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Role of matrix metalloproteinases in the inflammatory response in human airway cell based assays and in rodent models of airway disease

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Running title: Impact of an MMP inhibitor on airway inflammation

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Abbreviations: BAL, Bronchoalveolar lavage; BALF, Bronchoalveolar lavage fluid, LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PPE, porcine pancreatic elastase.

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Abstract

Since the discovery of the first matrix metalloproteinase (MMP), this ever growing family of proteinases has been the subject of intense research. Although it was initially believed that MMPs were solely involved in matrix turnover and degradation, there is now data suggesting MMPs are actively involved in the inflammatory process. In previous studies, we have demonstrated an increase in MMP expression in human cell based assays and in pre-clinical rat models of airway inflammation. Therefore, the aim of this study was to characterise the role of MMPs in these models by profiling the impact of a broad spectrum MMP inhibitor. In LPS stimulated THP-1 cells and primary human lung tissue macrophages, the MMP inhibitor had no significant effect on the release of TNFα, IL-8, IL-1β, GROα, MIP-1α or IL-6 whereas dexamethasone significantly impacted on all cytokines from both cell types. Similarly, in the more biologically complex LPS-driven rat model of airway inflammation the MMP inhibitor did not impact on mediator release and cellular burden. The compound did, however, significantly reduce levels of lung MMP-9. Furthermore, in a "disease" model the compound did not affect cellular inflammation but did significantly reduce elastase-induced experimental emphysema. In summary, this data demonstrates for the first time that MMPs do not play a role in the increase in inflammatory mediators or cellular burden observed in these pre-clinical models. However, they do appear to be involved in the elastase driven breakdown of airway structure, which is not due to a direct effect of the stimulus.

Introduction

Since the discovery of the first matrix metalloproteinase (MMP), this ever growing family of proteinases has been the subject of intense research. Although it was initially believed that MMPs were solely involved in the turnover and degradation of the extracellular matrix, there is now data suggesting MMPs act on cytokines, chemokines and protein mediators to regulate various aspects of inflammation and immunity (expertly reviewed by Parks *et al,* 2004). The exact role of most MMPs in inflammation / immunity is not known and, in fact, it is not clear whether they are involved in promoting or reducing these responses. MMPs are believed to impact on many of the cytokines / chemokines involved in the inflammatory response, often with apparently opposing outcomes. *In vitro,* MMPs have been shown to reduce the activity of chemokines such as ENA78 (Van Den Steen *et al,* 2003), whilst increasing the activity of other such as IL-8 (Van Den Steen *et al,* 2000). They have been shown to release immobilised chemokine complexes, such as syndecan-1/IL-8 (Li *et al,* 2002) but also convert others (i.e. MCP family) into chemokine receptor antagonists (McQuibban *et al,* 2000). IL-1β is activated by MMP driven proteolytic processing (Schonbeck *et al*, 1998); but then degraded by MMPs (Ito *et al,* 1996). Although it is known that TNFα is converted to an active form by TNF-converting enzyme (TACE) (Black *et al*, 1997), studies have shown that MMPs can also be involved (English *et al*, 2000). In addition, *in vitro* studies have shown that an MMP inhibitor can reduce trans-basement membrane neutrophil migration (Delclaux *et al*, 1996). *In vivo* studies have shown that a dual MMP/TACE inhibitor and dual MMP/ neutrophil elastase (NE) inhibitors reduced endotoxin driven airway cellular inflammation (Trifilieff *et al*, 2002; McCann *et al,* 1999). In other models of respiratory diseases such as

bleomycin induced fibrosis and antigen driven allergic inflammation, MMP inhibitors have been shown to inhibit cellular recruitment (Corbel *et al*, 2001a; Kumagai *et al*, 1999).

In previous studies, an increase in MMP expression has been described in our fully characterised human cell based assays and pre-clinical rodent models of airway inflammation. In cultured THP-1 cells and primary human lung tissue macrophages, we have shown that the expression of a range of MMPs is increased after stimulation (Wong *et al*, 2005). Furthermore, we have shown a similar increase in MMP expression at the gene and protein level in a rat model of LPS induced innate airway inflammation and an elastase driven disease model of airway inflammation / experimental emphysema (McCluskie *et al*, 2004; Birrell *et al*, 2005; Wong *et al*, 2005). Therefore, the aim of this study was to determine the role of these proteases in the models by utilising a broad spectrum MMP inhibitor, Bay 17-4003. Through this series of studies we aim to study, for the first time, the role of MMPs in the inflammatory response in human cell based assay systems. Finally we will use this inhibition to dissect out the role of MMPs in the more biologically complex innate response seen in the rodent airway following LPS challenge and in an elastase-driven "disease" model which exhibits inflammation and structural changes.

MATERIALS AND METHODS

Effect of the MMP inhibitor on MMP activity – fluorogenic assay

This assay used a fluorogenic MMP substrate (Bachem UK, Merseyside, UK) (10μM) for MMP 2 and 9, at a concentration slightly above the Km values for MMP-2 and MMP-9, and a concentration of MMP active enzyme (CN Biosciences UK Ltd) low enough to ensure the substrate was in excess (30μM for MMP-2 and 10μM for MMP-9). The inhibitor (structure as in Figure 1) was pre-incubated with MMP 2 or 9 for 20 minutes prior to addition of the substrate, which was then kept in the dark at room temperature for 2 hours and the fluorescence measured using a fluorometer set at 330±12nm excitation and 405±10nm emission.

Effect of the compound on LPS induced cytokine release from THP-1 cells

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 grown in RPMI 1640 with glutamax I (Invitrogen LTD, UK) supplemented with 10% FCS and 1% antibiotic and antimycotic solution (Penicillin/Streptomycin – Sigma-Aldrich Co., Poole, UK) at 37°C in a humidified atmosphere (95% air, 5% (v/v) CO_2).

On experiment days the cells were centrifuged at 1000rpm for 5 minutes, the supernatant discarded, and the pellet of cells were washed in serum free RPMI 1640 with glutamax I (Invitrogen LTD, UK) supplemented with 1% antibiotic and antimycotic solution (Penicillin/Streptomycin – Sigma-Aldrich Co., Poole, UK). After a second centrifugation the cells were resuspended in RPMI 1640 with glutamax I, supplemented with 3% FCS and 1% antibiotic and antimycotic solution,

and transferred to 24 well plates (400,000 per well). Vehicle (DMSO, 0.1% v/v, final concentration), Bayer compound (0.001-1μM; a gift from Bayer Plc) or dexamethasone (1μM) was added to the wells for 60 minutes at 37°C in a humidified atmosphere (95% air, 5% (v/v) CO_2). After which vehicle (RPMI 1640 with glutamax I, supplemented with 3% FCS and 1% antibiotic and antimycotic solution) or LPS (0.1μg/ml) was added, and the plate was then incubated at 37°C in a humidified atmosphere (95% air, 5% (v/v) CO_2) for 24 hours. The supernatants were retained for cytokine protein expression by ELISA (TNFα, IL-8, IL-1β, GROα, MIP-1α, IL-6; DuoSet, R&D Systems) and cell viability was assessed by Trypan Blue exclusion. TNFα convertase (TACE) inhibitor (TAP-1, Calbiochem) and IL-1β converting enzyme (ICE) inhibitor (ICE inhibitor II, Calbiochem) were included as positive controls and tested as above at 1μM.

MMP-9 levels in the cell supernatants were determined using zymography in accordance with manufacturer's instructions (Invitrogen) and detailed in McCluskie *et al* 2004.

Effect of the MMP inhibitor on LPS induced cytokine release from human lung tissue macrophages

Human lung tissue macrophages were obtained from non-diseased, 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 and flushed with phosphate buffered saline using a needle and syringe (without calcium and magnesium) (Sigma-Aldrich Co., Poole, UK). The pooled cell suspension were passed through a 70 μm cell sieve and centrifuged at 250 x *g* for 10

minutes at 4^{o}C, in a chill spin (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. After centrifugation, the macrophage enriched fractions were obtained from the 35% and the 60% Percoll interface, and washed twice with phosphate buffered saline. The cells were then resuspended with RPMI 1640 with glutamax I (Invitrogen LTD, UK) supplemented with 10% FCS (Invitrogen LTD, UK) and 1% antibiotic and antimycotic solution (Penicillin/Streptomycin – Sigma-Aldrich Co., Poole, UK). Trypan Blue exclusion was performed to assess cell viability, and 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 400,000 cells per well were added to 24 well plates (Costar). 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 by washing and fresh medium was added. The adherent, purified macrophages (consistently > 99%) were incubated overnight, for treatment the following day. The remainer of the experiment was performed as detailed above in the THP-1 experiments except we used 0.01µg/ml of LPS as a sub-maximal stimulant.

Effect of the MMP inhibitor on LPS induced airway inflammation in the rat.

Animals

Male Wistar rats (150-180g) 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 n = 8 animals.

Rats were orally dosed with vehicle (0.5% methylcellulose and 0.2% tween80 in distilled water, 2ml/kg) or Bayer compound (0.1, 1 or 10 mg/kg) one hour prior to an aerosol challenge of endotoxin free saline (for 30 minutes from Fresenius Kabi (Warrington, UK) or LPS (0.3 mg/ml, *Escherichia coli* serotype 0111:B4 from Sigma, UK) in a Perspex box (600 x 240 x 350mm). A positive standard, dexamethasone (1 mg/kg) was included in the experimental design.

Quantification of airway inflammation

Six hours after saline or LPS challenge animals were euthanised with sodium pentobarbitone (200 mg/kg, i.p.) and the trachea cannulated. Bronchoalveolar lavage (BAL) cells were recovered from the airway lumen by flushing the airways with 10 ml/kg of Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen, Paisley, UK) 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 Birrell *et al* (2005). Total white cell numbers in the BAL and lung tissue samples were determined on the Sysmex F820 haematology analyser (Linford Wood, Milton Keynes, U.K). Cytospins of these samples were prepared by centrifugation of

100 µl aliquots in a cytospin (Shandon, Runcorn, UK) at 700 rpm for 5 min, 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. Three part differential counts on 200 cells per slide were performed following standard morphological criteria and the percentage of eosinophils, lymphomononuclear (LMN) cells and neutrophils were determined.

Inflammatory biomarker level determination in the airways following LPS challenge

Levels of MMP-9 were determined using zymography as outlined above. The amount of TNFα, IL-6 and IL-1β in the BAL fluid (BALF) and lung tissue were determined using DuoSet ELISA's from R&D Systems according to manufacturer's instructions. The detection limit of these assays was determined to be in the range of 1 to 5 pg/ml. The cytokine levels in the lung tissue were corrected for total protein content which was measured using the Bradford assay. Myeloperoxidase activity was measured in lung tissue homogenate supernatants using o–dianisidine hydrochloride as the substrate/hydrogen donor as described in McCluskie *et al*, 2004.

Effect of the MMP inhibitor on porcine pancreatic elastase induced airway inflammation.

Prior to determining the impact of the MMP inhibitor on PPE driven airway inflammation we confirmed that the MMP inhibitor was indeed selective and did not have any direct affect on PPE. Bay 17-4003, or the positive control (Elastase Inhibitor III, Calbiochem), was prepared in DMSO (0.01, 0.1, 1 and 10 µM, final

concentration) and added to BMG labtechnologies black plates (Cat No 77600-04) along with buffer (Trizma base, 0.05M; NaCl, 0.5M; $CaCl_2$ 0.01M; pH 7.5) containing 1 U/ml (final concentration) of PPE (Calbiochem) and left in the dark for 30 minutes. Then the fluorogenic substrate (N-Methoxysuccinyl-Ala-Ala-pro-Val-7-amido-4-methylcoumarin, 0.1 mM final concentration from Sigma) was added and the plate left for 10 minutes. Fluorescence was determined with excitation and emission wavelengths set at 360 and 460 nm, respectively.

For profiling the impact of the MMP inhibitor in the PPE driven model 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 (0.5% methylcellulose and 0.2% tween80 in distilled water, 2 ml/kg) or Bayer compound (0.1, 1 or 10 mg/kg) one hour prior to and 8, 22 and 30 hours after challenge with either vehicle (endotoxin free saline, 1 ml/kg, dosed intratracheally) or PPE (120 U/kg, i.t.)

Forty-eight hours after PPE administration the lungs were assessed for cellular infiltration as above. Assessment of average airspace area was measured in formalin insufflated (pressure of 20 mmHg) fixed lung tissue by an experienced, group blinded, histologist as previously described by Birrell, *et al* (2005). Briefly, quantification was carried out on an Olympus BX40 microscope using Zeiss KS300 image processing software (Imaging Associates Ltd). Using colour threshold techniques the total parenchymal air spaces in square micrometers and the number of

air spaces were measured from 10 random fields per slide/lobe. From these figures the mean air space area for each field can be calculated. Any fields containing airways or vasculature were excluded.

ANALYSIS

Values are expressed as mean ± s.e.mean of n independent observations. Statistical analysis was assessed by one-way ANOVA using the appropriate post test analysis. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of the Bayer compound on MMP activity

To demonstrate the activity of the broad-spectrum MMP inhibitor it was profiled in fluorogenic assays. Active MMP-2 or MMP-9 increased the amount of fluorescence in the assay and addition of the compound reduced the amount of fluorescence to the basal level (Figure 2). The IC_{50} of the compound on both MMP-2 and MMP-9 activity was approximately 10 nM which is similar to that reported by Bayer Plc (Figure 1 legend).

Effect of the compound on LPS induced cytokine release from THP-1 cells and human lung tissue macrophages

LPS (0.1μg/ml) caused a sub-maximal release of TNFα, IL-8, IL-1β, GROα, MIP-1α, IL-6, and MMP-9 from THP-1 cells and 0.01μg/ml LPS caused a sub-maximal release of TNFα, IL-8, IL-1β, GROα, MIP-1α and IL-6 from human lung tissue macrophages (Figure 3, 4 and 5). Pre-treatment with the compound had no significant impact on cytokine release from THP-1 cells, whereas the intra-assay positive control, dexamethasone, inhibited the release of all cytokines (Figure 3 and 4). In comparison, the TACE inhibitor caused a significant 51% inhibition of TNFα release whereas the ICE inhibitor caused a significant 47% inhibition of IL-1β release. Neither inhibitor significantly impacted on the other cytokines measured (data not shown).

Unlike the intra-assay positive control, dexamethasone, the MMP inhibitor did not significantly impact on MMP-9 levels in the supernatant (Figure 4). There was no significant impact on cell viability by either the MMP inhibitor or dexamethasone (Figure 4).

Similar to the result with the THP-1 cells, only the intra-assay positive control had any significant affect on cytokine release from primary human lung tissue macrophages (Figure 5). In our hands the cultured human lung tissue macrophages do not release any more MMP-9 upon stimulation with LPS (data not shown).

Effect of the compound on LPS induced airway inflammation in the rat.

To determine if inhibition of MMP activity would impact on the inflammatory response in a more complex biological system, Bay 17-4003 was profiled in a fully characterised LPS-driven model of rodent airway inflammation (McCluskie *et al*, 2004). The MMP inhibitor caused a significant and dose related inhibition of MMP-9 activity/release in BALF samples as measured by zymography (Figure 6). However, figure 4 clearly shows that zymography is not appropriate for demonstrating the effect of the MMP inhibitor on MMP activity which suggests that, in this experiment, the MMP inhibitor has reduced the amount of MMP-9 in the lungs. This data demonstrates that this compound inhibits rat MMP-9, as well as human MMP activity, and that the dosing regimen employed was appropriate.

The LPS challenge caused a significant increase in BALF TNFα and IL-β, which unlike the positive control, dexamethasone, was not significantly affected by the MMP inhibitor (Figure 6). To confirm that the lack of effect of the MMP inhibitor was not restricted to cytokines in the airway lumen, levels of cytokines were determined in the lung tissue. Similar to the result in the BALF, unlike the glucocorticoid, the MMP inhibitor did not impact on the LPS-induced increase in IL-1β and IL-6 levels in the lung tissue (Figure 6). No significant increase in TNFα was observed in the lung tissue at this time point after LPS challenge (data not shown).

Aerosolised LPS caused a significant increase in BALF neutrophilia and eosinophilia and lung tissue neutrophilia (Figure 7). Treatment with the MMP inhibitor failed to have any significant impact on LPS induced lung cellular inflammation, whereas the positive control, dexamethasone, significantly inhibited neutrophilia and eosinophilia (Figure 7). To determine if the MMP inhibitor affected the activation status of the neutrophils MPO levels were determined. Figure 6F clearly shows that the activation status of the neutrophils in the lung tissue was not affected by the MMP inhibitor. There was no significant change in LMN cell number in both the BALF or lung tissue by any treatment (data not shown).

Effect of the compound on PPE-induced airway inflammation in the rat.

The MMP inhibitor failed to impact on LPS-induced inflammation in the human cell based assays or the more biologically complicated rodent model of lung inflammation. To determine if the compound would have an effect on a more severe disease model, the MMP inhibitor was profiled in a fully characterised PPE driven rodent model of lung inflammation (Birrell *et al*, 2005). Prior to initiating this experiment it was important to demonstrate that the selective MMP inhibitor did not affect the activity of the stimulus used in this pre-clinical model. Figure 8 clearly demonstrates that unlike the positive control compound, Elastase inhibitor III, Bay 17-4003 had no impact on the activity of PPE up to the highest concentration tested.

Challenge with PPE caused a significant increase in BALF and lung tissue neutrophilia and LMN cell number (Figure 9). The MMP inhibitor failed to significantly impact on the PPE-induced increase in lung cellular burden (Figure 9).

There was no glucocorticoid comparator in this study because we have previously shown this model to be steroid resistant (Birrell *et al*, 2005).

We have previously shown that after PPE challenge the model exhibits aspects of emphysema, as measured by increases in average airspace area (Birrell *et al*, 2005). Even at this early stage of experimental emphysema development, i.e. 2 days after challenge rather than 4 weeks, PPE challenge caused a small significant increase in average airspace area (Figure 10). Although the compound had no impact on inflammation, it appeared to cause a dose-related inhibition of experimental emphysema, which would suggest that the MMPs present are partially responsible for the change in lung structure (Figure 10). Interestingly, the effect of the MMP inhibitor appeared to be bell shaped with the highest dose tested having less impact. The high dose / vehicle challenge group had an increase in experimental emphysema compared with the vehicle dosed / vehicle challenge group (Figure 10). Whist the reason for this is not known, it might explain why the highest dose of MMP inhibitor appeared not to affect the experimental emphysema.

DISCUSSION

Matrix metalloproteinases (MMPs) are important for homeostasis and turnover of the extracellular matrix (ECM) in health and disease. Although it was initially believed that MMPs were solely involved in matrix turnover and degradation, there is now data suggesting MMPs are actively involved in the inflammatory process. Interestingly, it has been demonstrated that the ECM is a source for several inflammatory cell chemotactic factors. In fact, fragments of elastin (Senior et al., 1980; Houghton et al., 2006) and collagen (Weathington et al, 2006) have been shown to be chemotactic and could therefore play a role in the inflammatory response.

We have previously described an increase in MMP expression in fully characterised human cell based assays and pre-clinical rodent models of airway inflammation. More specifically, we have recently demonstrated in cultured THP-1 cells and primary human lung tissue macrophages an increased expression of a range of MMPs following LPS stimulation (Wong *et al*, 2005). Furthermore, in a rat model of LPS-induced innate airway inflammation and an elastase-driven disease model of airway inflammation and experimental emphysema we have demonstrated a similar increase in MMP expression both at the gene and protein level (McCluskie *et al*, 2004; Birrell *et al*, 2005; Wong *et al*, 2005). Therefore, the aim of this study was to determine the role of these proteases in the inflammatory responses in these models by using a broad spectrum MMP inhibitor. To our knowledge this is the first time the role of MMPs has been extensively profiled in a range of pre-clinical models of airway inflammation and the effect of an inhibitor assessed.

Interestingly, the MMP inhibitor failed to have any significant impact on the cytokine production from LPS stimulated THP-1 cells, whereas the intra-assay

positive control, dexamethasone, inhibited the release of all cytokines. In parallel studies, we confirmed that TNFα release was controlled by TACE and IL-1β release was controlled by ICE in this model system. In addition, the MMP inhibitor did not affect the MMP-9 zymography analysis of the cell supernatant from stimulated THP-1 cells (Figure 4). As we have shown that the inhibitor blocks the activity of MMP-9 (Figure 2), it would imply that the compound is being "removed" from the active site on MMP-9 during the zymography process, possibly during the denaturing steps or through some of the washing phases. Therefore, the zymography result suggests that the MMP inhibitor, unlike the intra-assay positive control, is not having any affect on the amounts of MMP-9 produced. Similar to the THP-1 results the MMP inhibitor did not have any significant effect on cytokine release from primary human lung tissue macrophages, although there did appear to be a small, non-significant reduction in some of the cytokines measured. We were not able to determine the effect on MMP-9 levels in the primary macrophages because, unlike the gene expression of other MMPs, we are unable to measure an increase in the release of this MMP after stimulation with LPS (Wong *et al*, 2005).

The absence of a significant effect of the MMP inhibitor on cytokine release from our cell based assays would appear to be at odds with some of the published data discussed in the introduction (English *et al*, 2000; Schonbeck *et al*, 1998). The reason for this is not clear but is likely to be due to the different cells and assay protocols employed. Zhang *et al* (2004) have recently shown a dual TACE / MMP inhibitor to reduce LPS induced TNFα secretion in THP-1 cells, human primary monocytes and human whole blood. Our data would suggest that, at least in THP-1

cells, the inhibition observed by Zhang *et al* (2004) was due to an effect on TACE activity.

When the impact of the MMP inhibitor was assessed on the inflammatory response observed in the more biologically complex in vivo LPS model we observed a dose dependent decrease in the MMP-9 zymography signal. This result would suggest that the MMP inhibitor is reducing the amount of MMP-9, presumably through inhibition of the *in vivo* activity of one or more MMPs. There is evidence for the activity of MMPs to be involved in the production of MMPs, for example, Nenan *et al* (2005) have shown that addition of MMP-12 can lead to an increase in gelatinase expression, and Selman *et al* (2003) have shown an MMP inhibitor to reduce the amount of MMP-9 in a tobacco smoke-induced model of emphysema. Although in this study it is not clear how the MMP inhibitor is causing the reduction in MMP-9 levels or indeed which MMP is responsible, it does clearly show that this compound inhibits rat MMP activity (as well as human MMP activity) and validates the use of the dosing regimen employed. This effect on MMP-9 levels was not associated with a reduction in any of the other "inflammatory" indices in this model. The compound appeared not to affect BALF and tissue cytokine levels, the increase in cellular burden in the airway lumen and tissue or activation status of the cells. The lack of effect on TNFα levels appears to be at odds with the data published by Corbel *et al* (2001b) who reported a reduction in a mouse model after treatment with batimastat (another MMP inhibitor). However, this data would suggest that LPS-driven TNFα processing in the rat lung does not involve MMPs and hence is likely to be solely controlled by TACE.

The lack of effect of MMP inhibition on cellular burden in the in vivo LPS model agrees with some published *in vivo* studies by Corbel *et al* (2001b) using an inhibitor and Betsuyaku *et al* (1999) with MMP-9 knockout mice. It appears, however, to contradict data published by Trifilieff *et al* (2002) with a dual TACE/MMP inhibitor and by McCann *et al*, (1999) with dual MMP/NE inhibitors. The dual inhibitors employed in these studies make determining the role of the MMPs in isolation on the cellular inflammation extremely difficult. In addition, it may also imply that it is TACE/NE activity or a combination of these activities in addition to the MMP activity that is involved in the innate inflammatory response to LPS in the lung. In summary, even though we have demonstrated an increase in MMP activity which was reduced by an inhibitor, it appears that MMPs play no role in the recruitment of response effector cells in the LPS-driven inflammation seen in this in vivo model.

However, it would appear from the literature that MMPs may play more of a role in cellular recruitment in "airway disease" models insomuch as reports demonstrate that a reduction in MMP activity can protect against lung injury (Li *et al*, 2002; Corbel *et al*, 2001b; Hautamaki *et al*, 1997; Kumagai *et al*, 1999). For example, mice lacking MMP-12 are protected against the development of cigarette smoke-induced emphysema and from the accumulation of lung macrophages normally induced by chronic smoke exposure; an effect thought to be due to the generation of elastin fragments (Houghton et al., 2006). We therefore tested the same compound in a model of steroid-resistant airway inflammation and experimental emphysema (Birrell *et al*, 2005). Even though we have previously demonstrated an increase in MMP expression in this elastase-driven model, MMPs do not appear to play a role in cellular trafficking in the airways (Wong *et al*, 2005; Birrell *et al*,

2005). Interestingly, although the MMP inhibitor did not block cellular inflammation, or have any direct effect on the stimulus used, it did cause a significant reduction in the early stage of experimental emphysema. This is a fascinating observation and would suggest that the increased MMP activity caused by the inflammation may be driving the airway damage. It would be interesting to determine if inhibition of MMP activity would have a significant effect on the fully developed emphysema lesion observed in this model at 4 weeks after the insult. However, unfortunately insufficient compound was available to perform these studies. One issue with this data that should be discussed is the bell shaped response curve exhibited by the MMP inhibitor on the elastase-induced increase in average airspace area such that at the top dose tested the inhibition was lost. The reason for this is not known, but as we observed an increase in experimental emphysema in the high dosed-vehicle challenged group, it is possible that the compound has “off target”, adverse side effects on lung structure at this dose.

In summary, the data presented here demonstrate that MMPs do not play a substantial role in inflammatory mediator production and cellular burden in a comprehensive array of pre-clinical models of airway inflammation. This study, however, does show for the first time that MMPs are integral in the elastase-induced, inflammation-driven experimental emphysema lesion.

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FIGURE LEGENDS

Figure 1: The structure of the Bayer broad spectrum MMP inhibitor.

The potency (Ki (nM)) of the compound on isolated enzyme is reported to be 16, 3.1, 3.7, 0.2, and 0.5 for MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12, respectively – personal communication from Professor Guenter Benz from Bayer.

Figure 2: Effect of the Bayer compound on MMP-2 and MMP-9 activity.

Vehicle (DMSO) or compound (0.01, 0.1 or 1μM) was pre-incubated with active MMP-2 (A) or MMP-9 (B) for twenty minutes prior to addition of the fluorogenic substrate. Fluorescence was measured 2 hours later using a fluorometer set at 330±12nm excitation and 405±10nm emission. The assay was performed in duplicate.

Figure 3: Effect of the compound on cytokine release from cultured THP-1 cells.

Vehicle (DMSO, 0.1% v/v/ final concentration), compound (0.001, 0.01, 0.1, 1μM) or dexamethasone (1μM) was added to the cells one hour prior to stimulation with LPS (0.1μg/ml). Twenty-four hours after, the supernatants were collected and assayed for cytokine levels by ELISA (A: TNFα; B: IL-8; C: IL-1β; D: GROα; E: MIP-1α; F: IL-6). TACE inhibitor (1μM) caused 51% inhibition of TNFα levels and ICE inhibitor (1μM) caused 47% inhibition of IL-1β levels (both $p < 0.05$, neither significantly impacted on the other cytokines measured). Results represent mean ± s.e.mean ($n = 6$). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

Figure 4: Effect of the compound on MMP-9 release from cultured THP-1 cells and cell viability.

Vehicle (DMSO, 0.1% v/v/ final concentration), compound (0.001, 0.01, 0.1, 1μM) or dexamethasone (1μM) was added to the cells one hour prior to stimulation with LPS (0.1μg/ml). Twenty-four hours after the supernatants were collected and assayed for MMP-9 levels by zymography (A). Cell viability was determined by Trypan Blue exclusion (B). Results represent mean ± s.e.mean (n = 6). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

Figure 5: Effect of the MMP inhibitor on cytokine release from cultured human lung tissue macrophages.

Vehicle (DMSO, 0.1% v/v/ final concentration), compound (0.0001, 0.001, 0.01, 0.1, 1μM) or dexamethasone (1μM) was added to the cells one hour prior to stimulation with LPS (0.01μg/ml). Twenty-four hours after the supernatants were collected and assayed for cytokine levels by ELISA (A: TNFα; B: IL-8; C: IL-1β; D: GROα; E: MIP-1α; F: IL-6). Results represent mean ± s.e.mean (n = 6). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

Figure 6: Effect of the compound on LPS-induced inflammatory mediators in the rat lung.

Rats were treated with vehicle (0.5% methylcellulose and 0.2% tween80 in distilled water, 2 ml/kg), compound (0.1, 1 or 10 mg/kg) or dexamethasone (1 mg/kg) orally 1

hour prior to aerosolised saline or LPS (0.3 mg/ml, 30 minutes). Six hours after challenge the animals were culled and the inflammatory status of the airways assessed. MMP-9 activity levels in the BALF were determined by zymography (A). The levels of TNFα (B) and IL-1β in the BALF and IL-6 (C) and IL-1β (D) in the lung tissue homogenate were assessed by ELISA (DuoSets from R&D systems) according to manufacturer's instructions. Figure 6F shows the levels of MPO in the lung tissue. Results represent mean ± s.e.mean (n = 8). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

Figure 7: Effect of the MMP inhibitor on LPS induced airway cellular accumulation.

Rats were treated with vehicle (0.5% methylcellulose and 0.2% tween80 in distilled water, 2 ml/kg), compound (0.1, 1 or 10 mg/kg) or dexamethasone (1 mg/kg) orally 1 hour prior to aerosolised saline or LPS (0.3 mg/ml, 30 minutes). Six hours after challenge the animals were culled and the inflammatory status of the airways assessed. The number of BALF neutrophils (A) and eosinophils (B) and tissue neutrophils (C) was determined. Results represent mean ± s.e.mean (n = 8). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

Figure 8: Effect of the MMP inhibitor on PPE activity in an *in vitro* assay.

The MMP inhibitor was incubated with PPE (1 U/ml) prior to addition of fluorgenic substrate. Assessment of to PPE activity was then assessed. An elastase inhibitor III was included as a positive control. Results represent mean ± s.e.mean (n = 4).

Figure 9: Effect of the MMP inhibitor on PPE induced airway cellular accumulation.

Rats were treated with vehicle (0.5% methylcellulose and 0.2% tween80 in distilled water, 2 ml/kg) or compound (0.1, 1 or 10 mg/kg) orally 1 hour prior to and 8, 22 and 30 hours after challenge with either vehicle (endotoxin free saline, 1 ml/kg, dosed intratracheally) or PPE (120 U/kg, i.t.). Forty-eight hours after challenge the animals were culled and the inflammatory status of the airways assessed. The number of neutrophils (A) and LMN cells (B) in the BALF and neutrophils (C) and LMN cells (D) in the lung tissue was determined. Results represent mean ± s.e.mean (n = 8). + indicates significantly different from vehicle stimulation control.

Figure 10: Effect of the MMP inhibitor on PPE induced experimental emphysema.

Rats were treated with vehicle (0.5% methylcellulose and 0.2% tween80 in distilled water, 2 ml/kg) or compound (0.1, 1 or 10 mg/kg) orally 1 hour prior to and 8, 22 and 30 hours after challenge with either vehicle (endotoxin free saline, 1 ml/kg, dosed intratracheally) or PPE (120 U/kg, i.t.). Forty-eight hours after challenge the animals were culled and the lungs insufflated with 10% formalin saline. Average airspace area was determined using an image analysis system. Results represent mean ± s.e.mean

(n = 8). + indicates significantly different from vehicle stimulation control and * indicates significantly different from LPS stimulation control.

The Bayer MMP inhibitor

Figure 1

JPET Fast Forward. Published on May 11, 2006 as DOI: 10.1124/jpet.106.105544
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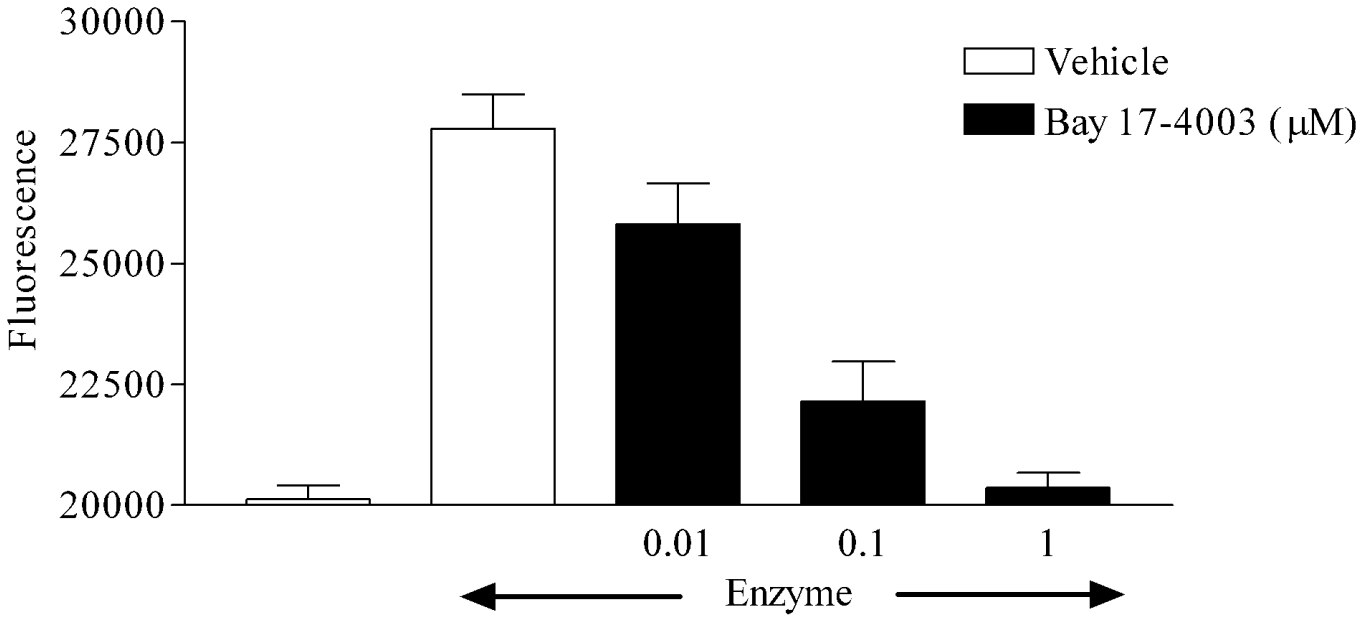
A: MMP-2 activity



B: MMP-9 activity

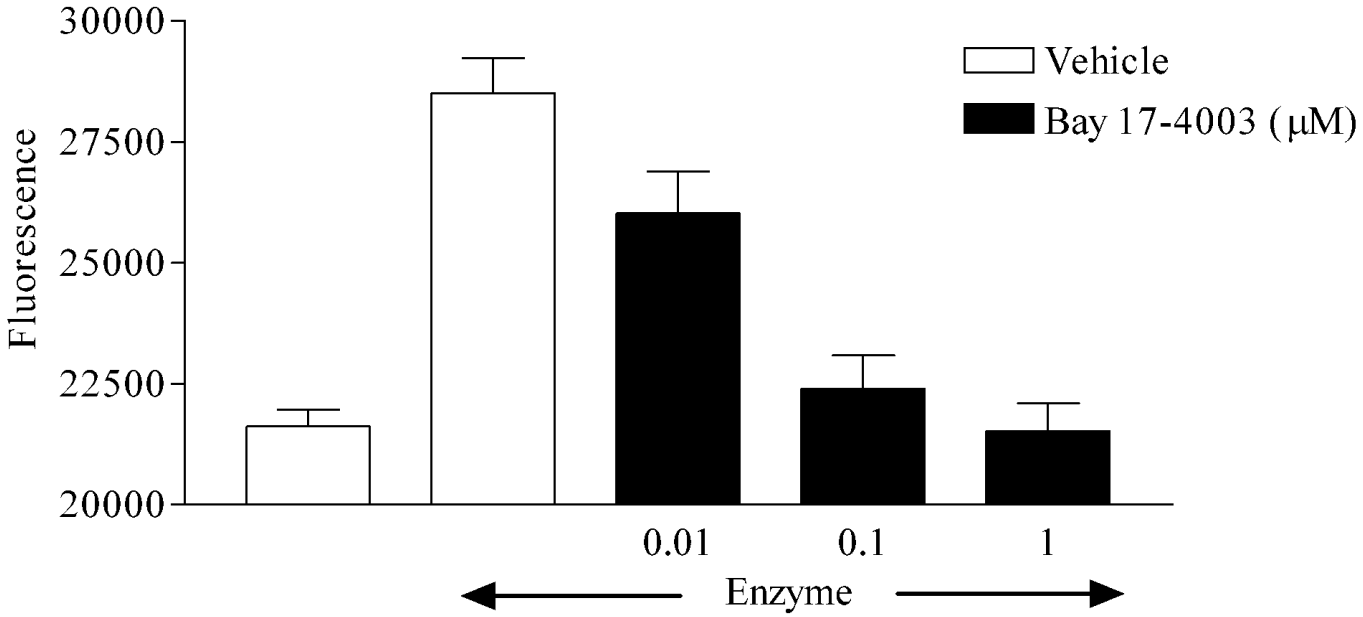


Figure 2

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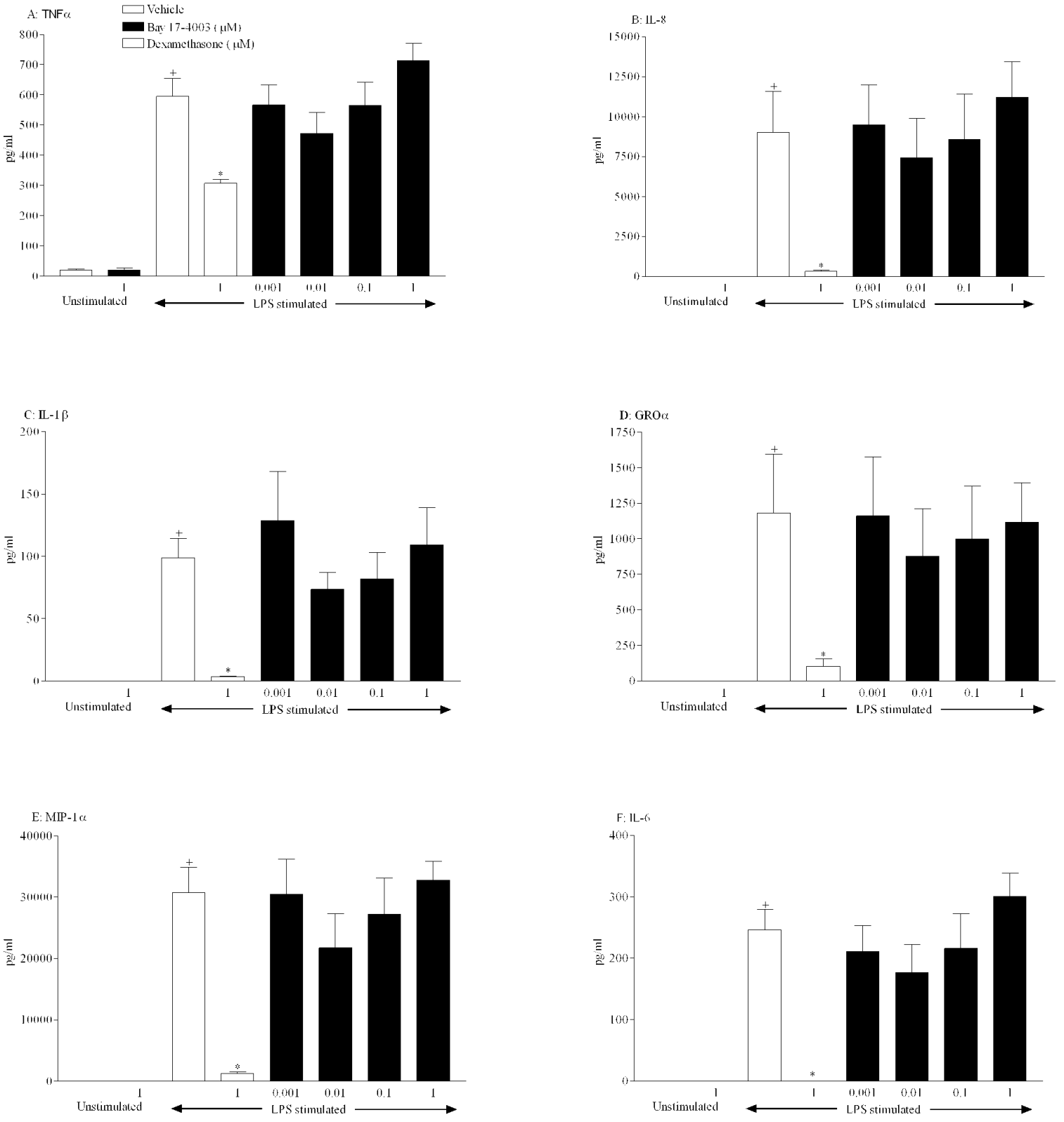

Figure 3

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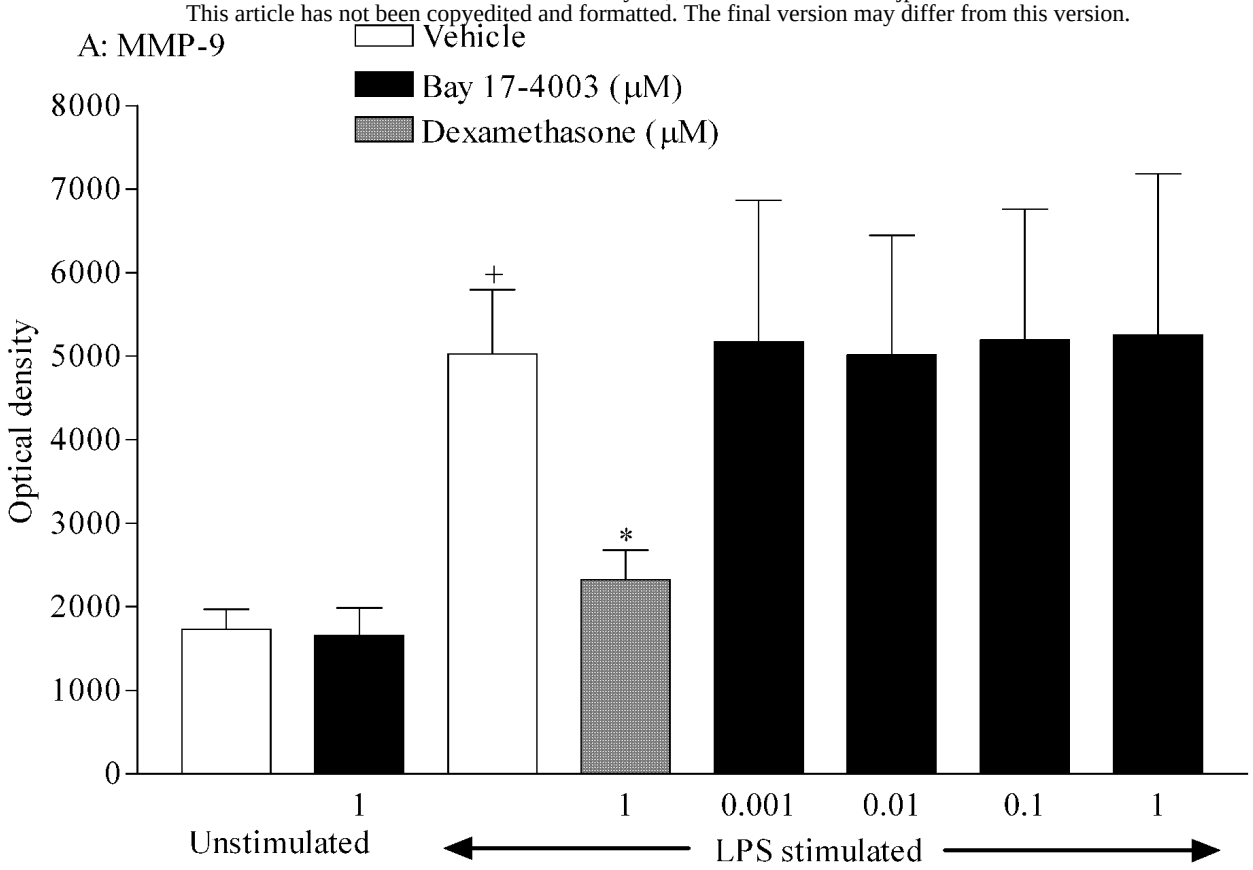

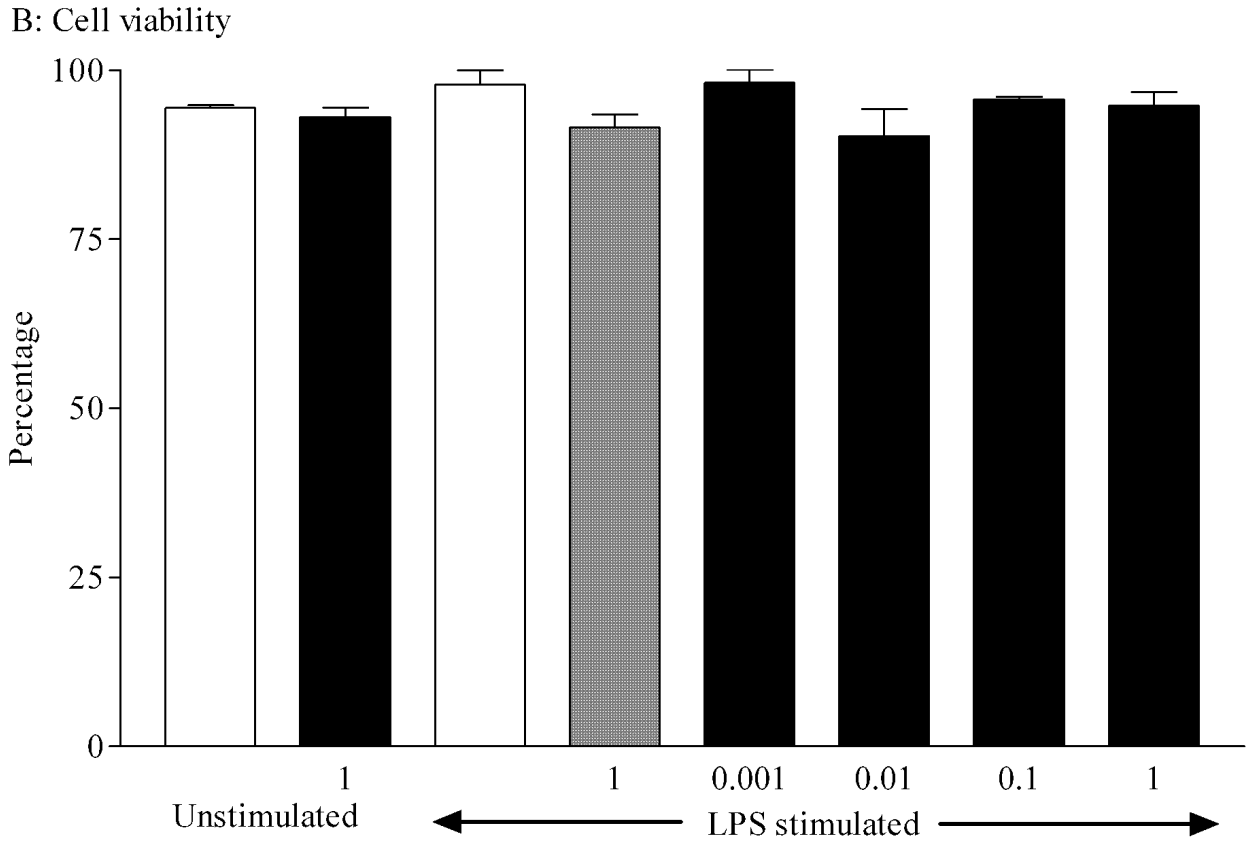

Figure 4

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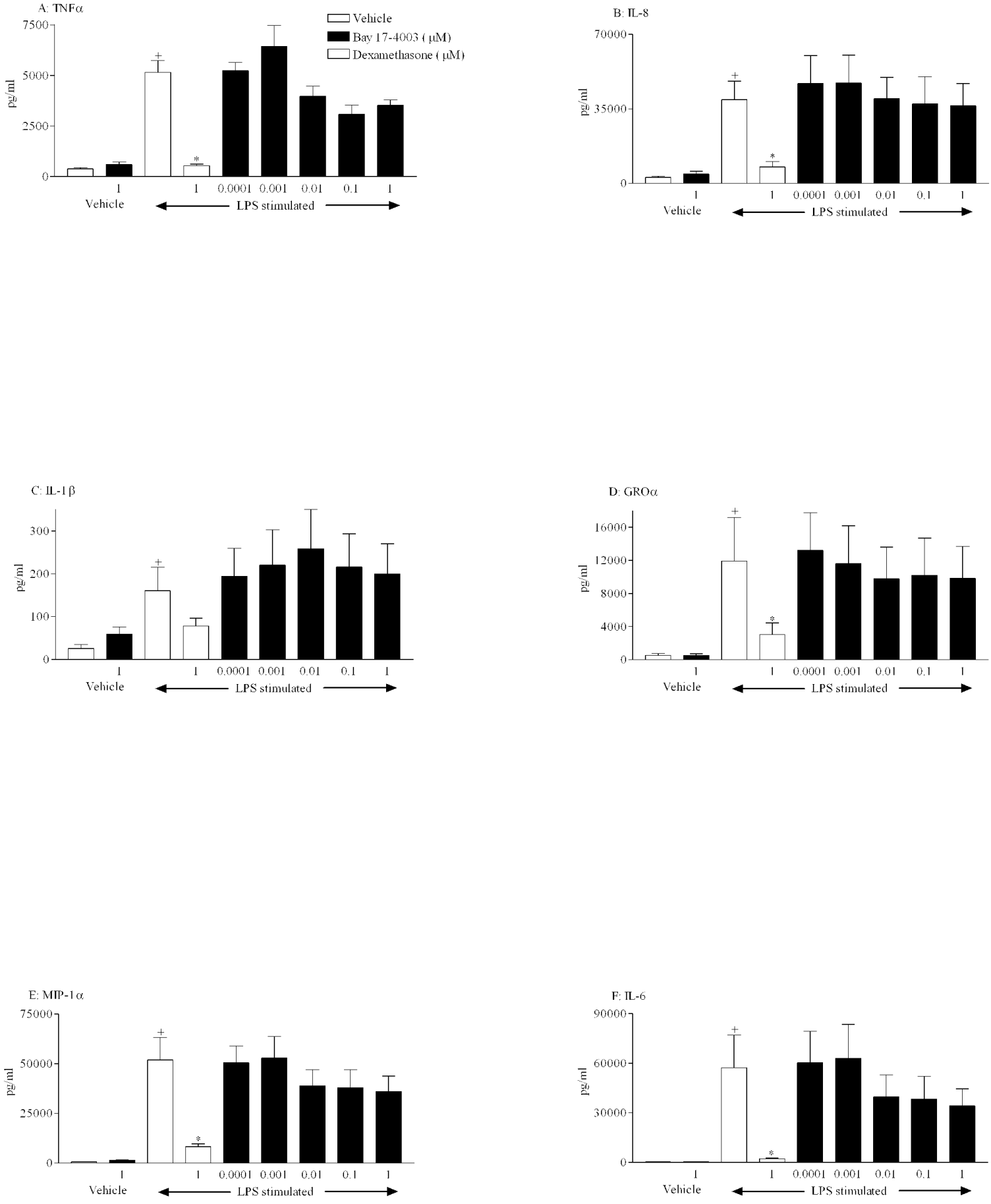

Figure 5

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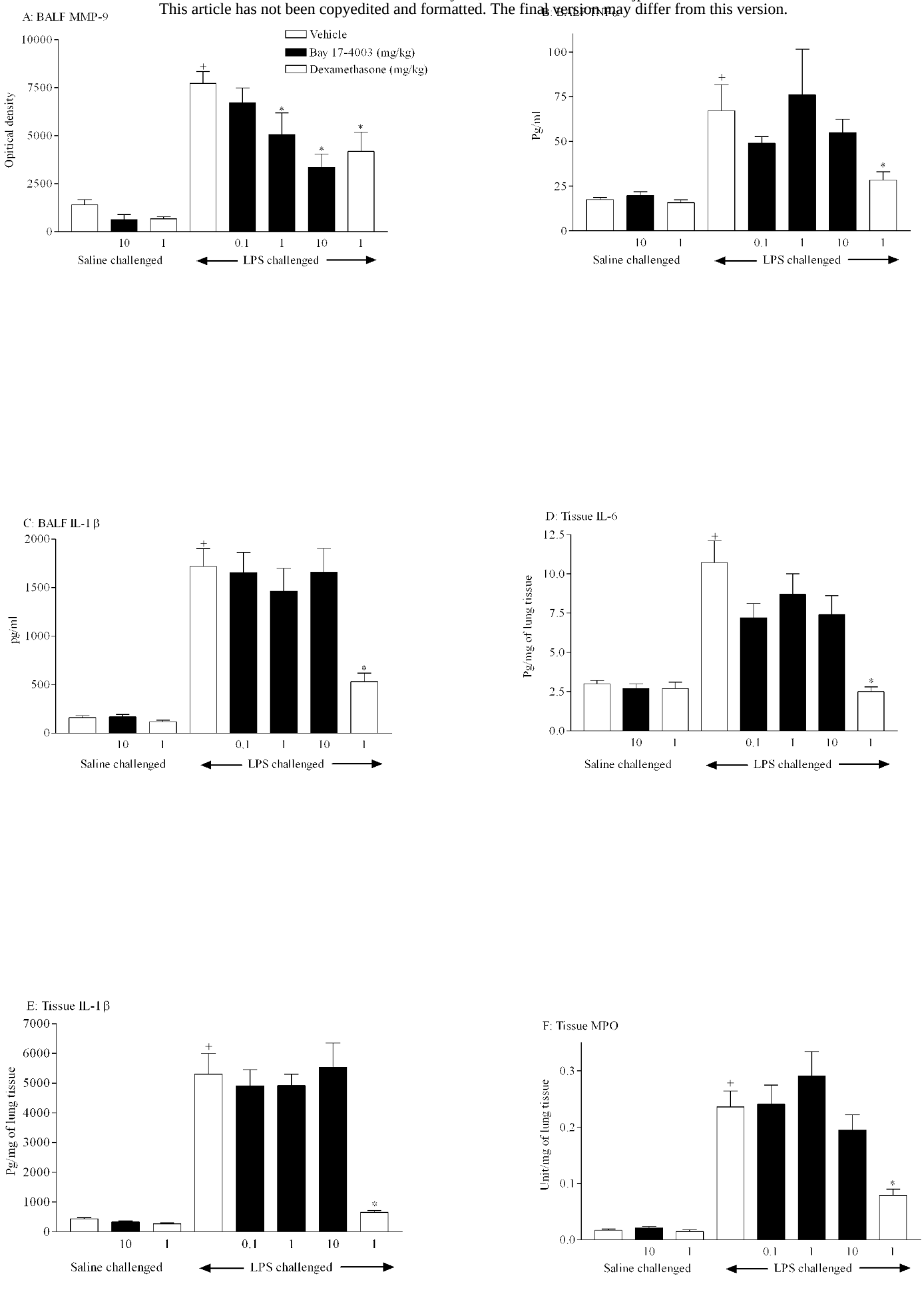
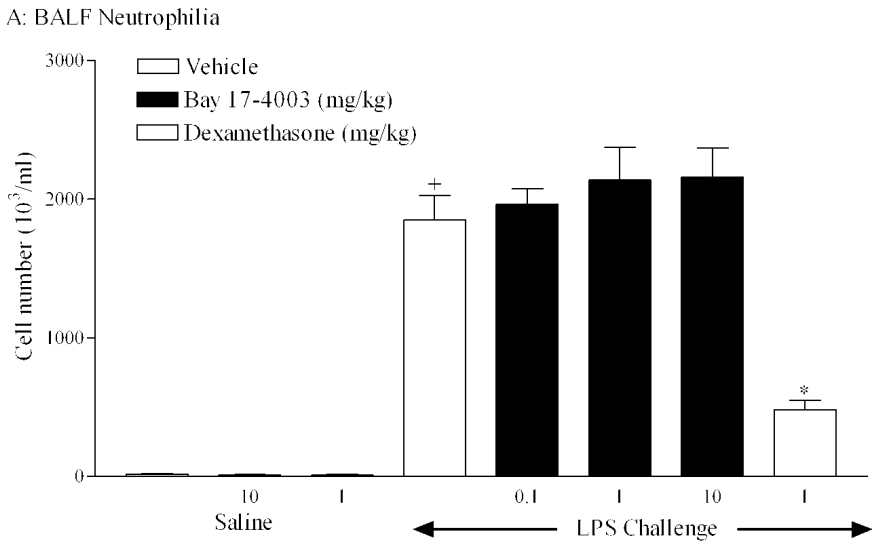
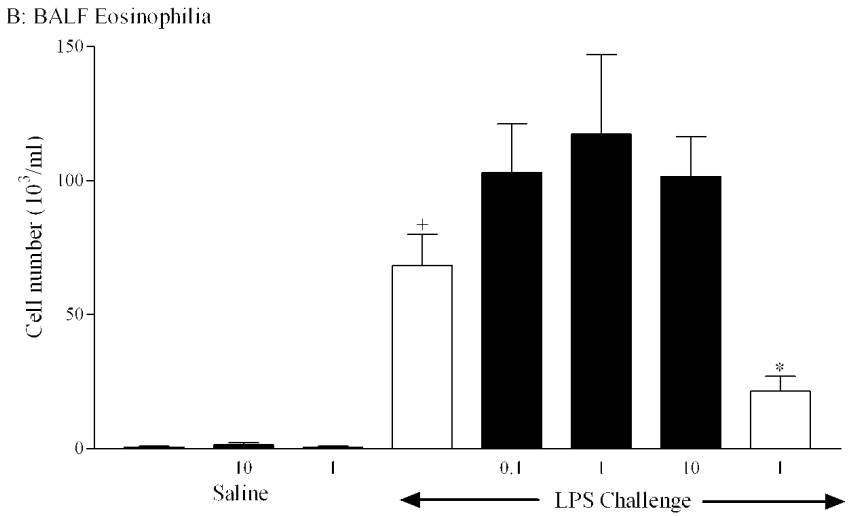


Figure 6





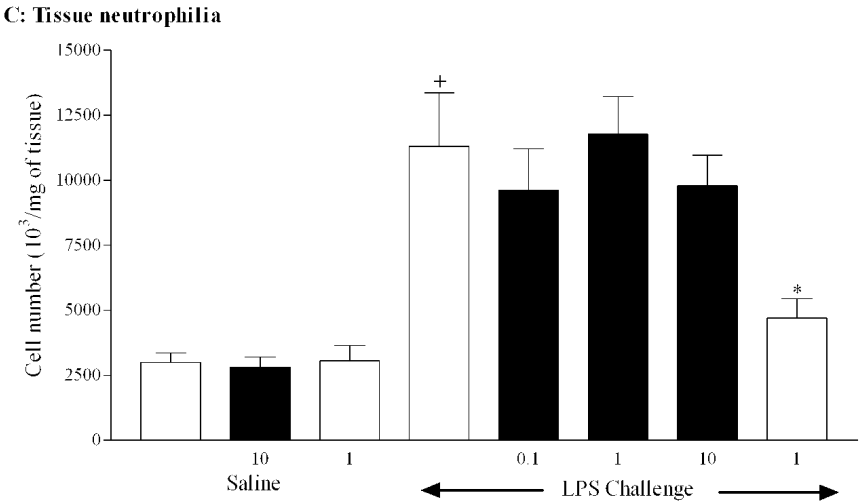
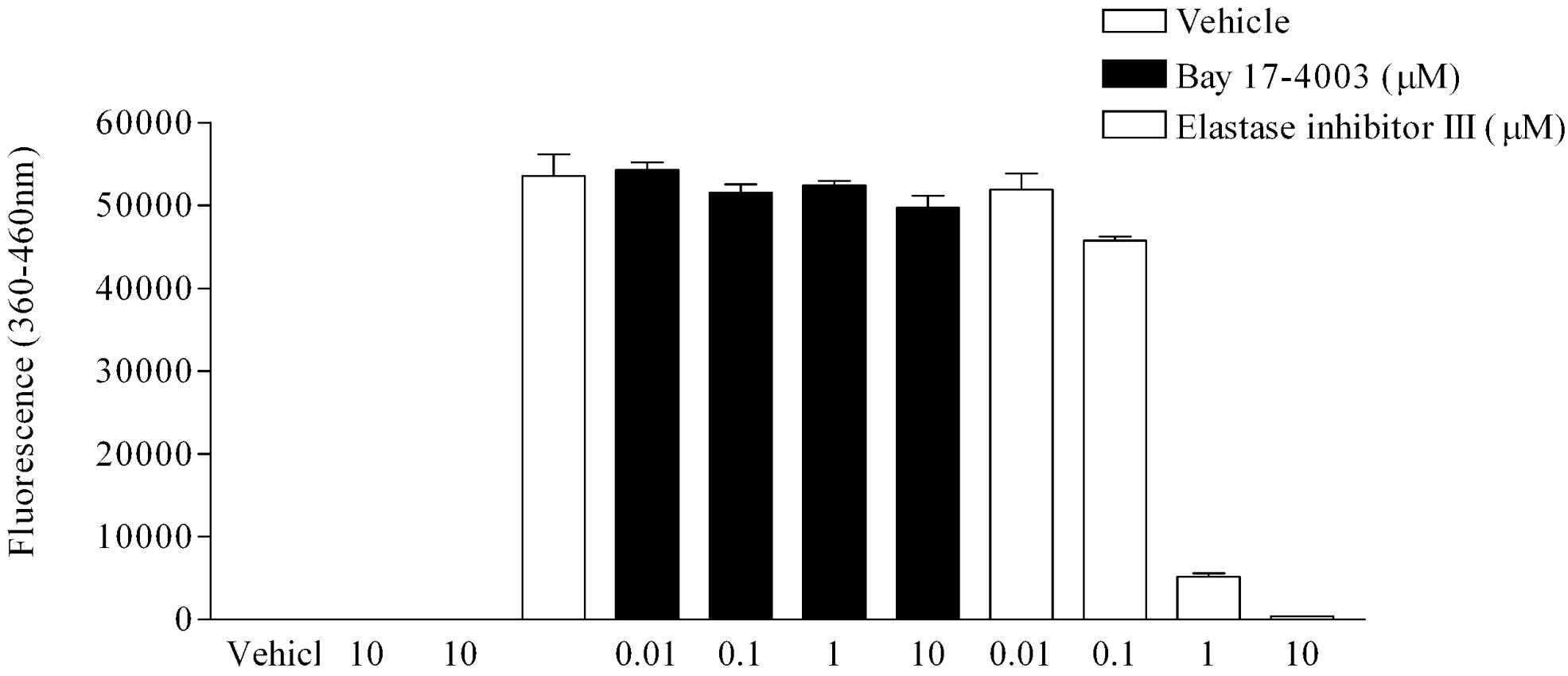


Figure 7

Figure 8



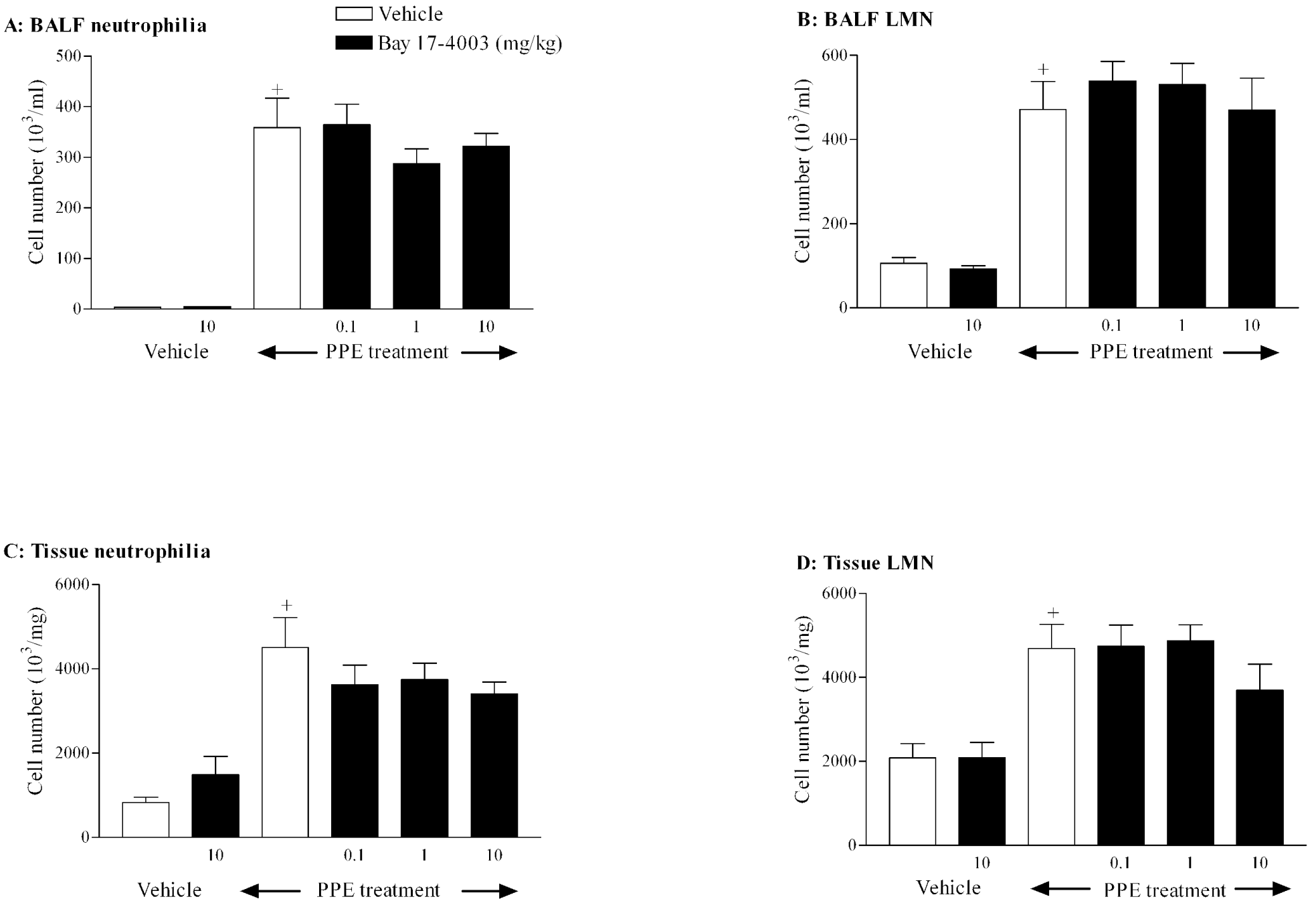


Figure 9

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Figure 10