and target values is known as delta ct. Due to the exponential nature of PCR, the delta ct values were converted to a linear form by $2^{-\text{delta ct}}$ i.e. $2^{-(\text{target ct} - \text{18s ct})}$, written as $2^{-\Delta ct}$. This calculation was used to enable relative quantitation analysis between samples.

*Effect of TPCA-1 on LPS induced cytokine release from human lung tissue macrophages*

Human lung tissue macrophages were obtained from non-diseased, lung transplant donor tissue that was not suitable for transplant as outlined below. Ethical approval for the study was obtained along with consent from the relatives. Lung tissue was cut into small pieces (approx. 3cm x 3cm) and lavaged with phosphate buffered saline (without calcium and magnesium) (Sigma-Aldrich Co., Poole, UK). The pooled cell suspensions were passed through a 70 µm cell sieve and centrifuged at 250 x *g* for 10 minutes at 4$^{o}$C, in a chilled centrifuge (Mistral 3000i, MSE). The supernatant was discarded and the cell pellets were resuspended in phosphate buffered saline (without calcium and magnesium) and layered onto six discontinuous Percoll gradients (60%/35%/25% v/v). These gradients were then centrifuged at 1200 x *g* for 25 minutes at 20$^{o}$C, with the brake set at 0. After centrifugation, the macrophage enriched fractions were obtained from the 35% and the 60% Percoll interface, and washed twice with Hanks balanced salt solution. The cells were then resuspended with RPMI 1640 with Glutamax I supplemented with 10% FCS (Invitrogen LTD,

UK) and 1% antibiotic and antimycotic solution. Trypan Blue exclusion was performed to assess cell viability, cell purity of the macrophage enriched fraction was determined with Kimura stain. The cell suspension was diluted in RPMI 1640 with glutamax I, supplemented with 10% FCS and 1% antibiotic and antimycotic solution, and 500µl of 400,000 cells per well were added to 24 well plates (Costar, UK). These plates were then incubated for 60 minutes at 37$^{o}$C in a humidified atmosphere (95% air, 5% (v/v) $CO_2$). After 60 minutes, the non-adherent cells were removed and fresh medium was added. The adherent purified macrophages were incubated overnight, for treatment the following day. After discarding the supernatant, the macrophages were treated in the similar way as the THP-1 cells, as detailed above.

*Effect of TPCA-1 on LPS induced airway inflammation in the rat.*

Male Wistar rats (175-200g) were purchased from Harlan-Olac (Bicester, U.K.) and kept for at least 5 days before initiating experiments. Food and water were supplied *ad libitum*. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. Experiments were conducted with groups of n = 8 animals.

Rats were orally dosed with vehicle (DMSO (2%), CremophorEL (10%) and ethanol (5%) in distilled water, at a dose volume of 3ml/kg) or TPCA-1 (3, 10, 30 or 60 mg/kg) one hour prior and 2 hours post an aerosol challenge of endotoxin free saline (for 30 minutes) or LPS (1mg/ml). This dosing regimen was used as it was found to give adequate compound exposure as assessed by pharmacokinetic studies and efficacy in an in-house antigen-driven model of allergic inflammation (Birrell *et al* 2005a). The glucocorticoid, Budesonide (3mg/kg), which is a commonly used oral

steroid in man, was used as a positive control in these *in vivo* experiments as it has previously been shown to inhibit LPS-induced neutrophilia in the rat (Birrell *et al*., 2005b). A similar dosing regimen was adopted which had been validated in our previous studies (Birrell *et al* 2005b; Belvisi *et al*, 2001).

*Quantification of airway inflammation*

Six hours after saline or LPS challenge animals were euthanised with sodium pentobarbitone (200mg/kg, i.p.) and the trachea cannulated. Cells were recovered from the airway lumen by bronchoalveolar lavage (BAL), this involves flushing the airways with 10 ml/kg of RPMI delivered through the tracheal cannula and removed after a 30 sec interval. This procedure was repeated and samples were then pooled for each animal. The inflammatory cells were extracted from the lung tissue by collagenase digest as described by Underwood *et al*, (1997). The remaining lung tissue was either flash frozen in liquid nitrogen for gene expression assessment or insufflated with 10% neutral buffered formalin at a pressure of 20mmHg for demonstration of inflammatory status in the lung. After remaining overnight in the fixative, the lungs were cleaned and processed into paraffin blocks. Paraffin sections (3µM) were cut and stained with Mayer's Haematoxylin and Eosin for assessment of cellular inflammation.

Total white cell numbers in the BAL and lung tissue samples were determined on the Sysmex F820 haematology analyser according to manufactures instructions and after calibration of the machine using a standard protocol and sample of standard whole blood (Linford Wood, Milton Keynes, U.K). Cytospins of these samples were prepared by centrifugation of 100µl aliquots in a cytospin (Shandon, Runcorn, UK) at 700 rpm for 5 min at low acceleration at room temperature. Slides were fixed and

stained on a Hema-tek 2000 (Ames Co., Elkhart, U.S.A.) with modified Wrights-Giemsa stain. Four part differential counts on 200 cells per slide were performed following standard morphological criteria and the percentage of eosinophils, lymphomononuclear cells and neutrophils were determined as described in Underwood *et al* (1997).

*Mediator determination in the lung following LPS challenge*

Levels of TNFα and IL-1β in the lung were determined by ELISA using rat DuoSets ® according to manufacturer's instructions. The levels of MMP-9 in the lung were determined by zymography as described by Birrell *et al* (2005b).

*Effect of TPCA-1 on porcine pancreatic elastase induced airway inflammation.*

Male Sprague Dawley rats (260-300g) were purchased from Harlan-Olac (Bicester, U.K.) and kept for at least 5 days before initiating experiments. Food and water were supplied *ad libitum*. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. Experiments were conducted with groups of n = 8 animals.

Rats were orally dosed with vehicle (DMSO (2%), CremophorEL (10%) and ethanol (5%) in distilled water, 3ml/kg) or TPCA-1 (10, 30 or 60mg/kg) one hour prior and 6, 22, 30 and 46 hours post an intratracheal dose of endotoxin free saline (1ml/kg) or porcine pancreatic elastase (PPE) (120U/kg). As above, budesonide (3mg/kg) was included in the study and dosed using the same regimen.

Forty-eight hours after PPE administration the lungs were assessed for cellular infiltration and inflammatory mediators as above. Assessment of airway tissue

inflammation was performed in formalin insufflated (pressure of 20mmHg) fixed lung tissue by an experienced histologist. Levels of overall inflammatory cell burden and macrophages were assessed using a doubling of severity scoring system of no score, focal inflammation, 1, 2, 3 and 4. These scores were then converted into arbitrary values (0, 10, 20, 40, 80 and 160).

Levels of inflammatory mediator gene expression (TNFα, IL-1β, iNOS and MMP-9) in the frozen lung tissue from the time course experiment performed in Birrell *et al* (2005c) were determined using TaqMan real-time PCR using the methods outlined above.

*Assessment of NF-κB pathway activation*

To assess the effect of the compound on NF-κB:DNA binding in the LPS driven *in vivo* model EMSA assays were carried out. Details of the assay used are outlined in Birrell *et al* (2005c), briefly nuclear extracts were prepared from the lung tissue using an NXTRACT kit (Sigma-Aldrich Ltd), and assessed for protein levels using a Bradford assay. EMSA oligonucleotide probes were labelled using 5' end labelling with T4 polynucleotide kinase. The labelling reactions, containing 1 × kinase buffer (Promega) supplemented with 3.5 pmol of double stranded probe NF-κB consensus oligonucleotide (Promega), 20 µCi [$\gamma_{32}$P] ATP and 10 units of T4 polynucleotide kinase, were incubated for 1 hour at 37$^{o}$C. Labelled oligonucleotide was separated from residual [$\gamma_{32}$P] ATP by G25 sephadex spin column.

EMSA reactions containing equal concentrations of nuclear extracts in 18 µl buffer D and 5 µl of 5× EMSA binding buffer (Promega) was incubated for 20 minutes on ice prior to the addition of 2 µl of labelled probe. Specificity was determined by the

addition of a 100-fold excess of unlabelled competitor consensus oligonucleotide. After an additional 1 hour on ice the reactions were stopped by the addition of 3 µl of EMSA loading buffer (50% (v/v) glycerol, 0.05% (w/v) bromophenol blue), loaded onto a 6% poly acrylamide gel and electrophoresed until the dye front runs to the end of the gel, typically about 2 hours. The experiments were carried out on ice to prevent protein degradation. The gels were then vacuum dried and exposed to BioMax MS-1 film (Sigma) at -80$^{o}$C until defined bands were visible.

*Statistical analysis*

Values are expressed as mean ± s.e.mean of n independent observations. Statistical comparisons were made using a Mann-Whitney test for two groups of non-parametric data; one-way analysis of variance followed by Dunnett's post test for multiple comparisons of parametric data or Kruskal-Wallis one-way analysis of variance followed by Dunn's post test for multiple comparisons of non-parametric data.

Results

*Effect of TPCA-1 on LPS induced cytokine release from cultured human cells.*

Stimulation with LPS of cultured THP-1 cells caused an increase in a range of inflammatory cytokines: TNFα, IL-8, IL-6, IL-1β and MIP-1α (Figure 1). Treatment with TPCA-1 caused a concentration related decrease in all cytokines measured, similar in magnitude to the intra-assay steroid positive control (Figure 1). The potency of the compound in this assay ($IC_{50}$ of approximately 100-300nM) appeared to be comparable with data reported by Podolin *et al* (2005). Assessment of the gene expression of the same cytokines would suggest that the IKK-2 inhibitor acts at the transcriptional level but post-transcriptional effects (eg. mRNA stability) can not be ruled out (Figure 2). In primary human lung tissue macrophages LPS stimulation increased the production of a similar range of inflammatory cytokines: TNFα, IL-8, IL-6, MIP-1α and GROα (Figure 3). Similar to the result with the THP-1 cells, treatment with TPCA-1 caused a concentration related decrease in all cytokines measured in macrophages, this was similar in magnitude to the intra-assay positive control, a steroid (Figure 3).

*Effect of the IKK-2 inhibitor on airway inflammation in two rodent models.*

To demonstrate that inhibition of IKK-2 by TPCA-1, using the dosing regimen stated in the methods, is impacting on NF-κB pathway activation an EMSA was carried out. As can be seen from the representative blot, in the steroid-sensitive LPS-driven model there was an increase in the amount of NF-κB:DNA binding which suggests an increase in NF-κB pathway activation (Figure 4). The IKK-2 inhibitor caused an inhibition of the LPS-induced increase in NF-κB:DNA binding, with the

profile of inhibition being similar to the impact on inflammatory mediators and cellular burden (Figure 4). Previously it has been demonstrated by our group that there is no increase in NF-κB:DNA binding over an extensive time course in the PPE driven, steroid-insensitive model (Birrell *et al.,* 2005c).

In the LPS-driven model we detected an increase in inflammatory mediators such as TNFα, IL-1β and MMP-9, which were inhibited by treatment with TPCA-1 and budesonide (Figure 5). In the PPE-driven model a similar increase in mediator release was detected but this was not affected by TPCA-1 or the steroid (Figure 6). The effect of TPCA-1 on mediator release was mirrored by its effect on inflammatory cell burden in these models in that it was reduced by the IKK-2 inhibitor and budesonide in the LPS model but not in the PPE model (Figures 7 and 8). Confirmatory evidence for these results is depicted in Figure 9 and 10 which shows the histological profile of the stained lung tissue from both the LPS and the PPE driven studies.

When the levels of TNFα, IL-1β, MMP-9 and iNOS gene expression were measured in a time course experiment performed in Birrell *et al* (2005c) a significant increase was observed (Table 1). This would suggest that there are some transcriptional events occurring in the elastase-driven model and that the inflammatory mediators measured after PPE insult may be from *de novo* synthesis.

Discussion

It is currently believed that chronic inflammation is not only central to the pathogenesis of many respiratory diseases but that it is also partly responsible for many of the symptoms. Hence a great deal of effort has, and is, being made to find anti-inflammatory therapies to treat these diseases. Inhibition of NF-κB pathway activation is a popular target for novel anti-inflammatory therapies. Activation of the NF-κB pathway is believed to play a key role in the induction of pro-inflammatory gene expression, leading to the synthesis of inflammatory mediators and recruitment/activation of inflammatory cells (Baldwin, 2001). As discussed in the introduction, our group has preliminary data to suggest that a similar profile of airway inflammation, characterised by airway neutrophilia, can be induced by NF-κB-dependent or -independent mechanisms. The data obtained previously demonstrated that NF-κB pathway activation was present in the LPS-driven model and that the inflammatory response was blocked by steroid treatment (Birrell *et al*., 2005b). Others have also shown, in a similar murine model, a prominent role for NF-κB activation in LPS-induced airway inflammation (Poynter *et al*., 2003). In the elastase-driven model a similar inflammatory response was observed despite the fact that an increase in NF-κB pathway activation was not observed (Birrell *et al*, 2005c). Steroid therapy is believed to impact on the actions of NF-κB and in the elastase-driven model the inflammatory response proved resistant to treatment with a steroid (Birrell *et al*., 2005c) and so perhaps the lack of effect of an IKK-2 inhibitor in this model could have been expected. However, although some groups have reported an inhibitory action of glucocorticoids on NF-κB translocation and DNA binding (Mukaida, *et al*, 1994; Cazes *et al*, 2001), we have evidence to suggest that steroids do not inhibit NF-

κB translocation and binding, and only partially impact on NF-κB reporter gene assays (Birrell *et al*, 2005a; Newton *et al*, 1998). It is not clear why these discrepancies exist but it may be due to the different cell types and/or stimulus employed in these studies and suggests that controversy still surrounds the action of steroids on NF-κB pathway activation. In our previous study we were not able to demonstrate an increase in NF-κB pathway activation in the elastase model when looking at gross changes in pathway activation in lung tissue samples at different time points following elastase administration (Birrell *et al*., 2005c). Even though the results of this study are compelling, it may be possible that, despite the extensive time course being studied, we may not have chosen the correct time point to demonstrate NF-κB pathway activation. In addition, it may be possible that local increases in NF-κB:DNA binding were lost when the whole lung tissue was assessed. One way in which to address this question would be to perform immunohistochemical studies to search for discrete areas of NF-κB pathway activation (e.g. by determining nuclear localisation of p65). However, in this study we have adopted a pharmacological approach by using a selective inhibitor of this signalling pathway. We have previously shown, in human cell based assays, that an IKK-2 inhibitor has a more comprehensive anti-inflammatory profile and different mechanism to that of a steroid (Birrell *et al*., 2005a). Therefore, the aim of this study was to compare the impact of an IKK-2 inhibitor with that of a steroid in the proposed NF-κB dependent and independent models. Although it is not the subject of this publication, it would be interesting to determine if the combination of an IKK-2 inhibitor with a steroid would result in any additive or synergistic effects.

The IKK-2 inhibitor blocked the release of a range of cytokines from LPS stimulated THP-1 cells (Figure 1) and human lung tissue macrophages (Figure 3).

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Assessment of gene expression (Figure 2) suggested that TPCA-1 impacted at the transcriptional level (i.e. percentage inhibition of TNFα / MIP-1α / IL-1β / IL-8 at the protein and gene levels with TPCA-1 (300 nM) were 68 versus 59; 89 versus 75; 67 versus 87 and 30 versus 86, respectively), although post-transcriptional events can not be ruled out. The reduction in inflammatory cytokine production observed in this cell system is similar to that seen in another study performed by our group, using the same compound, in which we demonstrated that TPCA-1 blocked IL-1β induced NF-κB reporter assay activity and mediator release in human airway smooth muscle cells ($IC_{50}$ between 100-1000 nM) (Birrell *et al*., 2005a).

In the pre-clinical rodent model of LPS-induced airway inflammation we observed an increase in NF-κB pathway activation, as measured by an increase in NK-κB:DNA binding (Figure 4). Treatment with TPCA-1 inhibited this increase in NF-κB:DNA binding, suggesting that the compound is impacting on NF-κB pathway activation (Figure 4). The profile of inhibition by TPCA-1 on the NF-κB pathway was very similar to the impact on inflammatory cytokines, MMP-9 levels and cellular recruitment, suggesting a causative role (Figures 5, 7 and 9). However, it is possible that some of the impact of the IKK-2 inhibitor is not through inhibition of NF-κB pathway activation. Recently, data has been published suggesting a role for IKK-2 in NF-κB independent events. For example, Sizemore *et al* (2004) have demonstrated an NF-κB independent role for IKK-2 in IFNγ stimulated gene expression.

It has previously been demonstrated by our group that there is no increase in NF-κB:DNA binding in the steroid-insensitive elastase driven model (Birrell *et al*., 2005c). In this model, the IKK-2 inhibitor did not impact on the airway inflammatory response, in contrast to the inhibitory effect observed in the LPS model (Figures 6, 8 and 10). This result by itself does not prove that elastase-induced inflammation is NF-

κB independent as it is now believed that IKK-2 is not central to all NF-κB pathway activation (Hayden *et al*., 2004). However, this data in conjunction with the absence of an increase in NF-κB:DNA binding and the lack of impact of a steroid makes for a persuasive argument. In fact, this data may suggest that, along with NF-κB dependent inflammation, aspects of the inflammatory profile in diseased patients could be independent of the NF-κB transcription factor. It is possible that this NF-κB-independent inflammation could be the reason for the limited impact of glucocortoid therapies in respiratory diseases such as COPD and severe asthma, especially as these compounds are thought to achieve efficacy through impacting on aspects of the NF-κB pathway. One may question the clinical relevance of the inflammation observed in the elastase model, especially considering the publications in which an increase in NF-κB:DNA binding has been shown in patients with COPD (Caramori *et al*., 2003; Di Stefano *et al.*, 2004) and severe asthma (Gagliardo *et al*., 2003). However, we speculate that, similar to the data we have generated in these animal models, these two alternative inflammatory pathways may co-exist in the human disease. Indeed, Caramori *et al* (2003) have published data which demonstrated an increase in nuclear p65 staining in macrophages from COPD patients but not in neutrophils from the same patients. In addition, Di Stefano *et al* (2004) demonstrated an increase in the expression of NF-κB in the lungs of 'healthy' smokers, suggesting that some of the increase observed may be related directly to smoking cigarettes, rather than a facet of disease *per se*. Furthermore, a recent publication described no significant difference in nuclear localisation of NF-κB in bronchial biopsies from healthy smokers compared with patients with COPD. Indeed, in the same study BAL fluid leucocytes from healthy smokers showed a significant reduction in NF-κB:DNA binding compared with non-smokers, while nuclear translocation of NF-κB during exacerbations of

COPD did not differ from that in non-smokers (Drost *et al*, 2005). However, regardless of whether this model represents the clinical situation at the very simplest level it provides us with a robust model for dissecting the signalling pathways behind this NF-κB-independent, steroid-resistant inflammatory response.

At the moment it is unclear which molecular mechanism(s) are responsible for the inflammation observed in the elastase-driven model. If the mechanism(s) could be elucidated, and be shown to be relevant to human diseases, it might highlight potential novel anti-inflammatory targets for the treatment of steroid-resistant diseases. It is possible that the inflammation observed in the elastase model is down stream of IKK-2/NF-κB i.e. driven by pre-existing mediators which are activated directly or indirectly by the elastase. The increase in gene expression (Table 1), however, would suggest that transcriptional events are occurring that are independent of IKK-2 and increased NF-κB:DNA binding and are not sensitive to treatment with a glucocorticoid. Although it may also be possible that the increase in inflammatory mediator gene expression is due to changes in the basal transcriptional machinery (i.e. altered chromatin configuration) or post-transcriptional modification (i.e. increase in mRNA stability).

In summary, we have demonstrated that similar profiles of airway inflammation, induced by different stimuli, can be NF-κB-dependent or -independent and IKK-2 inhibitor/steroid-sensitive or -insensitive. Elucidation of the molecular mechanisms behind this apparent NF-κB-independent inflammation could lead to the discovery of novel anti-inflammatory targets and the development of inhibitors which may be effective in diseases less sensitive to steroids such as COPD and severe asthma.

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**Footnote**

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Figure legends

Figure 1: Effect of IKK-2 inhibitor (shown as the negative Log of the Molar concentration) on cytokine production in THP-1 cells.

Supernatant cytokine levels from cultured human monocyte stimulated with LPS (0.1µg/ml) and treated with TPCA-1 or dexamethasone. A: TNFα, B: IL-8, C: IL-6, D: IL-1β, E: MIP-1α (n = 9). + = significant difference between vehicle stimulated / vehicle treated group and LPS stimulated /vehicle treated group, * = significantly different from LPS stimulated / vehicle treated group ($P<0.05$).

Figure 2: Effect of IKK-2 inhibitor (shown as the negative Log of the Molar concentration) on cytokine gene expression in THP-1 cells.

Gene expression ($2^{-\Delta ct}$ (x $10^6$)) levels in LPS (0.1µg/ml) stimulated human monocytes and treated with TPCA-1 (A: TNFα, B: MIP-1α, C: IL-8 and D: IL-1β (n = 9). + = significant difference between vehicle stimulated / vehicle treated group and LPS stimulated /vehicle treated group, * = significantly different from LPS stimulated / vehicle treated group ($P<0.05$).

Figure 3: Effect of IKK-2 inhibitor (shown as the negative Log of the Molar concentration) on cytokine production in macrophages.

Supernatant cytokine levels from primary human lung tissue macrophages stimulated with LPS (0.1µg/ml) and treated with TPCA-1 or dexamethasone. A: TNFα, B: IL-8, C: IL-6, D: MIP-1α, E: GROα (n = 5). + = significant difference between vehicle stimulated / vehicle treated group and LPS stimulated /vehicle treated group, * = significantly different from LPS stimulated / vehicle treated group ($P<0.05$).

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Figure 4: NF-κB pathway activation in the lung tissue.

A representative autoradiograph from EMSA analysis of NF-κB:DNA binding in the nuclear fraction extracted from the lung collected from the LPS study. To demonstrate specificity 100-fold excess of unlabelled competitor consensus oligonucleotides was added to a reaction (indicated with #).

Figure 5: Effect of IKK-2 inhibitor on LPS induced mediator release in the rat lung.

Impact of TPCA-1 or budesonide on lung levels of inflammatory mediators 6 hours after exposure to aerosolised LPS (1mg/ml for 30 minutes). A: BAL fluid TNFα levels; B: lung tissue IL-1β levels; C: BAL fluid MMP-9 levels. + = significantly different from saline challenged / vehicle treated group, * = significantly different from LPS challenged / vehicle treated group $P<0.05$).

Figure 6: Effect of IKK-2 inhibitor on elastase induced mediator release in the rat lung.

Impact of TPCA-1 or budesonide on lung levels of inflammatory mediators 48 hours after exposure to PPE (120U/kg, i.t.). A: BAL IL-1β levels; B: BAL fluid MMP-9 levels. + = significantly different from saline challenged / vehicle treated group, * = significantly different from PPE challenged / vehicle treated group $P<0.05$).

Figure 7: Effect of IKK-2 inhibitor on LPS induced cellular recruitment in the rat lung.

Number of neutrophils (A) and eosinophils (B) in the lavage fluid and neutrophils (C) and eosinophils (D) in the lung tissue after exposure to aerosolised LPS and treatment

with vehicle, TPCA-1 or budesonide. + = significantly different from saline challenged / vehicle treated group, * = significantly different from LPS challenged / vehicle treated group $P<0.05$).

Figure 8: Effect of IKK-2 inhibitor on elastase induced cellular recruitment in the rat lung.

Number of neutrophils (A) and lymphomononuclear cells (B) in the lavage fluid and total inflammatory cell burden (C) and macrophage (D) in the lung tissue after exposure to PPE (120U/kg, i.t.) and treatment with vehicle, TPCA-1 or budesonide. + = significantly different from saline challenged / vehicle treated group, * = significantly different from PPE challenged / vehicle treated group $P<0.05$).

Figure 9: Effect of IKK-2 inhibitor on cellular inflammation after LPS challenge.

Paraffin sections were cut from tissue taken from A: Vehicle challenged/ Vehicle dosed; B: LPS challenged/ Vehicle dosed; C: LPS challenged/ TPCA-1 (60mg/kg) dosed; D: LPS challenged/ budesonide (3mg/kg) dosed groups. These were then stained with Mayer's Haematoxylin and Eosin for demonstration of cellular inflammation.

Figure 10: Effect of IKK-2 inhibitor on cellular inflammation after PPE challenge.

Paraffin sections were cut from tissue taken from A: Vehicle challenged/ Vehicle dosed; B: PPE challenged/ Vehicle dosed; C: PPE challenged/ TPCA-1 (60mg/kg) dosed; D: PPE challenged/ budesonide (3mg/kg) dosed groups. These were then stained with Mayer's Haematoxylin and Eosin for demonstration of cellular inflammation.

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Table 1: Data from the elastase driven model time course

| Time post challenge | 2 hours | 6 hours | 24 hours | 48 hours |
|---|---|---|---|---|
| | | | | |
| TNFα | 1.2 ± 0.1 | 2.2 ± 0.6 | 2.2 ± 0.8 | 4.5 ± 1.6 |
| IL-1β | 1.4 ± 0.4 | 3.2 ± 0.7 | 3.3 ± 0.9 | 3.3 ± 0.7 |
| MMP-9 | 1.7 ± 0.6 | 1.7 ± 0.5 | 3.2 ± 0.9 | 5.2 ± 1.0 |
| iNOS | 1.9 ± 0.4 | 5.3 ± 2.2 | 4.2 ± 1.3 | 15.0 ± 10.9 |
| | | | | |

Data expressed as fold difference from time matched vehicle dosed control groups.

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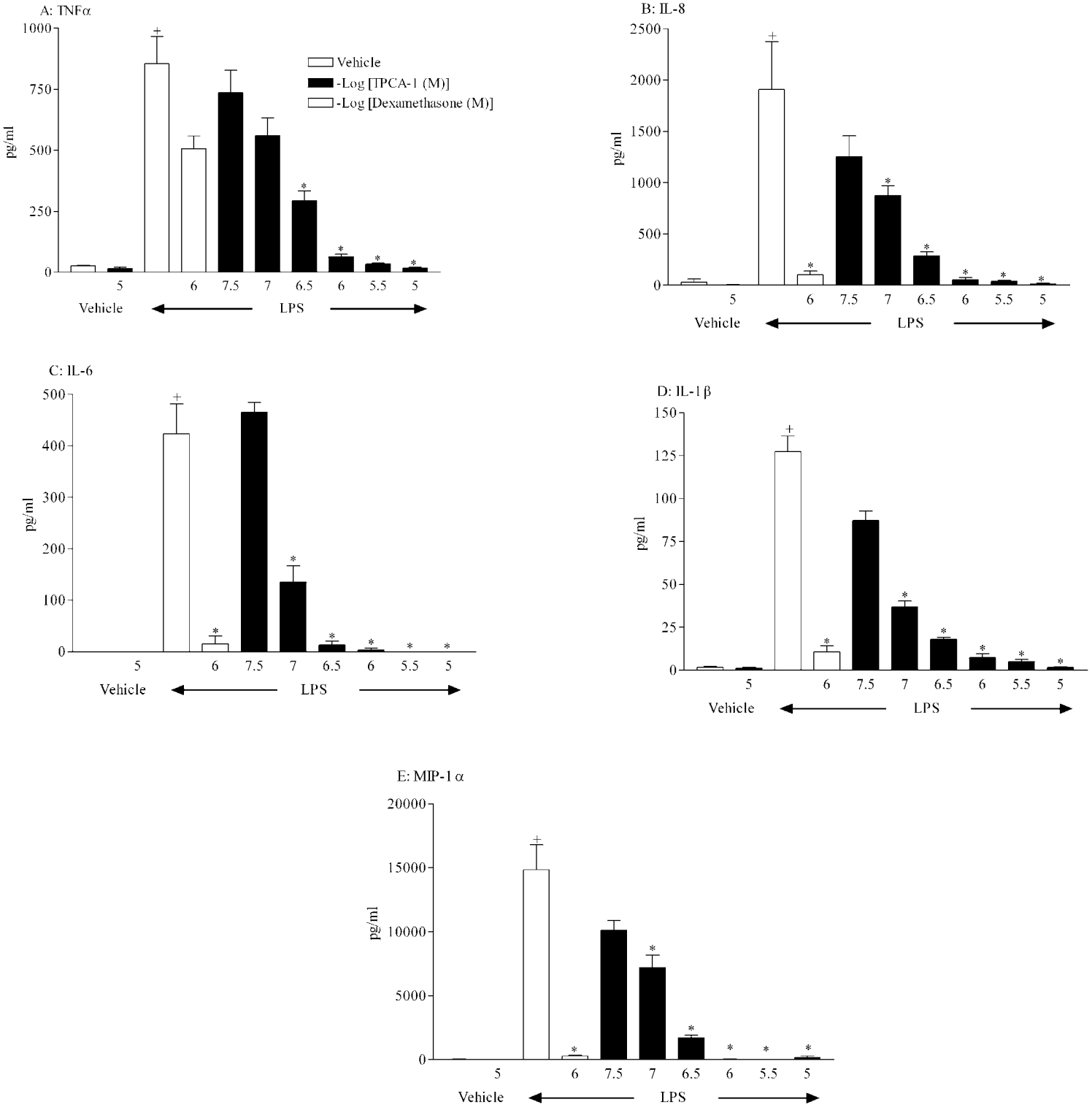
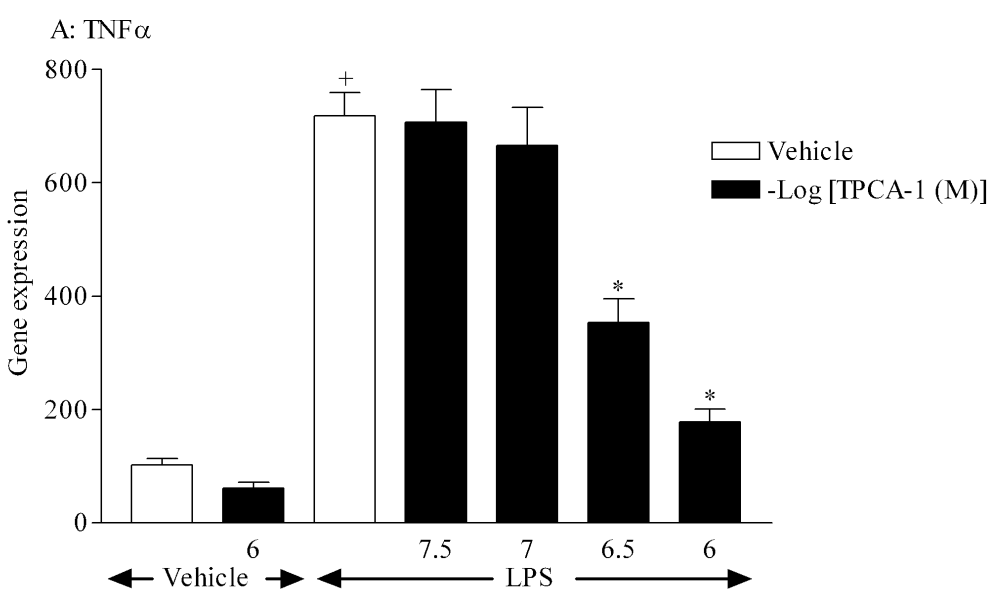
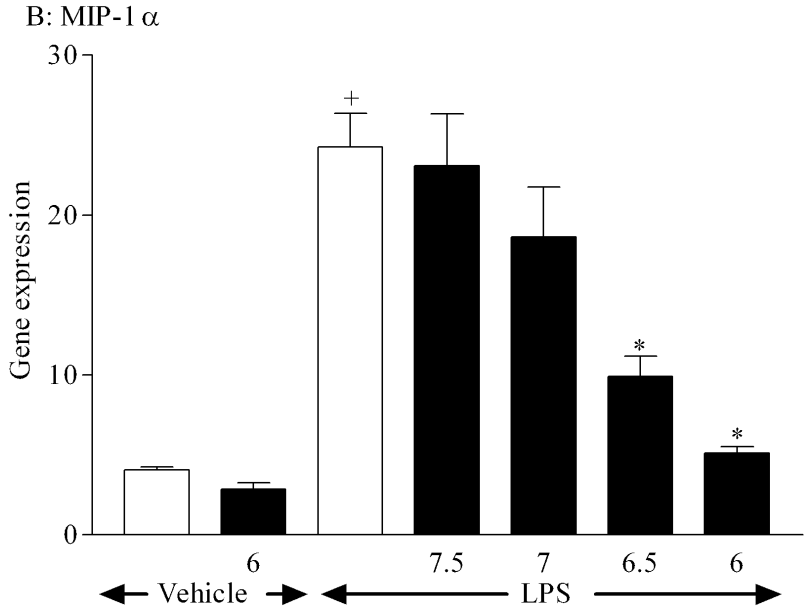
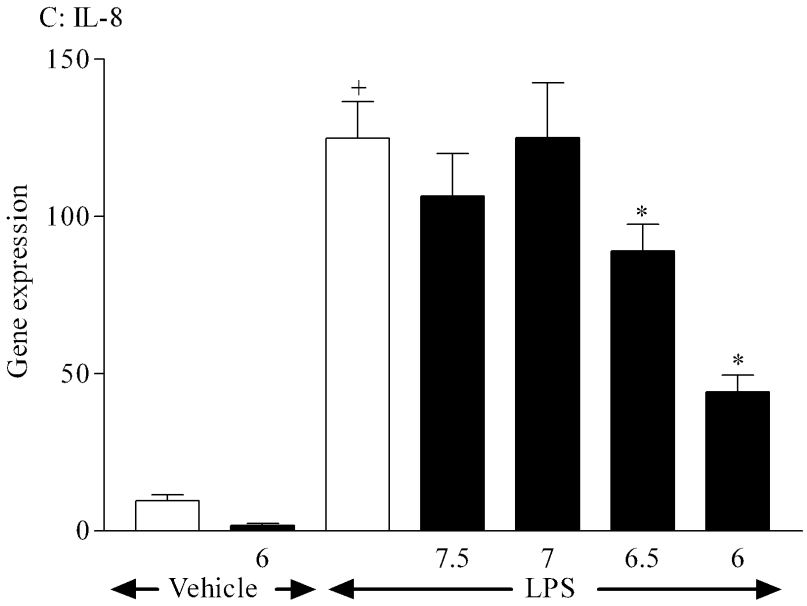


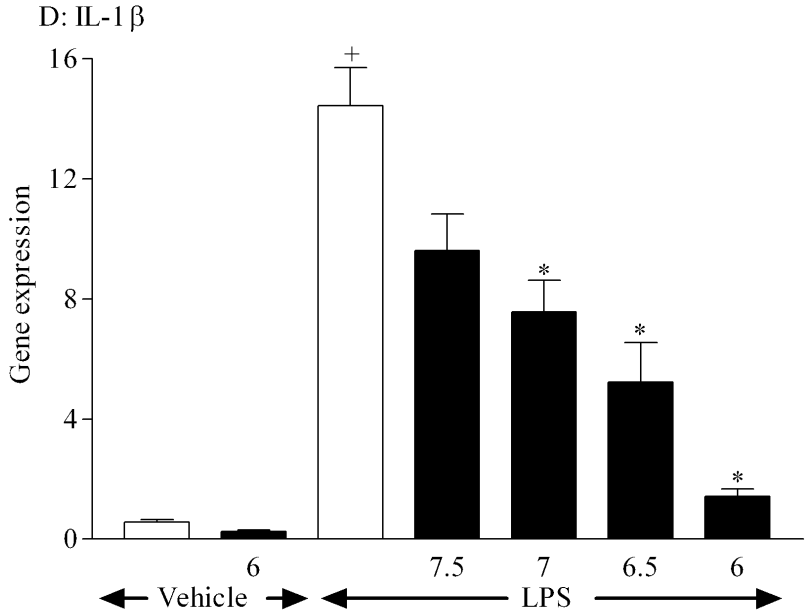

Figure 1

# Figure 2



# Figure 3

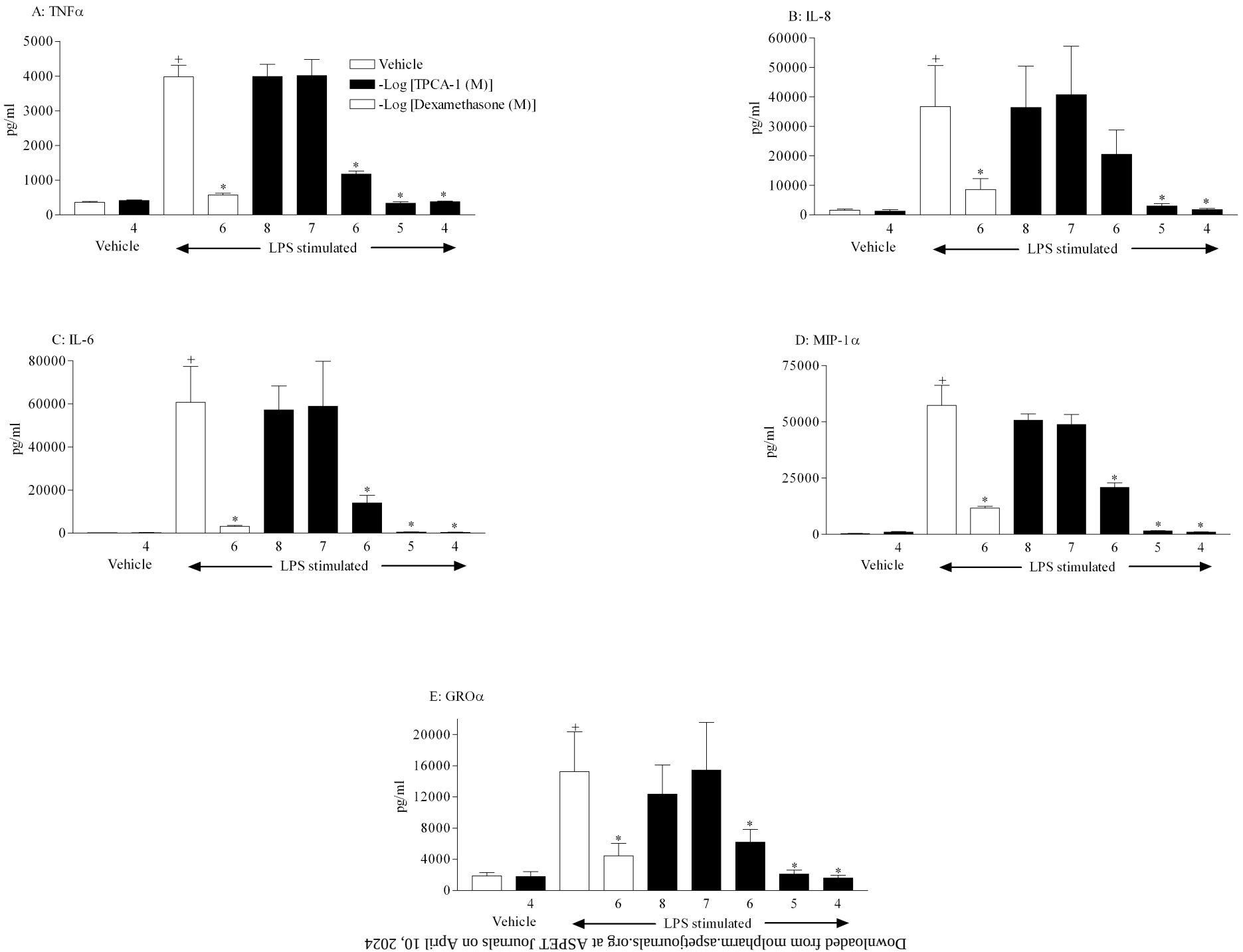


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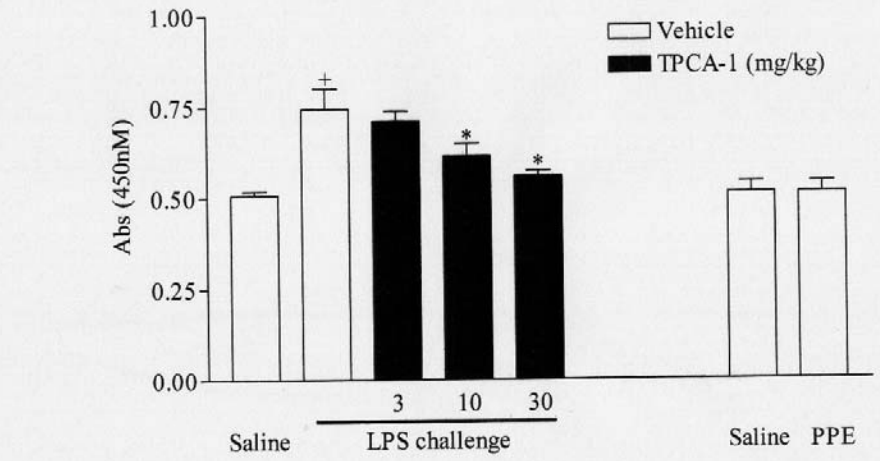
Figure 4




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| LPS | + | + | + | + | + | + | - | - | + |
| TPCA (mg/kg) | - | 3 | 10 | 30 | 60 | - | - | 60 | - |
| Budesonide (mg/kg) | - | - | - | - | - | 3 | - | - | - |

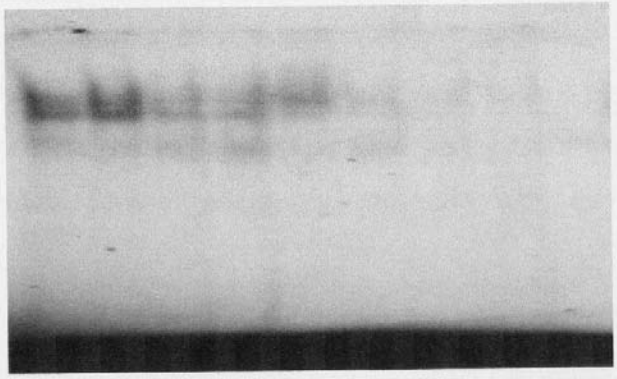


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Figure 5

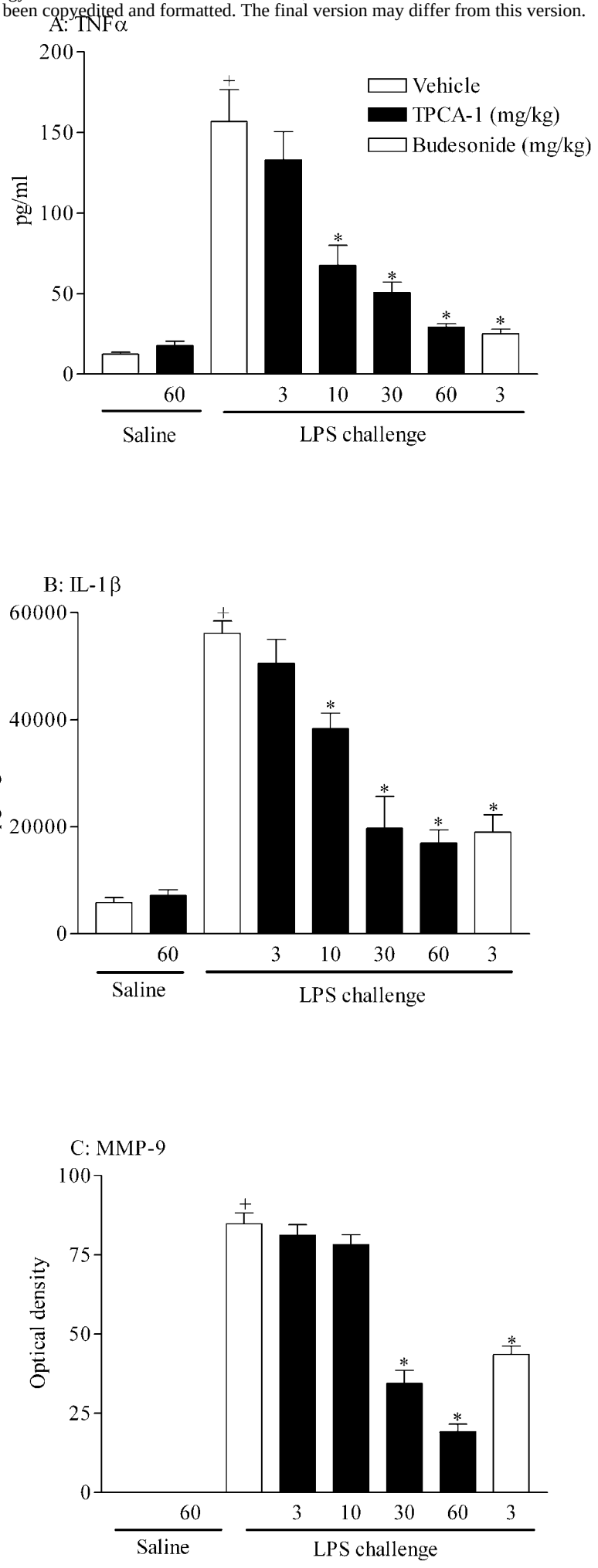

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Figure 6

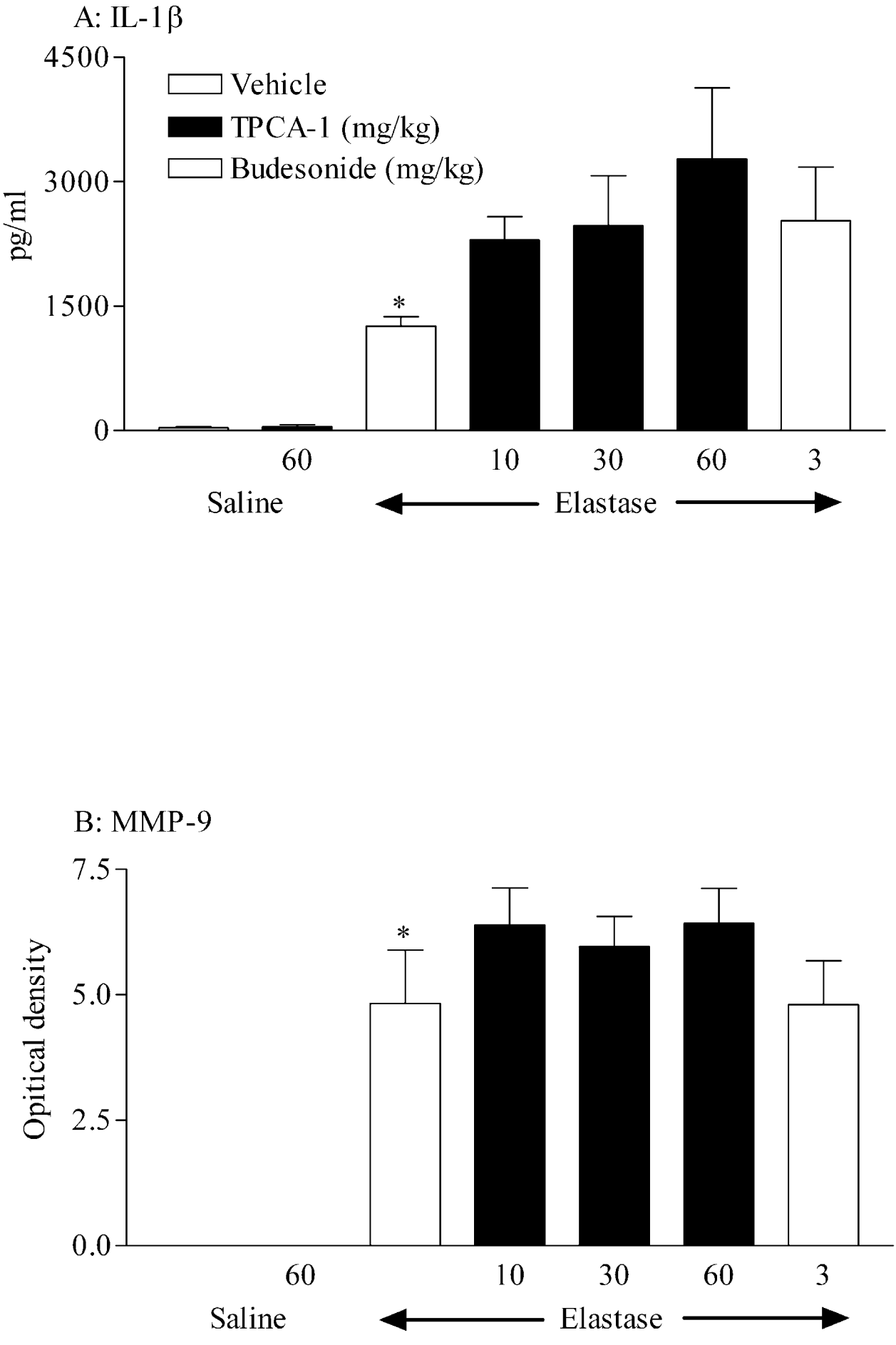


Figure 7

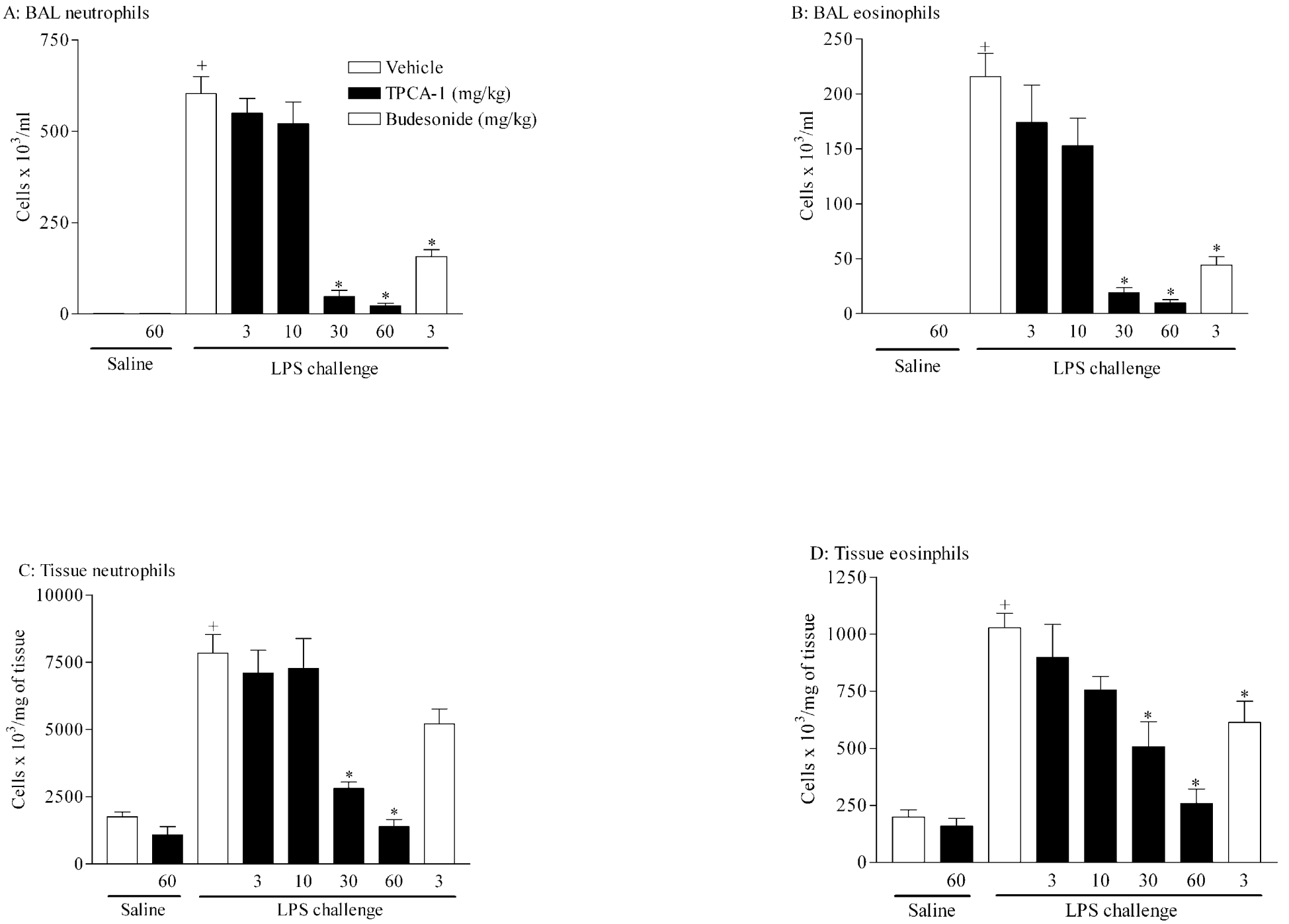
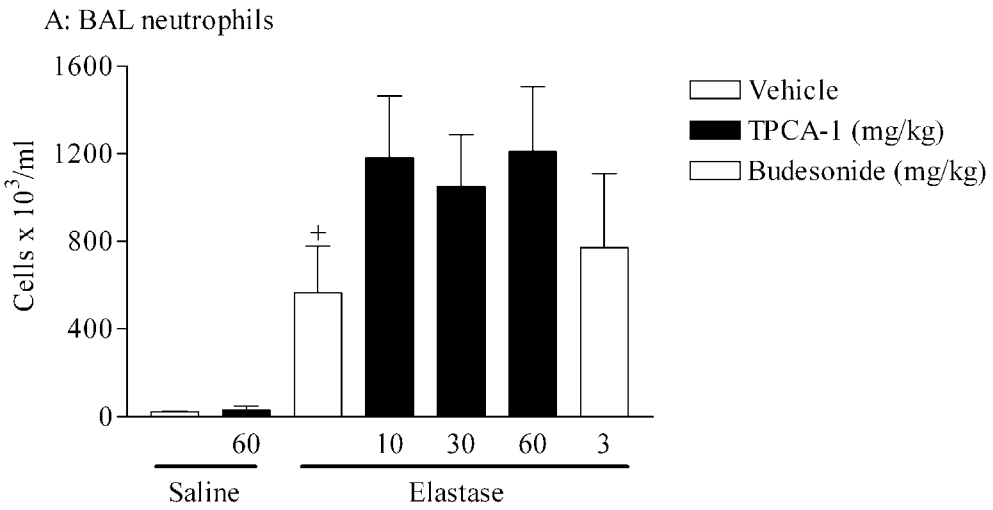
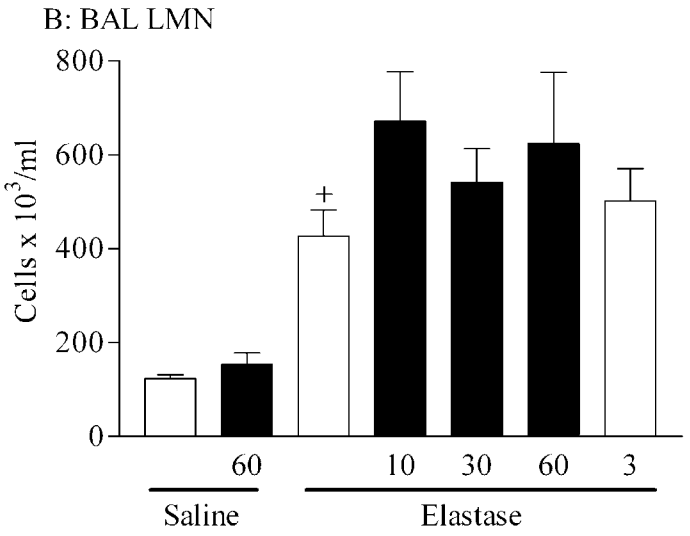
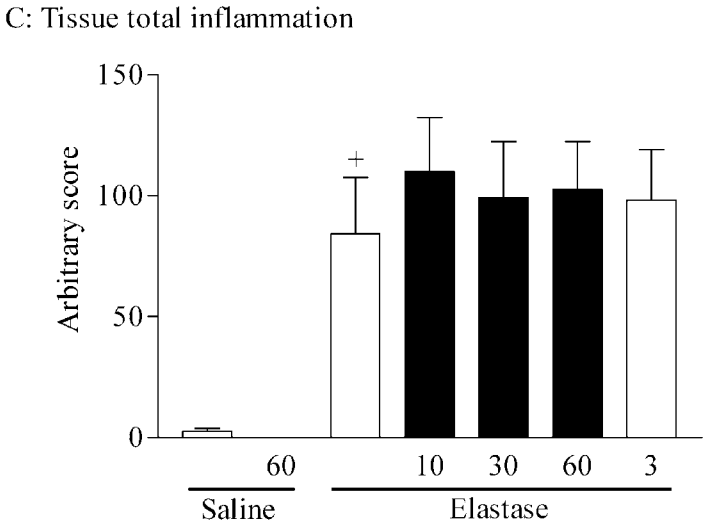
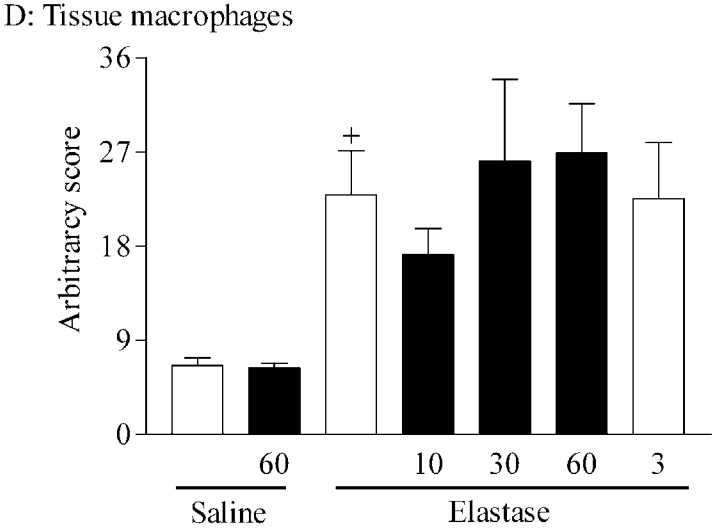


Figure 8



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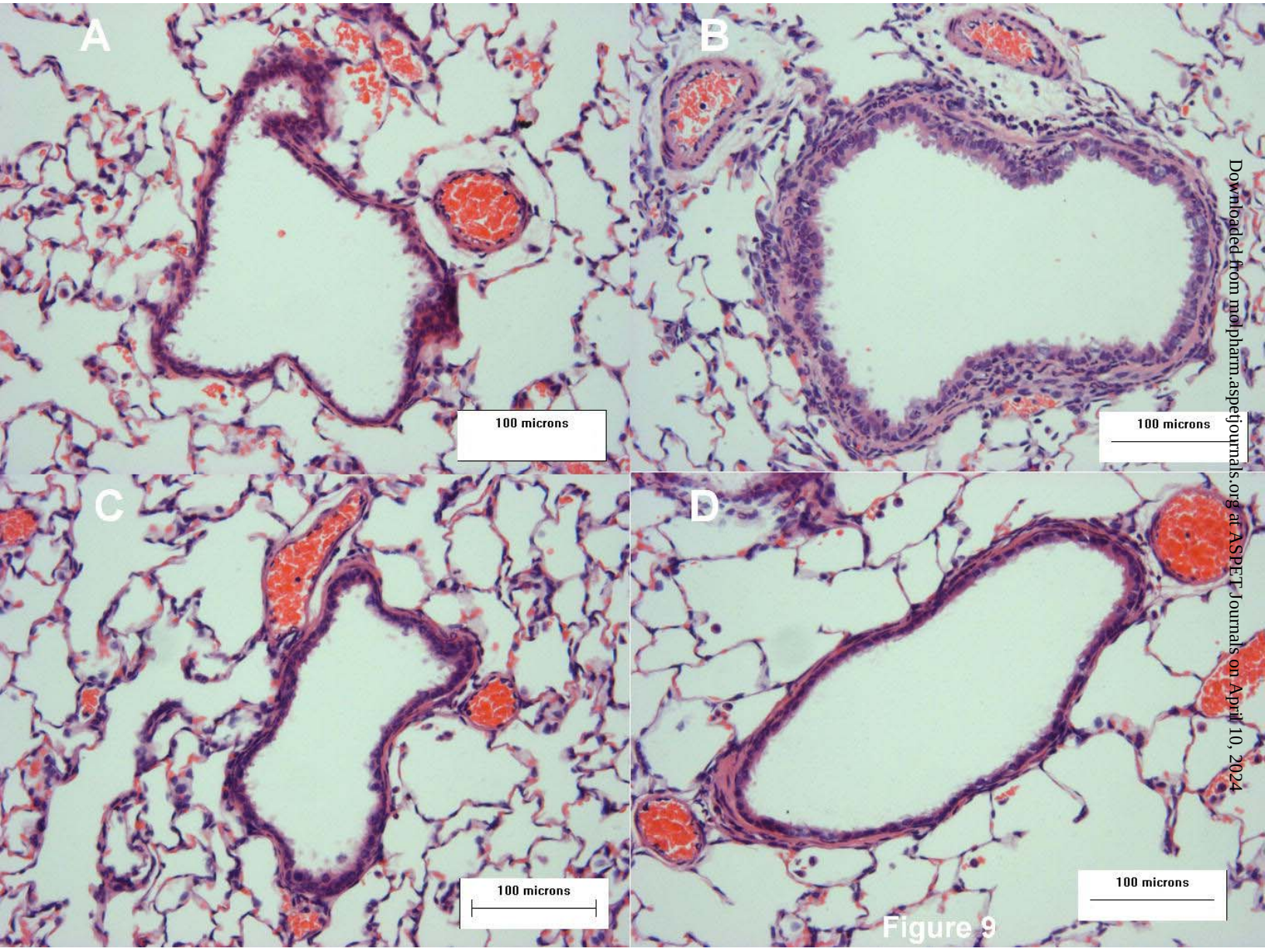


Figure 9

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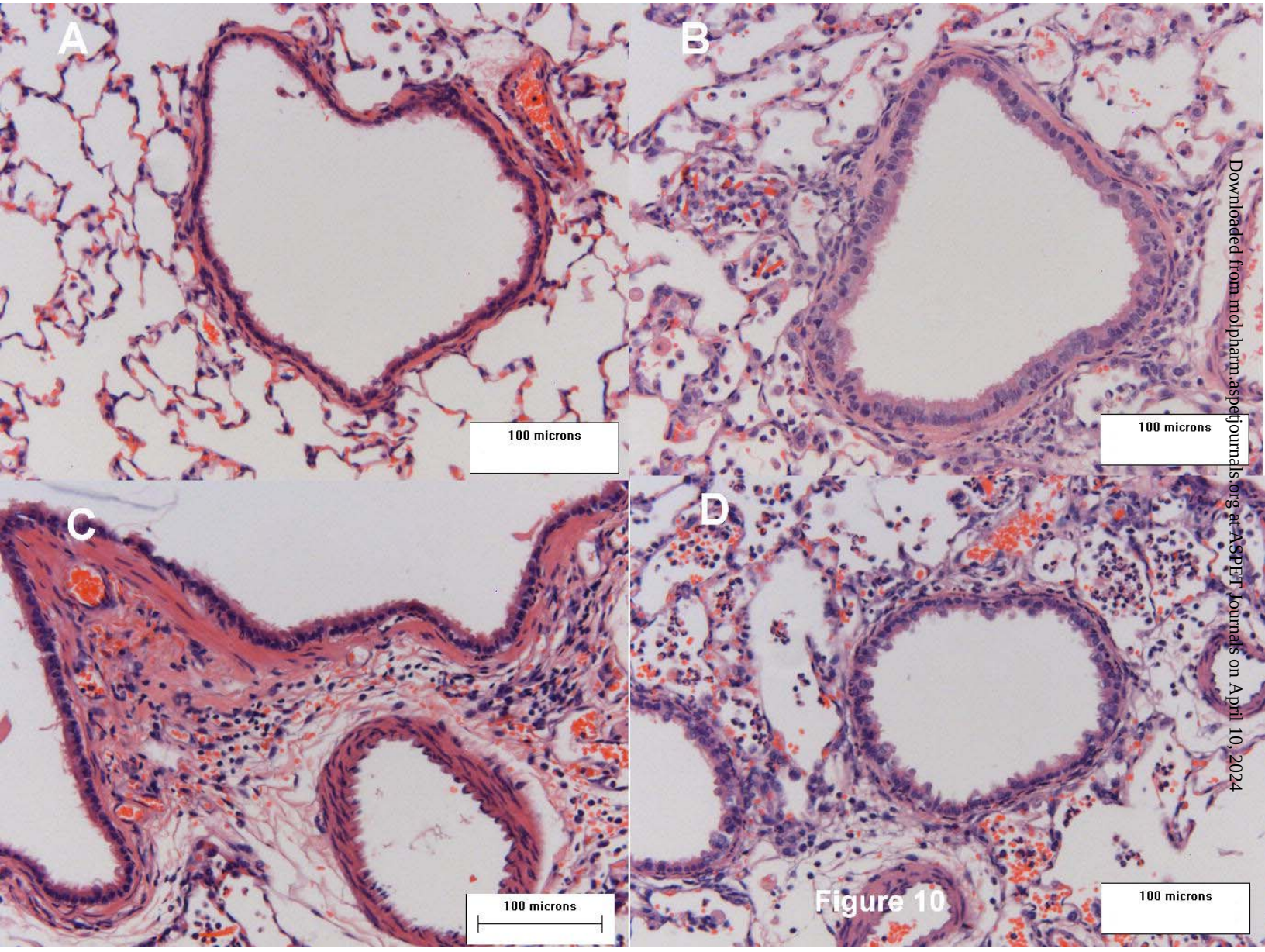


Figure 10.