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Involvement of MMP-12 and phosphodiesterase type 4 in cigarette Smoke-induced inflammation in mice

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Running title : Cigarette smoke –induced inflammation in mice

Abstract

The aims of this study were to characterize a mouse model of airways inflammation induced by cigarette smoke and to compare it with LPS model with regards to the efficacy of a PDE4 inhibitor, cilomilast, and a corticosteroid, dexamethasone and macrophage metalloelastase (MMP-12) gene deletion.

Cigarette smoke exposure for 3 days induced a time-dependent airway neutrophilia associated with an increased level of KC, MIP-2, MIP-1alpha and MMP-9 in the BAL. LPS exposure also induced an increase in the number of neutrophils in BAL. Studies in MMP12-/- mice showed that in contrast to the smoking model, MMP-12 did not have a critical role in LPS-induced inflammation. Both cilomilast and dexamethasone blocked LPS-induced neutrophilia in a dose-dependent manner. Cilomilast inhibited cigarette smoke-induced neutrophilia and MIP-1alpha but only 10 mg/kg of dexamethasone was effective. Both anti-inflammatory treatments had no effect on the levels of KC and MIP-2 in the BAL.

Although the inflammatory response was very similar in the smoking model and LPS, the pharmacological modulation and the MMP12 gene deletion highlighted the differences in the mechanisms involved. Furthermore, the cigarette smoke model seems to better represent the situation described in COPD patients. These differences underline the importance of using acute smoke exposure model to investigate potential new treatments for COPD.

Key words: Chronic obstructive pulmonary disease, Phosphodiesterase type 4 inhibitor, matrix metalloelastase , Corticosteroid, Cigarette smoke

Abbreviations

BAL: bronchoalveolar lavage

COPD: chronic obstructive pulmonary disease

FEV_1: force expiratory in 1 volume

IL: interleukin

KC: keratinocyte derived-chemokine

LPS: lipopolysaccharide

MIP-1α: macrophage inflammatory protein-1α

MIP-2: macrophage inflammatory protein-2

MMP : matrix metalloproteinase

PBS : phosphate buffer saline

PDE : phosphodiesterase

TNF: tumor necrosis factor

WT: wild type

Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by poorly reversible airflow limitation associated with an abnormal inflammatory response of the lung most commonly as a result of long term cigarette smoking [1-3]. The pathobiology of COPD includes chronic inflammation in the lung, which contributes to the hallmarks of the disease: chronic bronchitis, emphysema and a progressive decline in lung functions. The inflammatory reaction is characterised by neutrophilia in the bronchoalveolar lavage (BAL) fluid as well as by an increase of pro-inflammatory mediators, such as Interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) [4].

The pathology of COPD may involve the development of emphysema, characterized by a destruction of alveolar septae resulting in airspace enlargement, loss of surface area for gas exchange and loss of elasticity required for efficient contraction of the lung during the breathing cycle [4]. It is believed that the development of emphysema reflects a relative excess of cell-derived proteases, including serine proteases like neutrophil elastase and matrix metalloproteinases (MMPs) [4] that degrade the connective tissue of the lung and a relative paucity of antiproteolytic defenses. Macrophage metalloelastase (MMP-12) and gelatinase B (MMP-9), which are mainly produced by inflammatory cells, belong to the MMP-family of Zn^{2+}-dependent and Ca^{2+}-dependent proteinases [5] and seem to play a predominant role in the pathogenesis of COPD and particularly in emphysema. MMP-12 is able to degrade different substrates among which elastin is the major constituent of alveolar wall. MMP-9 is able to hydrolyze both gelatin and native elastin. More recently, an increase of MMP-9 and MMP-12 levels in the BAL from COPD patient has been reported [6, 7]. Knockout mice deficient in the gene encoding MMP-12 (MMP-12$^{-/-}$) show a diminished inflammatory response to cigarette smoke and are largely resistant to the development of emphysema [8].

Presently, only few therapies are available for COPD, with limited efficacy. These include bronchodilators, such as anti-cholinergics. Unlike in asthma, the use of inhaled glucocorticoids in the treatment of COPD is limited. While significant improvements have been reported in frequency of exacerbations and quality of life [9], no clinical benefit in either lung function or symptom scores has been shown. In most studies [10-12] with the exception of the study by Tashkin et al [13], inflammatory indices such as sputum neutrophilia and sputum mediators (*e.g.* IL-8 and TNF-α) did not change in response to treatment with glucocorticoids, suggesting that glucocorticoids have at least in the short term little anti-inflammatory effect in this group of patients, and therefore that the inflammatory process in COPD is resistant to glucocorticoids. Therefore, new anti-inflammatory drugs are needed to improve the control of symptoms and to prevent progression of COPD.

Such drugs may include inhibitors of phosphodiesterases (PDE), mainly selective PDE4 inhibitors that inactivate cyclic AMP. Indeed, PDE4 is the predominant PDE isoenzyme expressed in the inflammatory cells. The second generation of specific PDE-4 inhibitors, exemplified by cilomilast (Ariflo), has been shown to possess anti-inflammatory effects *in vitro* [14], *in vivo* [15] and in COPD clinical trials where 6 weeks treatment improves lung function such as force expiratory volume in 1 sec (FEV1) [16]. Additional candidate drugs include inhibitors of MMPs [5].

To date only few animal models of COPD have been developed that could be used to evaluate candidate therapeutic compounds or to identify new targets. This study was set up to develop an acute model of cigarette smoke-induced inflammation in mice which could highlight some of the key features of the inflammatory response observed in COPD patients. To further validate our model, we confirmed the involvement of MMP-12 in the development of airway inflammation. Moreover, we studied the effects of a glucorticosteroid , dexamethasone, and a PDE4 inhibitor, cilomilast, representatives of two classes of drugs used

in clinical trials in COPD. The smoking model was sensitive to cilomilast, but largely resistant to the effects of dexamethasone, which reflects the observations made in clinical trials with other glucocorticoids, notably prednisolone and fluticasone [17], and cilomilast itself [15]. Further confirmation of its value was obtained by comparing it with the classical model of neutrophilic inflammation in the lung, using inhaled exposure to lipopolysaccharide (LPS). In the LPS model both cilomilast and dexamethasone were active and gene deletion of MMP-12 had no effect.

Materials and Methods

Animals

Seven-week-old $C_{57}Bl_6$/J male mice were purchased from CERJ (Le Genest Saint Isle, France) and quarantined for 1 week before experiments. MMP-12$^{-/-}$ mice were obtained from Charles River laboratories following a transfer from Washington University and rederivation on $C_{57}Bl_6$ background. $C_{57}Bl_6$/J mice were used as wildtype control (wt) mice. Animals were handled in accordance with standards established by the European Animal Welfare acts.

Cigarette smoke exposure

Mice were exposed to the whole smoke of 2 Kentucky 1R3 cigarettes once or twice a day for 1, 2 or 3 days or to laboratory air (control mice). Mice were placed in a Plexiglas chamber cover by a disposable filter (Ingenia, Ivry, France). The smoke was produced by the burning of cigarettes and was introduced into the chamber with a constant airflow generated by a mechanical ventilator (7025, Ugo Basile, Comerio, Italy) at a rate of 25 ml/min.

LPS exposure

The mice were placed in a Plexiglas chamber and were exposed to 100µg/ml LPS for 1 hour or to the vehicle (NaCl, 0.9%) (control mice) as previously described [18] delivered using a small particle aerosol generator (SPAG-2 6000 series, ICN, CA, USA).

Bronchoalveolar lavage (BAL)

Sixteen hours after LPS challenge or the last cigarette smoke exposure, mice were anaesthetized by i.p. injection of sodium pentobarbital (60 mg/kg). Airspaces were washed

using 6 times 0.3ml of PBS solution, kept at 37°C. The samples collected were dispatched in 2 fractions: the first one (0.6ml corresponding to the 2 first lavages) was used for mediator measurement and the second one for the cell determination (0.9ml). The first fraction was centrifuged (600 g for 10 min) and kept at -80°C until mediator determination. The cell pellet was then resuspended in 0.6ml PBS and, pooled with the second fraction and maintained at 4°C until cell determination.

Cell count and determination

Total cell count in BAL fluid was determined using a particle counter (Z2 Coulter, Beckman Coulter). Differential cell counts were performed on cytospin preparations (Cytospin 3, Thermo Shandon) after staining with May-Grünwald-Giemsa stain (Sigma chemical, Saint Louis, USA).

Mediator measurements

KC, MIP-2 and MIP-1α levels in BAL fluid were determined using ELISA assay kits (Quantikine M Murine, R&D system, Minneapolis, USA) according to manufacturer's instructions. MMP levels in BAL fluid were determined by gelatin zymography.

Drug administration to mice

Dexamethasone (0.5 to 10 mg/kg) or cilomilast (3 to 100 mg/kg) were suspended in methylcellulose (MC) 0.5% (wt/vol) and administered orally (20ml/kg). Control mice received 0.5% MC only. Treatments were performed one hour before LPS, smoke or control exposure.

Materials

Lipopolysaccharide (LPS from *E. coli*, serotype 055:B5), Tris buffer, Coomassie stain solution, dexamethasone 21-posphate and methylcellulose were purchased from Sigma chemical, (Saint Louis, USA). Kentucky 1R3 cigarettes (Tobacco Health Research) were provided by University of Kentucky, USA. Sodium Pentobarbital was from Sanofi Santé Animal, France. PBS solution was from Dulbecco's, Gibco, UK. Standards for zymography and gelatin were from BioRad, Hercules, USA. Triton X-100 was from ICN Pharmaceuticals Inc, Ca, USA.

Statistical Analysis

Comparisons were made by analysis of variance (ANOVA). The results were expressed as means ± standard error of mean (S.E.M.). Statistical analysis was performed using the "Statview" software. Analysis of treatment effects across the various groups was performed using a two-way ANOVA. Comparison of treatment interaction was realized by Fischer tests. For each analysis, p values less than 0.05 was considered to be statistically significant.

Results

Effect of cigarette smoke exposure on the development of airway inflammation

$C_{57}Bl_6$/J mice exposed to two cigarettes once a day showed a moderate increase in the number of neutrophils in the BAL fluid only after 3 days of consecutive smoke exposure (Fig. 1A). In contrast, exposure of mice to two cigarettes, twice a day elicited a marked and significant neutrophil influx in the BAL fluid that appeared after 2 days and was further increased after 3 days of exposure (Fig. 1B). In the latter protocol, a moderate but significant increase in the number of eosinophils was also noted after 2 and 3 days of smoke exposure, whereas the number of macrophages and lymphocytes were unchanged. All further data were obtained in the three-day model.

Using ELISA, we found that keratinocyte derived-chemokine (KC), macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein-2 (MIP2) were markedly increased in the BAL fluid of mice exposed to cigarette smoke from 3±0.5 to 45±16 pg/ml ($P<0.01$), from 1± 0.1 to 13± 7 pg/ml ($P<0.05$) and from 0.5±0.5 to 17.7±4.7 µg/ml ($P<0.01$), respectively.

Using zymography, we observed 3 bands corresponding to gelatinase activities of 130 kDa, 96 kDa, and 88 kDa (Fig. 2A) in the BAL fluid from smoking mice compared to control mice. These bands were not present after treatment of the gel with 10 mM EDTA (data not shown) suggesting that these bands corresponded to metalloproteases. The activities observed at 96 kDa and 88 kDa may correspond to MMP-9 pro- (latent) and active- forms. The analysis of the intensity of the spot by densitometry showed a marked and significant increase in the pro- and active form of MMP-9 in mice exposed to cigarette smoke after three days in comparison to control (laboratory air-exposed) mice (Fig 2B).

Effect of cigarette smoke exposure in MMP-12$^{-/-}$ mice

Figure 3 shows the effects of exposure to cigarette smoke in MMP-12$^{-/-}$ and wt mice. As previously observed in $C_{57}Bl_6$/J, cigarette smoke exposure (two cigarettes, twice a day) to wt mice elicited an increase of neutrophils after 2 and 3 days of exposure. In contrast, in MMP-12$^{-/-}$ mice, the neutrophil influx was significantly reduced after both 2 and 3 days of smoke exposure. A non-significant reduction was noted on the increased eosinophilia. Using zymography, we observed an important decrease of MMP-9 activity in MMP-12$^{-/-}$ mice after smoke exposure compared to the wt mice (Fig. 4). The relative intensity of the pro-MMP-9 in MMP-12$^{-/-}$ mice exposed to cigarette smoke appeared equivalent to that in the control mice exposed to laboratory air.

To investigate whether the deletion of MMP-12 affected the general ability of the mice to respond to an inflammatory stimulus involving neutrophil influx, we investigated the effect of exposure to LPS by aerosol on wt and MMP-12$^{-/-}$ mice. LPS exposure elicited a marked enhancement in the number of neutrophils in both WT and MMP-12$^{-/-}$, which was not significantly different (3.43±1.47 and 4.25±0.91 cells(x100000)/ml respectively), suggesting that MMP-12 is specifically involved in smoke-induced inflammation.

Effect of dexamethasone and cilomilast on lung inflammation induced by cigarette smoke exposure

Treatment of mice with 2.5 or 5 mg/kg dexamethasone did not significantly reduce the increase in neutrophil number in the BAL of mice after 3 days of cigarette smoke exposure (Fig. 5A). Only a moderate but significant reduction of neutrophil influx was noted at the highest dose of dexamethasone (10mg/kg). However, treatment with dexamethasone elicited a complete inhibition of the eosinophil influx at all concentrations tested (Fig 5A). Cilomilast

(3, 10 or 30 mg/kg) dose-dependently prevented the neutrophil influx induced by cigarette smoke exposure (Fig. 5B). Cilomilast (3 and 30 mg/kg) also reduced the eosinophil influx.

The levels of MIP1-α were markedly increased in the BAL fluid of mice exposed to cigarette smoke after three days in comparison to control mice (Fig 6). Interestingly, cilomilast, but not dexamethasone was able to dose-dependently prevent the increase of MIP1-α levels (Fig. 6A/B) whereas KC and MIP-2 were not affected by either even at the highest dose (data not shown).

Effect of dexamethasone and cilomilast on neutrophil influx by LPS exposure

To compare the potential anti-inflammatory role of dexamethasone and cilomilast in the smoking model to that in a well-known airway inflammatory model, we investigated the potency of both treatments on neutrophil influx in the BAL fluid of $C_{57}Bl_6$/J mice upon exposure to LPS (Fig. 7). In contrast to the observations in the smoking model, neutrophilia in the LPS model is sensitive to all concentrations of dexamethasone tested (0.5, 1, 2.5 and 5 mg/kg) as well as to cilomilast (10, 30, 60 and 100 mg/kg). The effect of dexamethasone was already noted with the dose of 0.5 mg/kg with a maximum inhibition already achieved at 2.5mg/kg. Cilomilast reduced neutrophilia after LPS exposure with similar efficacy to the cigarette smoke exposure experiments.

Discussion

The present study reports the development of an acute model of cigarette exposure in mice, in which a marked neutrophila develops after 2-3 days. This airway inflammation was sensitive to MMP-12 gene deletion and the PDE-4 inhibitor cilomilast, but largely insensitive to the glucocorticoid dexamethasone. In this, it was significantly different from the traditional model of neutrophilic lung inflammation using inhalation of LPS, which was sensitive to both cilomilast and dexamethasone, but in which MMP-12 deletion had no effect.

Cigarette smoking is the prevalent predisposing factor to chronic bronchitis, emphysema and COPD. However, the mechanisms by which exposure of the lung to cigarette smoke causes the inflammatory process and pathological alterations of the tissue seen in COPD are not completely understood. Glucocorticoids are commonly used as treatment of COPD and show benefits in particular on frequency and severity of exacerbations [19,20]. However, most studies suggest that there is little evidence of significant anti-inflammatory activity with regards to neutrophils or macrophages [17], although there is an effect on tissue mast cells [21]. Data exists on inhibitors of PDE4 suggesting that the anti-inflammatory activity is limited to CD8+ and CD68+ cells in tissue [15].

The development of new therapies for COPD is hindered due in part to the lack of relevant animal models that could be used for increasing our understanding of the inflammatory mechanisms and the study of new candidate drugs. Although cigarette smoke is the most relevant stimulus to reproduce pathology seen in human COPD disease, the length of time required to induce significant emphysematous changes limits the usefulness of such an emphysema model as a pharmacological screening tool. As an alternative, acute models that reflect the early inflammatory response to cigarette smoke may provide a first step in the selection of candidate mechanisms and drugs [21].

Therefore, this study was designed to set up an acute model of cigarette smoke-induced inflammation in mice to investigate mechanisms of COPD and to test candidate drugs. We focused the read-out on cell influx in BAL, cytokine levels and MMP activity, all already employed in clinical trials, and compared the smoking model to a model of LPS inhalation.

Here, we show that once a day exposure to two cigarettes was largely without effect, but exposure to smoke from two cigarettes twice a day during three days elicited a dramatic influx of neutrophils in the BAL fluid. In addition we observed an increase in eosinophils and lymphocytes. Although no increase in macrophages was observed in the BAL, phenotypic changes of lung macrophages suggested an enhanced state of activation and are subject of further study (data not shown). The inflammatory cell recruitment was associated with an increased level of chemokines MIP1-α, KC and MIP-2, which have been described to be involved in the chemotaxis and activation of neutrophils and monocytes [22]. A similar pattern of cellular inflammation has been reported in bronchoalveolar lavage fluid and sputum [23,24] of COPD patients, while IL-8 is increased in induced sputum [11]. IL-8 is a human ligand for CXCR-1/2 and as such an equivalent of KC in mice.

MMP-9 is elevated in bronchoalveolar lavage fluid of subjects with emphysema, suggesting that MMP-9 may be important in the pathogenesis of the disease [25]. These observations have been supported and extended by Russell and colleagues [6], who showed that more MMP-9 was secreted from macrophages from COPD patients compared to macrophages from healthy volunteers when stimulated with IL-1β, endotoxin or cigarette smoke-conditioned medium. In our experimental model, we also found that MMP-9 activity was increased. This increased activity may be linked to activation of macrophages and/or the neutrophil influx as previously suggested in studies involving LPS-exposed mice [26].

Cigarette smoke exposure in mice is associated with MMP-12 expression in macrophages and the development of emphysema [27]. Moreover, MMP-12$^{-/-}$ mice exposed to cigarette smoke for 6 months demonstrated a central role for MMP12 in cell recruitment and the development of emphysema [8]. In an acute model of smoke exposure, neutrophil influx in BAL fluid, desmosine and hydroxyproline (markers for elastin and collagen breakdown respectively) were greatly reduced in MMP-12$^{-/-}$ mice as compared to control mice [28]. In this study we confirmed this observation using the smoke exposure protocol that we developed. Interestingly, we also observed a very marked reduction of MMP-9 activity in BAL fluid, suggesting that the increase in MMP-9 activity in response to cigarette smoke depends almost entirely on MMP-12 expression. In contrast to the observations in the smoking model, MMP-12$^{-/-}$ mice developed a similar airway neutrophilia when exposed to LPS as wt mice. This indicates clear differences between the two models and that the early inflammatory process after cigarette smoke exposure, although similar in profile, has different causal mechanisms than the LPS-induced inflammation.

Several clinical trials have been performed using glucocorticoids and PDE4 inhibitors in COPD patients. The clinical benefits of glucocorticoids were mainly limited to effects on exacerbations; while in the majority of studies no effect was seen on inflammatory cell numbers in induced sputum, specifically neutrophils, and on IL-8 levels and MMP-9 activity [10-12]. *Ex vivo* studies on cells from COPD patients [6] suggest that the inflammatory response seen in COPD may at least in part be resistant to glucocortocoids. More limited data are available on the effects of inhibition of PDE4 in man but clinical trials reported an improvement in lung function after treatment with cilomilast in COPD patients [16] and in a small study it was shown to have anti-inflammatory effects in biopsies [15]. *Ex vivo* studies with cilomilast suggest a potent anti-inflammatory effect [29] on cytokine expression.

Glucocorticoids as well as PDE4 inhibitors have been reported to be potent anti-inflammatory compounds *in vitro* and *in vivo* in various animal models [for review see 30]. To establish the importance of the smoking model in predicting efficacy in human disease, we tested representatives from these two drug classes, dexamethasone and cilomilast, in our experimental model and compared it to the LPS model. Neutrophilic inflammation in both models is sensitive to the PDE-4 inhibitor cilomilast, but while the LPS model is very sensitive to treatment with the glucocortocoid dexamethasone, neutrophilia in the smoking model is largely insensitive up to concentrations of 10 mg/kg, which is the upper tolerated limit for use in mice. It is difficult to explain the difference of the inflammatory process induced by LPS or cigarette smoke. Various hypotheses regarding the involvement of different resident cells and/or pro-inflammatory mediators may be proposed. Experiments in vitro are in progress to dissect the cellular signalling induced by either cigarettes smoke condensate or LPS.

We found an increase of MIP-1 alpha chemokine after cigarette smoke and a significant reduction after the treatment with cilomilast, but not with dexamethasone, which agrees with the data on neutrophilia and suggests a possibly critical role of MIP-1alpha in cigarette smoke-induced neutrophil influx.

One hypothesis for the lack of efficacy of the glucocorticoid in the smoking model suggests that oxidative stress generated by cigarette smoke inhibits the anti-inflammatory activity of the glucocorticoid receptor [4]. In addition, it is known that glucocorticoids may prolong neutrophil survival. Further work is required to investigate the causes of steroid insensitivity in our smoking model. It is of interest to note that the relatively modest eosinophilic inflammation seen in the model did respond to treatment with corticosteroids, suggesting a neutrophil- rather than a glucocorticoid-specific effect. A similar hypothesis has been proposed for the reduced steroid sensitivity seen in severe asthma [31].

In conclusion, we have developed an acute smoking mouse model, in which several features of the airway inflammation observed in COPD patients were observed. We report that cigarette smoke-induced inflammation was reduced by the PDE4 inhibitor cilomilast, but was largely unresponsive to the glucocorticoid dexamethasone. Thus, the smoking model shows significant differences with, and potential advantages over the traditional model of airway neutrophilia using LPS, as was stressed by the differences in response to gene deletion of MMP-12. This model may therefore be used to explore the inflammatory mechanisms induced by cigarette smoke, the efficacy of new compounds, and for the discovery of potential new targets for drug discovery in COPD.

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Legends for figures:

Figure 1: Time-course of inflammatory cell recruitment in the BAL of C57bl6 mice after cigarette smoke exposure or laboratory air (Control mice), (A) 2 cigarettes once a day and (B) 2 cigarettes twice a day. Data shown are means ± SEM. The number of macrophages (*black columns*), neutrophils (*white columns*), lymphocytes (*doted columns*) and eosinophils (*dashed columns*) were determined by morphometric analysis under microscopic examination. No difference in the number of cells in the BAL of control mice was noted throughout the experiment. (n = 8, * $p<0.05$ vs. control, ** $p<0.01$).

Figures 2: Zymographic analysis of the BAL of $C_{57}bl_6$ mice after 2 cigarettes twice a day for 3 days smoke exposure. Data shown are BAL from two control mice (lanes 1 and 2) and BAL from two mice exposed to cigarette smoke (lanes 2 and 3). This zymography is representative of 3 independent experiments. (B) Relative intensity of the bands. Data shown are means ± SEM. (n=6, * $p<0.05$ vs. control, ** $p<0.01$).

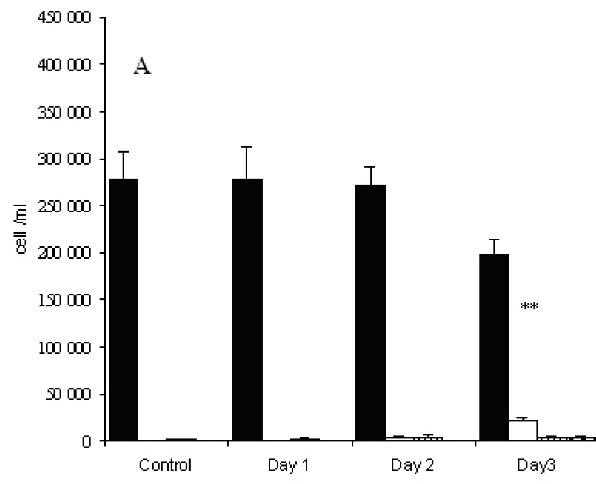
Figure 3: Time-course (2 and 3 days smoke exposure) of inflammatory cell recruitment in the BAL of WT or MMP-$12^{-/-}$ mice after exposure of 2 cigarettes twice a day or laboratory air (Control mice). No difference in the number of cells in the BAL of control mice were noted throughout the experiment. The number of macrophages (*black columns*), neutrophils (*white columns*), lymphocytes (*doted columns*) and eosinophils (*dashed columns*) (means ± SEM) were determined by morphometric analysis under microscopic examination. (n = 8, * $p<0.05$ and ** $p<0.01$ vs. control, # $p<0.05$ and WT## $p<0.01$ *vs.* WT).

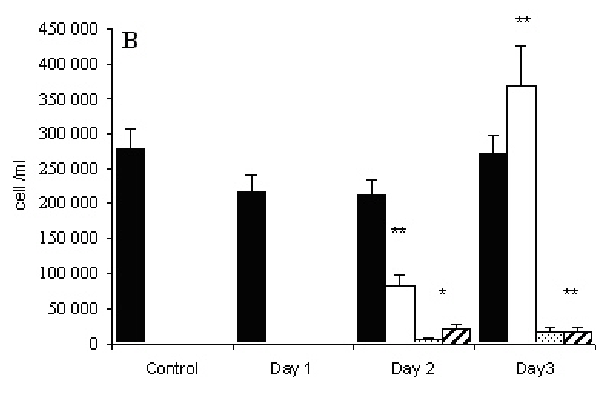
Figures 4: Zymography of the BAL fluids of WT and MMP-$12^{-/-}$ mice exposed to 2 cigarettes smoke twice a day for 3 days or laboratory air (Control mice). The relative intensity of the 96 kDa MMP-9 is also presented. Data shown are representatives of 3 experiments.

Figures 5: Dose-response effect of dexamethasone (dexam; 2.5, 5 and 10 mg/kg) (A) and cilomilast (3, 10 and 30 mg/kg) (B) on cigarette smoke induced inflammatory cell recruitment in BAL of $C_{57}bl_6$ mice. Mice were exposed to 2 cigarettes smoke twice a day for 3 days or laboratory air (Control mice). The number of macrophages (*white columns*), neutrophils (*black columns*), and eosinophils (*dashed columns) and* lymphocytes (*dotted columns*) (means ± SEM) were determined by morphometric analysis. (n = 8, * $p<0.05$, ** $p<0.01$vs vehicle).

Figures 6: Dose-response effect of dexamethasone (dexam; 2.5, 5 and 10 mg/kg) (A) and Cilomilast (3, 10 and 30 mg/kg) (B) on cigarette smoke exposure induced MIP-1α (means ± SEM) increase in BAL fluids of $C_{57}bl_6$ mice. Mice were exposed to 2 cigarettes smoke twice a day for 3 days or laboratory air (Control). Methylcellulose: MC, 0.5% is the vehicle of dexamethasone and cilomilast. n = 8, ** $p<0.01$ vs. control+ mice.

Figures 7: Dose-response effect of dexamethasone (0.5, 1, 2.5, 5mg/kg) (A) and Cilomilast (10, 30, 60 and 100 mg/kg) (B) on LPS (100 µg/ml for 60 min) induces neutrophil recruitment in BAL of $C_{57}bl_6$ mice. Control mice are treated with the vehicle of LPS (NaCl, 0.9%). The number of macrophages (*White columns*) and neutrophils (*black columns*), (means ± SEM). Methylcellulose: MC, 0.5% is the vehicle of dexamethasone and cilomilast. n = 10, * $p<0.05$, ** $p<0.01$ vs. vehicle (MC, 0.5%).


450 000
400 000
350 000
300 000
250 000
200 000
150 000
100 000
50 000
0
A
cell /ml
**
Control
Day 1
Day 2
Day3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B
cell /ml
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*
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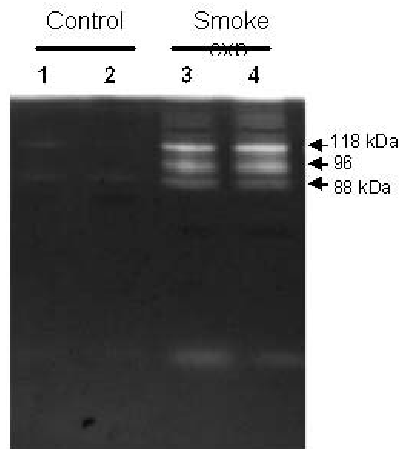
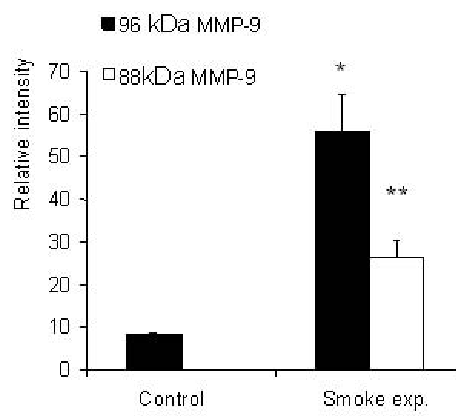
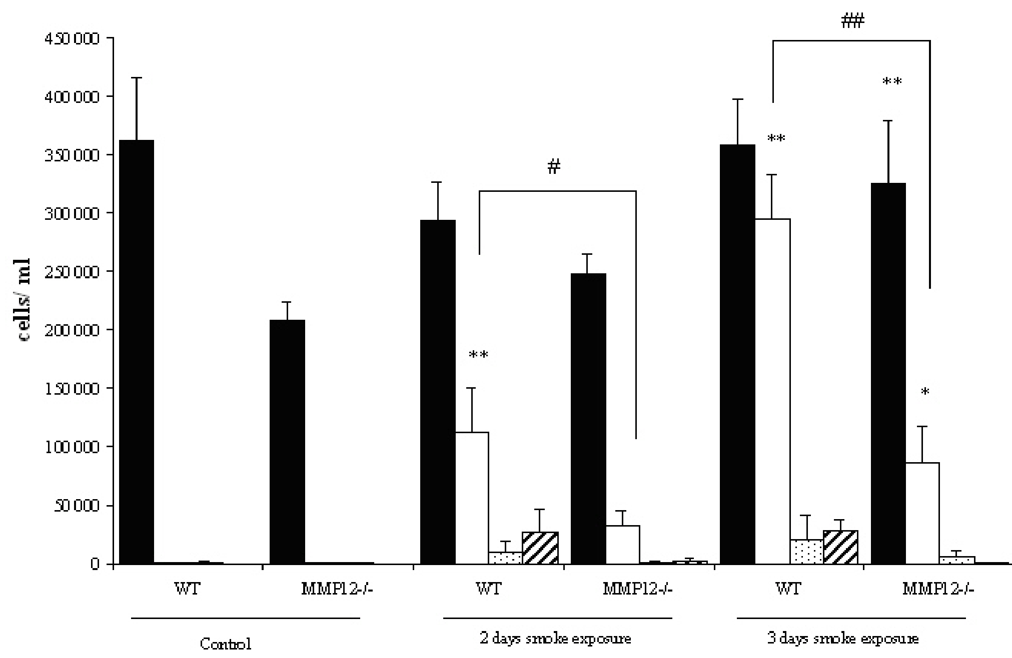
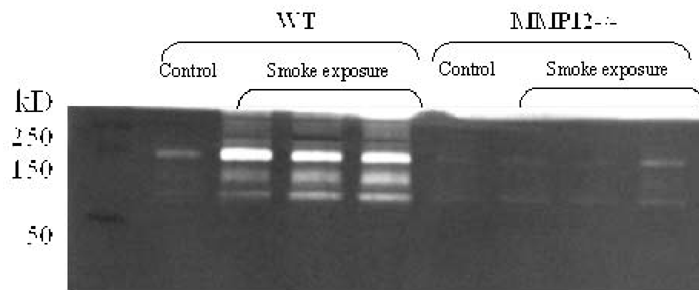
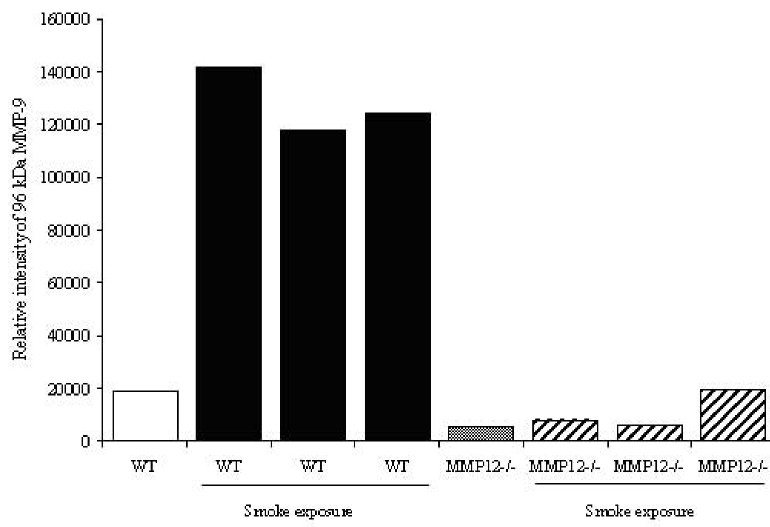
Day 1
Day 2
Day3


A
Control
Smoke exp
1
2
3
4
118 kDa
96
88 kDa


B
96 kDa MMP-9
88kDa MMP-9
Relative intensity
70
60
50
40
30
20
10
0
*
**
Control
Smoke exp.


450 000
400 000
350 000
300 000
250 000
200 000
150 000
100 000
50 000
0
cells/ ml
#
##
**
*
WT
MMP12-/-
WT
MMP12-/-
WT
MMP12-/-
Control
2 days smoke exposure
3 days smoke exposure


WT
MMP12-/-
Control
Smoke exposure
Control
Smoke exposure
kD
250
150
50


Relative intensity of 96 kDa MMP-9
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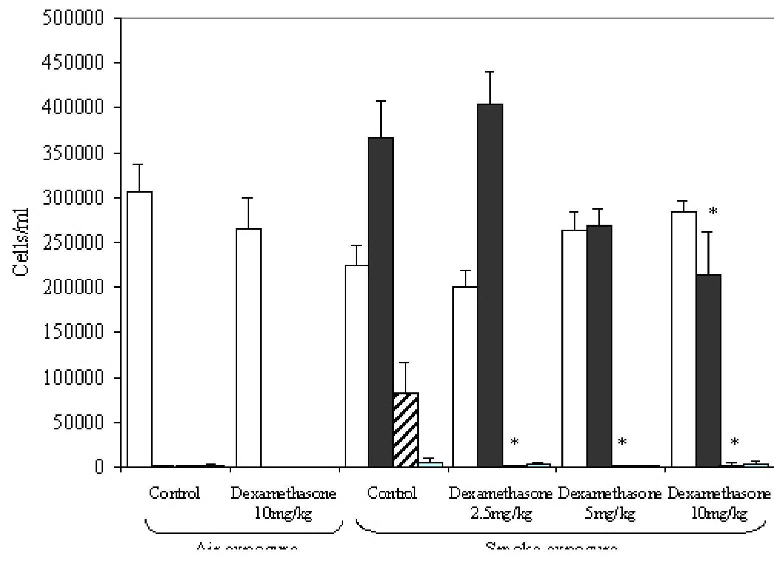
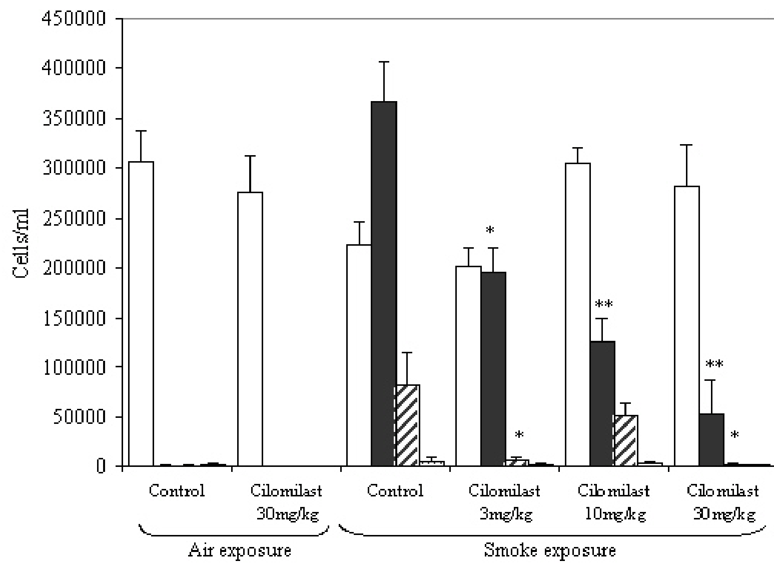
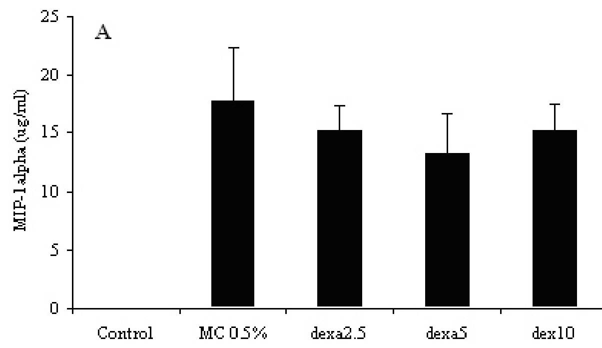
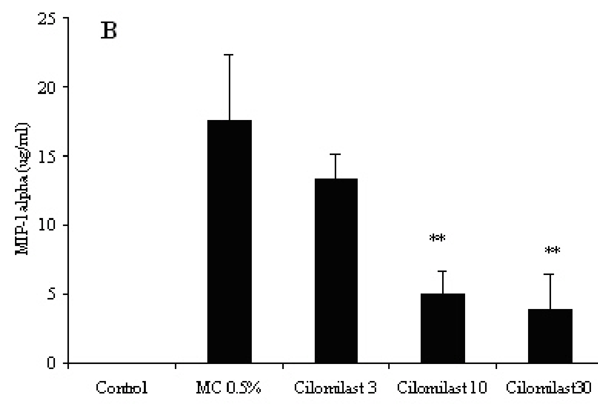
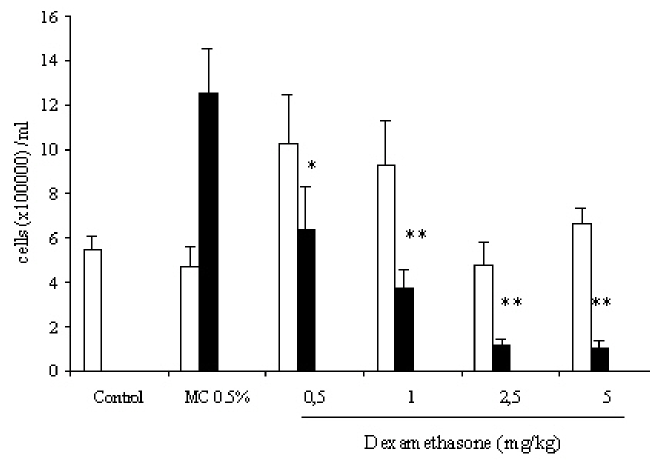
140000
120000
100000
80000
60000
40000
20000
0
WT
WT
WT
WT
MMP12-/-
MMP12-/-
MMP12-/-
MMP12-/-
Smoke exposure
Smoke exposure

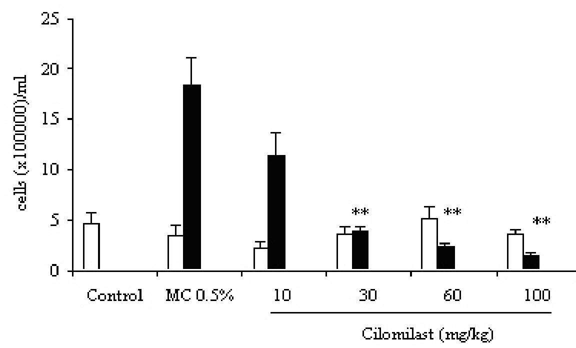

Cells/ml
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
*
*
*
*
Control
Dexamethasone 10mg/kg
Control
Dexamethasone 2.5mg/kg
Dexamethasone 5mg/kg
Dexamethasone 10mg/kg


Cells/ml
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
*
*
**
**
*
Control
Cilomilast 30mg/kg
Control
Cilomilast 3mg/kg
Cilomilast 10mg/kg
Cilomilast 30mg/kg
Air exposure
Smoke exposure


A
MIP-1alpha (ug/ml)
25
20
15
10
5
0
Control
MC 0.5%
dexa2.5
dexa5
dex10


B
MIP-1alpha (ug/ml)
25
20
15
10
5
0
**
**
Control
MC 0.5%
Cilomilast 3
Cilomilast 10
Cilomilast30


cells (x100000) /ml
16
14
12
10
8
6
4
2
0
*
**
**
**
Control
MC 0.5%
0,5
1
2,5
5
Dexamethasone (mg/kg)


cells (x100000)/ml
25
20
15
10
5
0
**
**
**
Control
MC 0.5%
10
30
60
100
Cilomilast (mg/kg)