ERJ Express. Published on February 8, 2013 as doi: 10.1183/09031936.00112412

Muscarinic receptor subtype-specific effects on cigarette smoke-induced inflammation in mice

L.E.M. Kistemaker[1,6*], I.S.T. Bos[1,6], M.N. Hylkema[2,6], M.C. Nawijn[2,6], P.S. Hiemstra[3], J. Wess[4], H. Meurs[1,6], H.A.M. Kerstjens[5,6], R. Gosens[1,6]

[1] Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands.

[2] Department of Pathology and Medical Biology, University Medical Centre Groningen, Groningen, The Netherlands

[3] Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

[4] Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA

[5] Department of Respiratory Medicine, University Medical Center Groningen, Groningen, The Netherlands

[6] GRIAC Research Institute, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

* Author for correspondence
Loes E.M. Kistemaker
Department of Molecular Pharmacology
University of Groningen
A. Deusinglaan 1
9713 AV Groningen
The Netherlands
Tel. + 31 50 363 8680
Fax. +31 50 363 6908
Email: l.e.m.kistemaker@rug.nl

Abstract

Cholinergic tone contributes to airflow obstruction in COPD. Accordingly, anticholinergics are effective bronchodilators by blocking the muscarinic M_3 receptor on airway smooth muscle. Recent evidence indicates that acetylcholine also contributes to airway inflammation. However, which muscarinic receptor subtype(s) regulate(s) this process is unknown.

In this study, the contribution of the muscarinic M_1, M_2 and M_3 receptor subtypes to cigarette smoke-induced airway inflammation was investigated by exposing muscarinic receptor subtype deficient mice to cigarette smoke for four days.

In wild-type mice, cigarette smoke induced an increase in macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid. Neutrophilic inflammation was higher in $M_1^{-/-}$ and $M_2^{-/-}$ mice compared to wild-type mice, but lower in $M_3^{-/-}$ mice. Accordingly, the release of KC, MCP-1 and IL-6 was higher in $M_1^{-/-}$ and $M_2^{-/-}$ mice and reduced in $M_3^{-/-}$ mice. Markers of remodeling were not increased after cigarette smoke exposure. However, $M_3^{-/-}$ mice had reduced expression of TGF-β1 and matrix proteins. Cigarette smoke-induced inflammatory cell recruitment and KC release were also prevented by the M_3–receptor selective antagonist 4-DAMP in wild-type mice.

Collectively, our data indicate a pro-inflammatory role for the M_3 receptor in cigarette smoke-induced neutrophilia and cytokine release, yet an anti-inflammatory role for M_1 and M_2 receptors.

Keywords: acetylcholine; airway inflammation; airway pharmacology; anticholinergics; COPD; non-neuronal

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by a progressive airflow limitation that is not fully reversible [1]. The most common cause of COPD in the Western world is tobacco smoking. Inflammation plays a central role in the disease, and contributes to airway fibrosis, mucus hypersecretion and emphysema that is observed in patients with COPD [1]. Macrophages and neutrophils are particularly increased in patients with COPD and this increase is related to an increased production of specific cytokines and chemokines, including interleukin (IL)-8, IL-6, IL-1β, IL-17, monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) [2]. Moreover, COPD is associated with an increased production of growth factors, including transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF), which are thought to contribute to the remodeling of the airways [3].

Acetylcholine is the primary parasympathetic neurotransmitter in the airways that induces bronchoconstriction. Parasympathetic activity is increased in patients with COPD, and this appears to be the major reversible component of airway obstruction [4]. Therefore, treatment with anticholinergics, inhibiting muscarinic receptor activation, is an effective bronchodilator therapy in COPD.

Five muscarinic receptor subtypes are present in the human genome (M_1-M_5). The muscarinic M_1, M_2 and M_3 receptors are abundantly expressed in the lungs and have been extensively studied in the context of vagal neurotransmission. Their primary roles are bronchoconstriction and mucus secretion, which are mainly regulated via muscarinic M_3 receptors on airway smooth muscle and glands, respectively. Further, the muscarinic M_1 receptor facilitates neurotransmission in the parasympathetic ganglia and regulates electrolyte and water

secretion by mucus producing cells. The muscarinic M_2 receptor is an auto-inhibitory prejunctional receptor on vagal nerves inhibiting acetylcholine release, and an abundant postjunctional receptor on airway smooth muscle [5-7].

It is now known that acetylcholine can exert many additional, non-neuronal effects in the airways. Muscarinic receptors are expressed by almost all cell types in the lungs, including epithelial and inflammatory cells [8,9]. Strikingly, these cells express all necessary components to synthesize and release acetylcholine by themselves, including ChAT, the synthesizing enzyme of acetylcholine. This is referred to as non-neuronal acetylcholine and may contribute to airway inflammation [9-11]. Indeed, *in vitro* studies revealed a variety of effects of acetylcholine on these cell types [11], including an induced release of the potent neutrophil chemoattractants IL-8 and leukotriene B_4 (LTB_4) from airway epithelial, smooth muscle and inflammatory cells [12-15]. Recent evidence from *in vivo* studies also demonstrated a pro-inflammatory role for acetylcholine under pathophysiological conditions. In a cigarette smoke-induced mouse model of COPD, tiotropium partly prevented the increase in total cells and neutrophils in the bronchoalveolar lavage fluid (BALF). Furthermore, the release of various cytokines, including IL-6, keratinocyte-derived chemokine (KC, the mouse orthologue of IL-8), LTB_4 and MCP-1, was inhibited by tiotropium [16]. Our group recently demonstrated that LPS-induced neutrophilic inflammation could be completely prevented by tiotropium in a guinea pig model of COPD [17]. Similar findings indicating a pro-inflammatory role for acetylcholine have been observed in animal models of asthma, acute lung injury and fibrosis (see [6] for review).

Together, these studies clearly indicate a role for acetylcholine in inflammation, which may have implications for anticholinergic therapy in patients with COPD. Therapy with anticholinergics is presently focused on the muscarinic M_3 receptor, since this receptor subtype

mediates bronchoconstriction. Although *in vitro* studies suggest a role for the muscarinic M_3 receptor in cytokine release [12,18], no information is available with respect to the muscarinic receptor subtypes involved in the pro-inflammatory effects of acetylcholine *in vivo*. Therefore, the aim of this study was to investigate the role of the muscarinic M_1, M_2 and M_3 receptor subtypes in cigarette smoke-induced airway inflammation using muscarinic receptor subtype-deficient mice. We hypothesized that the muscarinic M_3 receptor plays a predominant pro-inflammatory role. In order to study this, we assessed inflammatory cell counts and mediator release in the lavage fluid. Furthermore, we analyzed the expression of genes associated with remodeling.

Methods

Animals

Homozygous, inbred, specific-pathogen-free breeding colonies of $M_1^{-/-}$, $M_2^{-/-}$ and $M_3^{-/-}$ mice and C57Bl/6NTac wild-type (WT) mice with the same genetic background were obtained from Taconic (Ry, Denmark). The $M_1^{-/-}$, $M_2^{-/-}$ and $M_3^{-/-}$ mice used were generated on a 129 Sv/J background and backcrossed for at least 10 generations onto the C57Bl/6NTac background [19-21]. Knock-out animals did not differ from WT controls in overall health, fertility and longevity [19-21], although the weight of $M_3^{-/-}$ mice was less compared to WT mice (Table E1). Exposure to cigarette smoke (CS) did not affect the weight of the mice (Table E1). Animals were housed conventionally under a 12-h light-dark cycle and received food and water ad libitum. All experiments were performed in accordance with the national guidelines and approved by the University of Groningen Committee for Animal Experimentation.

Animal model

Male mice (n=8-9 per group, 10 – 12 weeks old) were exposed to CS from Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, USA) on 4 consecutive days by whole body exposure. Each cigarette was smoked without a filter in 5 minutes at a rate of 5L/hr in a ratio with 60L/hr air using a peristaltic pump (45 rpm, Watson Marlow 323 E/D, Rotterdam, The Netherlands). CS was directly distributed into a 6-liter perspex box. On day 1, mice were exposed to the mainstream smoke of one cigarette in the morning and three cigarettes in the afternoon. On day 2 to 4, mice were exposed to five cigarettes in the morning and five in the afternoon (Figure 1). Control animals were handled in the same way but exposed to fresh air only. Because of the capacity of the experimental set-up, not all animals

used for this study could be included in a single experiment. Therefore, experiments were performed on 3 occasions (n=19-24 animals per experiment). Air and cigarette smoke exposed WT mice were included in every experiment to minimize variability. Sixteen hours after the last CS exposure, animals were euthanized by intraperitoneal pentobarbital injection (400 mg/kg, hospital pharmacy, University Medical Center Groningen), after which the lungs were immediately lavaged, resected and snap frozen in liquid nitrogen.

Muscarinic antagonist administration

In a sub-study, the muscarinic M_3–receptor selective antagonist 4-DAMP (1mg/kg) was administered to WT mice (n=7) by intraperitoneal injection, 30 minutes prior to each CS exposure. The same experimental protocol was used as described above.

Analysis of the bronchoalveolar lavage fluid

After euthanizing the mice, the lungs were gently lavaged through a tracheal cannula with 1 ml PBS containing 5% BSA and protease inhibitors (inhibiting chymotrypsin, thermolysin, papain, pronase, pancreatic extract and trypsin; F. Hoffman-La Roche, Basel, Switzerland) and another 4 times with 1 ml PBS. Cells were pelleted and the supernatants of the first fraction were stored at -20°C for measurement of cytokines and growth factors by ELISA. For each animal individually, BAL cells of the different fractions were combined, resuspended in 500 µl PBS, and total cell numbers were determined. For cytological examination, cytospin-preparations were stained with May–Grünwald and Giemsa (both Sigma, St. Louis) and a differential cell count was performed by counting at least 400 cells in duplicate in a blinded fashion. KC, MCP-1, IL-6, IL-1β, IL-17, TNF-α and VEGF release was determined in BALF supernatants by a MILLIPLEX assay

(Millipore, Billerica, USA). TGF-β in BALF was determined by an ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

ChAT staining

To identify ChAT expression, 5-µm-thick cryo-sections (n=4) were stained with a specific rabbit anti-ChAT-antibody provided by Prof. Kummer (Institute for Anatomy and Cell Biology, Justus-Liebig-University, Giessen, Germany). The antibody was visualized using a horseradish peroxidase-linked secondary antibody and diaminobenzidine (1 mg/ml). Airways within each section were digitally photographed.

Analysis of gene expression in lung tissue

Total RNA was extracted from lung tissue (right superior lobe) or BAL cells using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Lung homogenates were prepared by pulverizing the tissue under liquid nitrogen. Equal amounts of total mRNA were then reverse transcribed and cDNA was subjected to real-time qPCR (Westburg, Leusden, The Netherlands). Real time PCR was performed with denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 10 minutes at 72°C. Real-time PCR data were analyzed using the comparative cycle threshold (Ct: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA. Several other housekeeping genes, including β2-microglobulin and GAPDH, were tested for the influence of the experimental procedure on the expression. The expression of all housekeeping genes, including 18S, was stable in the tested conditions. The specific forward and reverse primers used are listed in Table E2.

Statistical analysis

Data are presented as mean ± s.e. of the mean. Statistical differences between means were calculated using one- or two-way ANOVA, followed by Newman Keuls multiple comparison tests. Differences were considered significant at $p<0.05$.

Results

Characterization of the mice

The genotypes of the knock-out mice were confirmed by PCR analysis of mouse ear DNA (Figure 2A, table E3). We also examined whether the deletion of one muscarinic receptor gene affected the expression levels of the two other muscarinic receptors expressed in the lung. As shown in Figure 2B, no such compensatory changes were observed. Muscarinic receptors were highly expressed in lung tissue homogenates, with $M_2R > M_3R > M_1R$ (Figure 2B).

The non-neuronal cholinergic system

It has been proposed that the non-neuronal cholinergic system (NNCS) might contribute to airway inflammation [11]. To investigate the effect of CS exposure, gene expression of different components of the cholinergic system was analyzed in lung tissue homogenates of WT mice, including the choline transporters CHT1 and CTL1, the acetylcholine synthesizing enzyme ChAT, the acetylcholine degrading enzyme AChE and the different muscarinic receptor subtypes. As depicted in Figure 3A, these components were expressed in the murine lungs and CS exposure did not affect their expression level. Similarly, the muscarinic receptor expression in the BAL cells from WT mice was not altered after CS exposure (Figure 3B). In lung tissue, muscarinic M_2 receptors were expressed at the highest levels ($M_2R > M_3R > M_1R$), whereas in the cells from the lavage fluid muscarinic M_3 receptors were expressed at the highest levels ($M_3R > M_1R > M_2R$; figure E1). ChAT expression, detected by immunohistochemical staining, was localized to the epithelium and smooth muscle layer of the airway wall in WT mice (Figure 3C). Exposure to CS did not alter the expression or localization of ChAT (Figure 3C).

Cigarette smoke-induced inflammatory cell recruitment

To study the contribution of muscarinic receptor subtypes to CS-induced airway inflammation, cell counts were determined in the BALF of WT, $M_1^{-/-}$, $M_2^{-/-}$ and $M_3^{-/-}$ mice. All mice exposed to CS had a 2-fold increase in the number of inflammatory cells compared to air-exposed control animals (Figure 4A). The predominant cell type after CS exposure was the macrophage, which almost doubled in number in all strains (Figure 4B). Only small increases in lymphocytic infiltration were observed after CS exposure, which were comparable in all strains (Figure 4C). Neutrophilic infiltration however, showed significant differences among the strains (Figure 4D). A 4-fold increase in the amount of neutrophils was observed in WT mice compared to air exposed animals. In $M_1^{-/-}$ and $M_2^{-/-}$ mice however, the increase in neutrophil number upon CS exposure was much higher, up to a 24-fold in $M_1^{-/-}$ mice. In striking contrast, no significant increase in neutrophil numbers was observed in $M_3^{-/-}$ mice after CS exposure compared to air-exposed control animals (Figure 4D).

Cigarette smoke-induced cytokine release

Subsequently, inflammatory cytokine release in BALF was determined. Levels of KC (the mouse orthologue of IL-8) in BALF of WT mice exposed to CS were 15-fold higher compared to air-exposed animals (Figure 5A). No significant differences in MCP-1 and IL-6 release were observed in CS-exposed WT mice (Figure 5B and C). In $M_1^{-/-}$ mice, CS-exposed mice had higher levels of KC, MCP-1 and IL-6 compared to air-exposed mice. Interestingly, the concentration of all these cytokines was significantly higher when compared to WT CS-exposed mice. In CS-exposed $M_2^{-/-}$ mice, KC release was also significantly higher, both compared to air-exposed mice and to WT CS-exposed mice, whereas MCP-1 and IL-6 release were not significantly different. In $M_3^{-/-}$ mice, no increase in the release of any of these cytokines was observed after CS exposure, and KC release was significantly lower compared to WT CS-

exposed mice (Figure 5). Levels of IL-17, IL-1β and TNF-α were below detection limit in all strains (not shown).

Cigarette smoke-induced growth factor and extracellular matrix expression

We next determined the release of the growth factors TGF-β1 and VEGF in the BALF. Small increases in TGF-β1 protein release were observed in all strains after CS-exposure (1.4 – 2.0-fold), which was significant in WT mice (Figure 6A). Remarkably, there was significantly less TGF-β1 in the BALF of $M_3^{-/-}$ mice compared to WT mice, irrespective of air or CS exposure (56% and 46% lower, respectively). VEGF release was not altered (Figure 6A). Expression of TGF-β1 at the mRNA level in lung tissue of $M_3^{-/-}$ mice was reduced to a similar extent compared to protein levels (Figure 6B). At the transcriptional level, TGF-β1 expression was significantly increased by 1.4 fold in $M_1^{-/-}$ mice and by 1.7 fold in $M_2^{-/-}$ mice after CS exposure (Figure 6B).

In addition, we analyzed the gene expression of the matrix proteins collagen Iα1 and fibronectin in lung tissue. No increased expression after CS exposure was observed (Figure 6C), with the exception of collagen Iα1 in $M_2^{-/-}$ mice, which was increased after CS exposure. Furthermore, expression levels of collagen Iα1 and fibronectin were higher in CS-exposed $M_2^{-/-}$ mice compared to CS-exposed WT mice. In line with the findings on TGF-β1, both matrix proteins were expressed at a significantly lower level in $M_3^{-/-}$ mice compared to WT mice, irrespective of air or CS exposure (68 and 65% lower, respectively). This was confirmed at the protein level for fibronectin, which was 38.6 ± 8.9% lower in $M_3^{-/-}$ mice than in WT mice ($p<0.05$). Finally, CS exposure had no significant effect on MUC5AC gene expression in any of the analyzed strains (Figure 6D).

Inhibition of CS-induced inflammation by a muscarinic M_3 antagonist

To investigate whether the pro-inflammatory role of the muscarinic M_3 receptor can also be observed using a pharmacological intervention, WT mice were pretreated with the muscarinic M_3–receptor selective antagonist 4-DAMP 30 minutes prior to every CS exposure. This resulted in an inhibition of inflammatory cell number in the BALF by 30% compared to untreated animals (Figure 7A). Similar inhibitory effects of pretreatment with 4-DAMP were observed on macrophage accumulation (Figure 7B), whereas CS-induced lymphocytic and neutrophilic inflammation were completely prevented (Figures 7C and D). This was accompanied by the absence of CS-induced KC release after pretreatment with 4-DAMP (Figure 7E).

Discussion

In this study we demonstrate that the muscarinic M_3 receptor plays a profound pro-inflammatory role in CS-induced inflammation and that this is the primary muscarinic receptor subtype involved in the pro-inflammatory effects of acetylcholine. Inhibition of the muscarinic M_3 receptor, by total knock-out of the receptor or by a pharmacological approach, prevented neutrophilic inflammation and cytokine release in the lavage fluid of CS-exposed mice. In striking contrast, knock-out of the muscarinic M_1 and M_2 receptors resulted in increased neutrophils and cytokine release in the BALF, indicating an anti-inflammatory role of these receptor subtypes in CS-induced inflammation. This study is the first to demonstrate the differential regulation of inflammation by muscarinic receptors *in vivo* and implies an anti-inflammatory role for muscarinic M_3 selective anticholinergics.

Neutrophils are considered as one of the major cell types involved in COPD [2].Various studies suggest an important role for acetylcholine in regulating neutrophilic inflammation. Activation of muscarinic receptors can contribute to neutrophil influx by inducing neutrophil chemotactic activity from macrophages [22,23] and LTB4 release from sputum cells of COPD patients [15]. Furthermore, IL-8 is released from epithelial and airway smooth muscle cells in response to muscarinic receptor stimulation [12,14]. These findings are supported by *in vivo* studies in which CS- and LPS-induced neutrophilia was inhibited by the muscarinic receptor antagonist tiotropium [16,17].

The regulatory effects of muscarinic receptors appeared to be specific for neutrophils in the muscarinic receptor deficient mice, whereas macrophages and lymphocytes were not altered. Our results on neutrophilic inflammation are in line with various *in vitro* studies demonstrating

that the pro-inflammatory effects of muscarinic receptor activation are mainly dependent on the muscarinic M_3 receptor subtype. Thus, it has been shown that 4-DAMP and DAU5884, muscarinic M_3 receptor selective antagonists, inhibited methacholine and CS-induced IL-8 release from airway smooth muscle cells [12]. In addition, alveolar macrophage mediated migration of neutrophils from COPD patients was inhibited by 4-DAMP [18]. Moreover, the muscarinic M_3 receptor was the primary receptor subtype expressed by inflammatory cells within the BALF as shown in our study. It is also known that inflammatory cells express muscarinic M_3 receptors and that this receptor mediates pro-inflammatory effects [8]. We therefore believe that the pro-inflammatory effect of the muscarinic M_3 receptor as found in our study is dependent on regulation of cytokine release by structural cells, in combination with direct activation of muscarinic M_3 receptors on inflammatory cells.

In contrast to the findings in $M_3^{-/-}$ mice, neutrophilic inflammation was increased in $M_2^{-/-}$ mice compared to WT mice. The muscarinic M_2 receptor is located prejunctionally on pre- and postganglionic nerves and acts as an inhibitory autoreceptor limiting acetylcholine release [5]. Furthermore, the muscarinic M_2 receptor is expressed postjunctionally by smooth muscle cells and fibroblasts [6,24]. With acetylcholine acting as a pro-inflammatory mediator inducing chemokine release from structural and inflammatory cells via the M_3 receptor, increased levels of acetylcholine in the $M_2^{-/-}$ mice, due to loss of its autoinhibitory role, may therefore explain the observed aggravated neutrophilia. In support of such a role, M_2 receptor expression appeared low on inflammatory cells in the BALF, but high in lung tissue, suggesting that the effects of M_2 receptor deficiency are not due to direct effects on inflammatory cells. The literature supports this notion, indicating that the pro-inflammatory effects of acetylcholine in macrophages, epithelial cells and airway smooth muscle cells are not mediated by M_2 receptors [12,23,25].

A role for muscarinic M_2 receptors as prejunctional autoreceptors driving exaggerated acetylcholine release and inflammation has significant implications. Acetylcholine has long been known as a classical neurotransmitter. More recent findings suggest that acetylcholine can also be released from non-neuronal origins, including epithelial, airway smooth muscle and inflammatory cells [9,10]. It is not yet known to which extent this non-neuronal acetylcholine affects airway inflammation [11]. The release of non-neuronal acetylcholine is not known to be affected by muscarinic M_2 receptors in an auto-inhibitory way. Our data therefore imply an important role for neuronal acetylcholine in CS-induced inflammation. Further, we did not find any upregulation of expression of components of the NNCS in the lungs of CS-exposed mice, in contrast to the previously reported increase in cultured human airway epithelial cells after exposure to CS extract [13]. Future studies investigating the contribution of neuronal and non-neuronal acetylcholine to inflammation are clearly warranted.

Surprisingly, neutrophilic inflammation was also enhanced in $M_1^{-/-}$ mice. It is well known that muscarinic M_1 receptors facilitate neurotransmission in the parasympathetic ganglia [5]. Based on this however, one would expect that muscarinic M_1 receptor deficiency causes reduced acetylcholine release leading to inhibition of inflammation. Reinheimer et al. reported that the muscarinic M_1-receptor selective antagonist pirenzepine can antagonize the inhibitory effect of acetylcholine on histamine release from human mast cells [26]. Lack of this inhibitory muscarinic M_1 receptor might explain the increased neutrophil chemotaxis, since mast cell numbers are increased upon smoking and higher in patients with COPD [27,28]. Alternatively, as the muscarinic M_1 receptor also controls electrolyte and water secretion by airway epithelial cells [29], lack of muscarinic M_1 receptor expression may result in a reduced ability of $M_1^{-/-}$ mice to clear their lungs of smoke particles after CS exposure, leading to aggravated inflammatory and

injury responses. The sharp induction of the damage response-associated cytokines IL-6 and MCP-1 in $M_1^{-/-}$ mice, which is absent in WT mice, supports this hypothesis.

The results of this study on transgenic mice suggest a primary role for the muscarinic M_3 receptor in regulating CS-induced inflammation, implying that muscarinic M_3 subtype selectivity of anticholinergics would be beneficial. Indeed, we show that pharmacological inhibition of the muscarinic M_3 receptor using 4-DAMP partly prevented accumulation of inflammatory cells in BALF, accompanied by a strong inhibition of CS-induced KC release. 4-DAMP is selective for muscarinic M_3 receptors over muscarinic M_2 (~16-fold) [7] and to a lesser extent muscarinic M_1 receptors (~3-fold) [30]. Interestingly, the anti-inflammatory effects of pharmacological inhibition of the muscarinic M_3 receptor are more pronounced than effects of knock-out of this receptor. Similar discrepant data are reported on muscarinic receptor mediated contraction *ex vivo*, which can be fully inhibited with a muscarinic M_3-receptor selective antagonist in wild-type mice, whereas only partial inhibition of contraction is observed in muscarinic M_3 knock-out mice [31,32]. Although the mechanism behind this discrepancy is unclear it thus appears that compensating mechanisms are operative in the knock-out mice that limit the impact of the muscarinic M_3 receptor deficiency.

Although tiotropium is known to be kinetically selective for the muscarinic M_3 receptor (dissociation half-life = 27h), it still has a dissociation half-life from the muscarinic M_1 receptor of 10.5 hours [33]. Steady-state binding affinity of tiotropium for the muscarinic M_1, M_2 and M_3 receptors is not different [34]. The half-life ratio of ipratropium and of aclidinium and glycopyrrolate, two anticholinergics under development, for the muscarinic M_3 versus M_1 receptor is comparable to tiotropium [33]. Our study suggests that an even more selective

compound solely inhibiting muscarinic M_3 receptors is desirable and may lead to improved effects on CS-induced inflammation.

Interestingly, in our study basal expression of TGF-β1, collagen Iα1 and fibronectin was significantly lower in $M_3^{-/-}$ mice compared to WT mice. In $M_2^{-/-}$ mice, expression of these components was increased after CS exposure. This suggests that in addition to inflammation, acetylcholine regulates important aspects of lung structure via the muscarinic M_3 receptor. Indeed, muscarinic M_3 receptors are expressed by structural cells in the airways, including epithelial cells and airway smooth muscle cells [24]. Moreover, *in vitro* studies have demonstrated a role for the muscarinic M_3 receptor in the regulation of airway smooth muscle proliferation [35] and *in vivo* studies have shown a protective effect of anticholinergics on matrix protein deposition [17,36]. The exact roles of the individual muscarinic receptor subtypes in this process are not yet clear and remain to be elucidated [6].

Evidence for the pro-inflammatory role of acetylcholine from *in vitro* and *in vivo* studies is increasing [6]. However, the translational utility of these observations is not yet clear, since in patients with COPD, effects on inflammation or on the rate of decline in lung function after anticholinergic therapy have not been demonstrated. It is known from the UPLIFT (Understanding Potential Long-Term Impacts on Function with Tiotropium) study that the use of tiotropium is associated with a reduction in the number of exacerbations, generally seen as inflammatory events [37]. Patients who have more exacerbations demonstrate increased levels of inflammatory markers at stable state [38,39]. In two recent studies however, no direct evidence for an anti-inflammatory effect was found, since IL-6 and IL-8 levels in the sputum of patients with COPD were not decreased after anticholinergic therapy [40,41]. However, both studies have substantial limitations as discussed by the authors. In the study of Perng et al., the treatment

group was small and patients were only treated with tiotropium for 12 weeks [41]. In the study of Powrie et al., the amount of sputum was reduced after tiotropium treatment, which might have resulted in increased cytokine concentrations [40]. Future studies using different methods to assess inflammation could therefore resolve the question whether anticholinergics indeed have anti-inflammatory properties in patients with COPD. At present, there is no evidence for such a role.

In conclusion, the results of our study demonstrate that inhibition of the muscarinic M_3 receptor prevents inflammation in response to CS-exposure in mice. This confirms the previously established pro-inflammatory role of acetylcholine in the pathophysiology of airway diseases, and demonstrates that this is solely mediated via muscarinic M_3 receptors, since knock-out of the muscarinic M_1 and M_2 receptor aggravated inflammation compared to WT mice. This study therefore opens new perspectives on muscarinic M_3 receptor selective anticholinergics to specifically target airway inflammation.

Acknowledgements

We would like to thank the Netherlands Asthma Foundation for financial support (Grant: 3.2.08.014).

References

1. Barnes PJ. Chronic obstructive pulmonary disease. *N Engl J Med* 2000; 343: 269-280.

2. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; 8: 183-192.

3. Postma DS, Timens W. Remodeling in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006; 3: 434-439.

4. Gross NJ, Skorodin MS. Role of the parasympathetic system in airway obstruction due to emphysema. *N Engl J Med* 1984; 311: 421-425.

5. Lee AM, Jacoby DB, Fryer AD. Selective muscarinic receptor antagonists for airway diseases. *Curr Opin Pharmacol* 2001; 1: 223-229.

6. Kistemaker LE, Oenema TA, Meurs H, Gosens R. Regulation of airway inflammation and remodeling by muscarinic receptors: Perspectives on anticholinergic therapy in asthma and COPD. *Life Sci* 2012.

7. Roffel AF, Elzinga CR, Van Amsterdam RG, De Zeeuw RA, Zaagsma J. Muscarinic M2 receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur J Pharmacol* 1988; 153: 73-82.

8. Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir.Res.* 2006; 7: 73.

9. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *Br J Pharmacol* 2008; 154: 1558-1571.

10. Kummer W, Lips KS, Pfeil U. The epithelial cholinergic system of the airways. *Histochem Cell Biol* 2008; 130: 219-234.

11. Gwilt CR, Donnelly LE, Rogers DF. The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? *Pharmacol Ther* 2007; 115: 208-222.

12. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J, Kolahian S, Halayko AJ, Hiemstra PS, Zuyderduyn S. Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. *Eur Respir J* 2009; 34: 1436-1443.

13. Profita M, Bonanno A, Montalbano AM, Ferraro M, Siena L, Bruno A, Girbino S, Albano GD, Casarosa P, Pieper MP, Gjomarkaj M. Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro. *Life Sci* 2011; 89: 36-43.

14. Profita M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Pieper MP, Gjomarkaj M. Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism. *Eur J Pharmacol* 2008; 582: 145-153.

15. Profita M, Giorgi RD, Sala A, Bonanno A, Riccobono L, Mirabella F, Gjomarkaj M, Bonsignore G, Bousquet J, Vignola AM. Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. *Allergy* 2005; 60: 1361-1369.

16. Wollin L, Pieper MP. Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. *Pulm Pharmacol Ther* 2010; 23: 345-354.

17. Pera T, Zuidhof A, Valadas J, Smit M, Schoemaker RG, Gosens R, Maarsingh H, Zaagsma J, Meurs H. Tiotropium inhibits pulmonary inflammation and remodelling in a guinea pig model of COPD. *Eur Respir J* 2011.

18. Vacca G, Randerath WJ, Gillissen A. Inhibition of granulocyte migration by tiotropium bromide. *Respir Res* 2011; 12: 24.

19. Fisahn A, Yamada M, Duttaroy A, Gan JW, Deng CX, McBain CJ, Wess J. Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. *Neuron* 2002; 33: 615-624.

20. Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H, Wess J. Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci U S A* 1999; 96: 1692-1697.

21. Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, Felder CC, Deng CX, Wess J. Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature* 2001; 410: 207-212.

22. Buhling F, Lieder N, Kuhlmann UC, Waldburg N, Welte T. Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro. *Respir Med* 2007; 101: 2386-2394.

23. Sato E, Koyama S, Okubo Y, Kubo K, Sekiguchi M. Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. *Am J Physiol* 1998; 274: L970-L979.

24. Meurs H, Dekkers BG, Maarsingh H, Halayko AJ, Zaagsma J, Gosens R. Muscarinic receptors on airway mesenchymal cells: Novel findings for an ancient target. *Pulm Pharmacol Ther* 2012.

25. Koyama S, Rennard SI, Robbins RA. Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *Am J Physiol* 1992; 262: L466-L471.

26. Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, Wessler I. Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. *Am J Respir Crit Care Med* 2000; 162: 534-538.

27. Lamb D, Lumsden A. Intra-epithelial mast cells in human airway epithelium: evidence for smoking-induced changes in their frequency. *Thorax* 1982; 37: 334-342.

28. Jeffery PK. Structural and inflammatory changes in COPD: a comparison with asthma. *Thorax* 1998; 53: 129-136.

29. Ishihara H, Shimura S, Satoh M, Masuda T, Nonaka H, Kase H, Sasaki T, Sasaki H, Takishima T, Tamura K. Muscarinic receptor subtypes in feline tracheal submucosal gland secretion. *Am J Physiol* 1992; 262: L223-8.

30. Boddeke HW, Buttini M. Pharmacological properties of cloned muscarinic receptors expressed in A9 L cells; comparison with in vitro models. *Eur J Pharmacol* 1991; 202: 151-157.

31. Schlenz H, Kummer W, Jositsch G, Wess J, Krasteva G. Muscarinic receptor-mediated bronchoconstriction is coupled to caveolae in murine airways. *Am J Physiol Lung Cell Mol Physiol* 2010; 298: L626-36.

32. Garssen J, Van Loveren H, Gierveld CM, Van der Vliet H, Nijkamp FP. Functional characterization of muscarinic receptors in murine airways. *Br J Pharmacol* 1993; 109: 53-60.

33. Casarosa P, Bouyssou T, Germeyer S, Schnapp A, Gantner F, Pieper M. Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. *J Pharmacol Exp Ther* 2009; 330: 660-668.

34. Barnes PJ. The pharmacological properties of tiotropium. *Chest* 2000; 117: 63S-6S.

35. Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H. Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol* 2003; 28: 257-262.

36. Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J* 2007; 30: 653-661.

37. Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, Decramer M, UPLIFT Study Investigators. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med* 2008; 359: 1543-1554.

38. Liesker JJ, Bathoorn E, Postma DS, Vonk JM, Timens W, Kerstjens HA. Sputum inflammation predicts exacerbations after cessation of inhaled corticosteroids in COPD. *Respir Med* 2011; 105: 1853-1860.

39. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* 2000; 55: 114-120.

40. Powrie DJ, Wilkinson TM, Donaldson GC, Jones P, Scrine K, Viel K, Kesten S, Wedzicha JA. Effect of tiotropium on sputum and serum inflammatory markers and exacerbations in COPD. *Eur Respir J* 2007; 30: 472-478.

41. Perng DW, Tao CW, Su KC, Tsai CC, Liu LY, Lee YC. Anti-inflammatory effects of salmeterol/fluticasone, tiotropium/fluticasone or tiotropium in COPD. *Eur Respir J* 2009; 33: 778-784.

Figure legends

Figure 1. Experimental procedure. Male C57Bl/6NTac mice were exposed to cigarette smoke twice daily on 4 consecutive days by whole body exposure. Sixteen hours after the last smoke exposure a bronchoalveolar lavage is performed and lungs are harvested for lung tissue homogenates and cryo-sections.

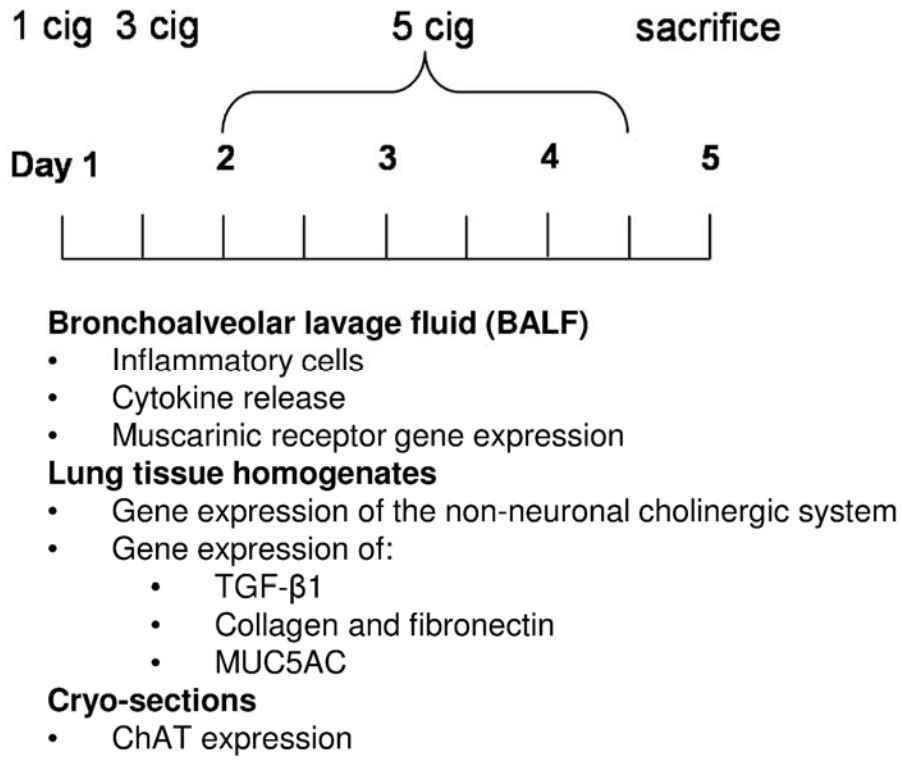

Figure 2. Characterization of the mice. A) SYBR Safe-stained agarose gel showing the PCR products for genotyping, including the WT, muscarinic $M_1^{-/-}$, $M_2^{-/-}$ and $M_3^{-/-}$ band for $M_1^{-/-}$, $M_2^{-/-}$ and $M_3^{-/-}$ mice, respectively. B) Gene expression of muscarinic receptors in lung tissue homogenates depicted as Ct values corrected for 18S (n=5 mice per group), n.a.: not applicable.

Note that data are expressed as Ct values, thus lower values mean higher expression levels and every unit lower on the y-axis represents a two-fold increase in expression.

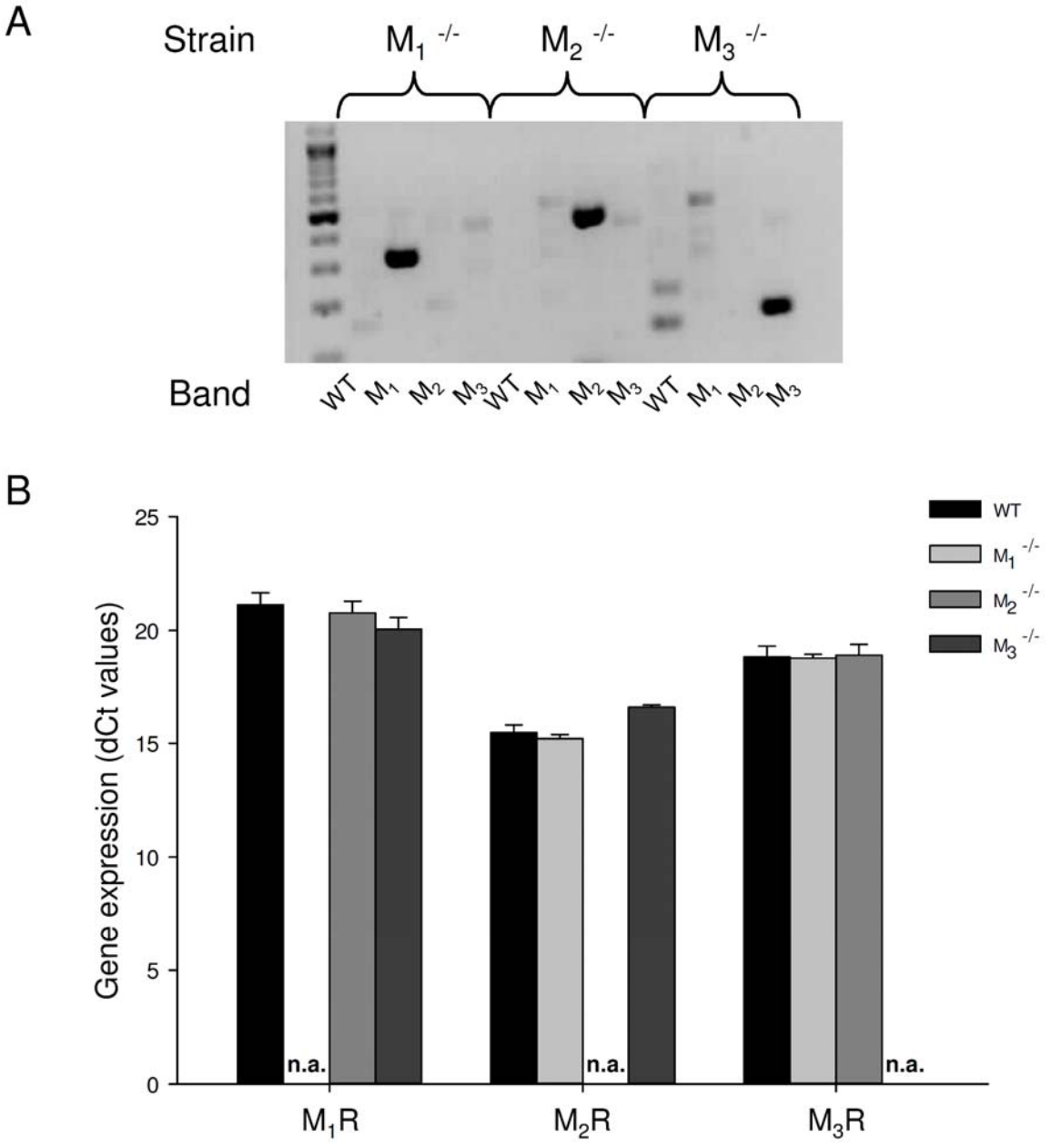

Figure 3. Effect of cigarette smoke exposure on the expression of the non-neuronal cholinergic system. Mice were treated as described in Figure 1. Sixteen hours after the last smoke exposure

lung tissue and bronchoalveolar lavage fluid (BALF) were harvested. Gene expression in lung tissue homogenates (A) and in BAL cells (B) was analyzed (n=3-5 mice per group). Ct values corrected for 18S are depicted, expressed as mean ± s.e. of the mean. Note that data are expressed as Ct values, thus lower values mean higher expression levels and every unit lower on the y-axis represents a two-fold increase in expression. High-affinity choline transporter-1 (CHT1), choline transporter like protein-1 (CTL1), choline acetyl transferase (ChAT), acetylcholine-esterase (AChE), muscarinic M_1 receptor (M1R), M_2 receptor (M2R) and M_3 receptor (M3R). (C) Cryo-sections of WT mice exposed to air and smoke stained for ChAT. A representative picture of n=4 animals is shown. Photographs were taken at 200x and 400x magnification. Lu: airway lumen, ep: epithelium, asm: airway smooth muscle.

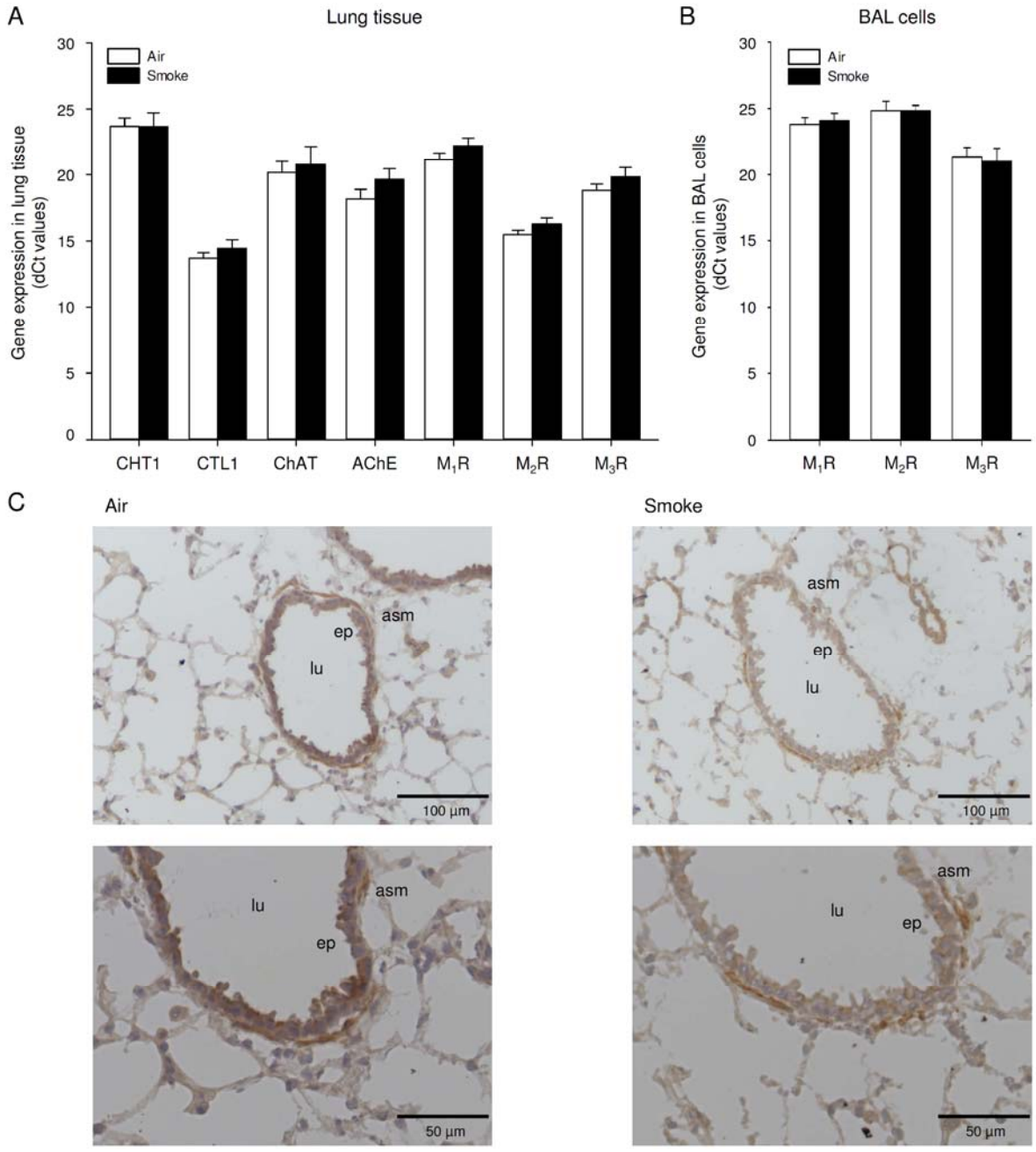

Figure 4. Inflammatory cell counts after cigarette smoke exposure. Mice were treated as described in Figure 1. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed and total cells (A), macrophages (B), lymphocytes (C) and neutrophils (D) were determined in the BALF. Results are expressed as mean ± s.e. of the mean. Data were analyzed

using two-way ANOVA; total cells: $F[1,56]=89.25$, $p<0.001$ for sham vs. smoke treatment. $F[3,56]=1.233$, $p=0.307$ for the muscarinic receptor subtypes. $F[3,56]=0.0493$, $p=0.985$ for the interaction. Macrophages: $F[1,56]=78.60$, $p<0.001$ for sham vs. smoke treatment. $F[3,56]=1.93$, $p=0.135$ for the muscarinic receptor subtypes. $F[3,56]=0.27$, $p=0.849$ for the interaction. Lymphocytes: $F[1,56]=12.70$, $p<0.001$ for sham vs. smoke treatment. $F[3,56]=0.587$, $p=0.587$ for the muscarinic receptor subtypes. $F[3,56]=0.05$, $p=0.984$ for the interaction. Neutrophils: $F[1,56]=17.65$, $p<0.001$ for sham vs. smoke treatment. $F[3,56]=6.37$, $p<0.001$ for the muscarinic receptor subtypes. $F[3,56]=5.90$, $p=0.001$ for the interaction. Individual comparisons were made using a Student-Newman-Keuls multiple comparisons post-hoc test; * $p < 0.05$; *** $p < 0.001$ compared to air exposed control mice, # $p < 0.05$ compared to WT CS-exposed mice, n=8-9 mice per group.

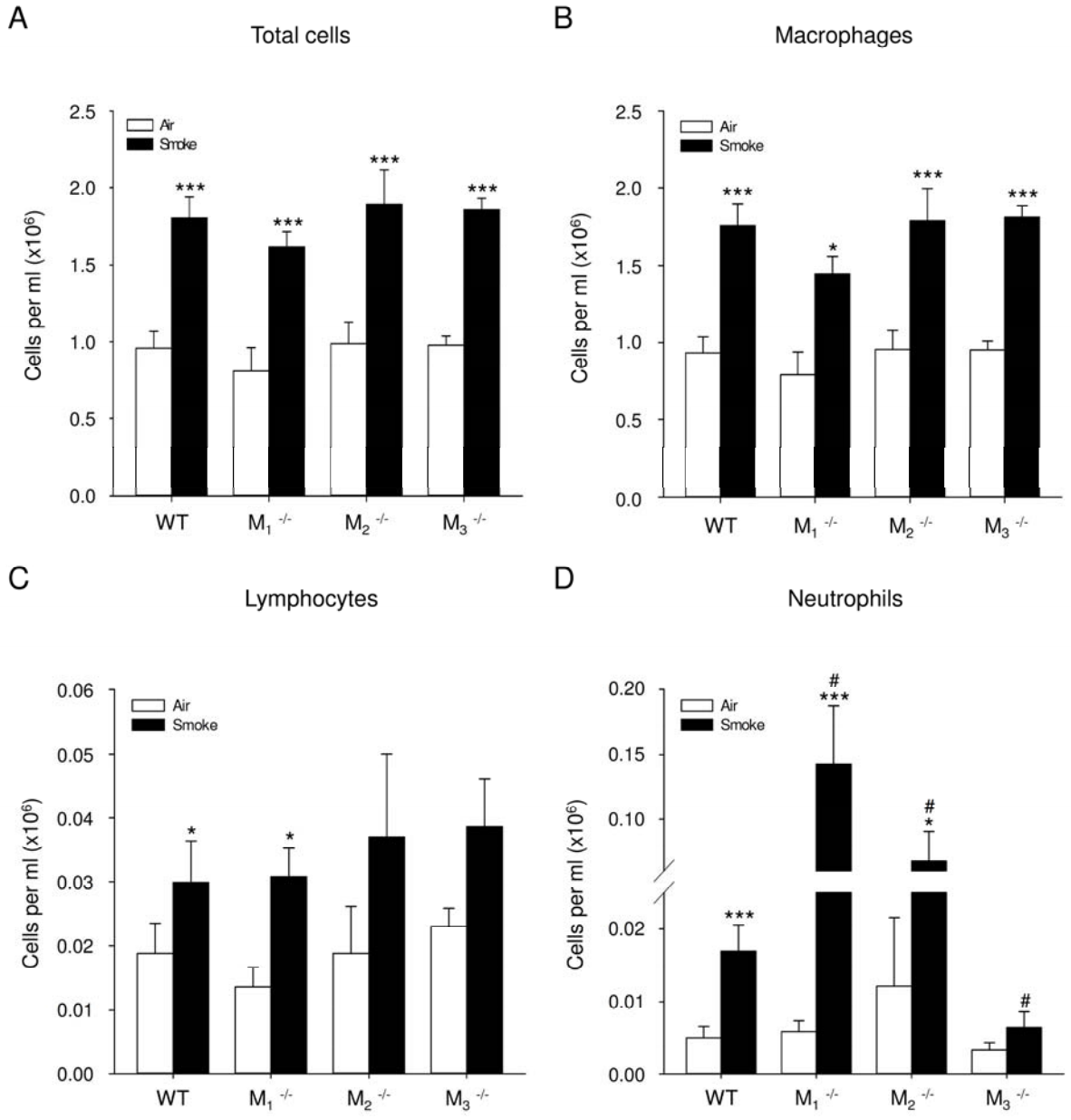

Figure 5. Inflammatory cytokine release after cigarette smoke exposure. Mice were treated as described in Figure 1. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed. Release of keratinocyte-derived chemokine (KC; A), monocyte chemotactic protein-1 (MCP-1; B) and interleukin-6 (IL-6; C) in BALF was determined. Results are expressed as mean ± s.e. of the mean. N.d.: not detectable. Data were analyzed using two-way ANOVA; KC:

F[1,56]=32.85, p<0.001 for sham vs. smoke treatment. F[3,56]=4.40, p=0.008 for the muscarinic receptor subtypes. F[3,56]=3.61, p=0.019 for the interaction. MCP-1: F[1,56]=13.91, p<0.001 for sham vs. smoke treatment. F[3,56]=7.49, p<0.001 for the muscarinic receptor subtypes. F[3,56]=5.89, p=0.001 for the interaction. IL-6: F[1,56]=9.32, p=0.003 for sham vs. smoke treatment. F[3,56]=8.69, p<0.001 for the muscarinic receptor subtypes. F[3,56]=5.63, p=0.002 for the interaction. Individual comparisons were made using a Student-Newman-Keuls multiple comparisons post-hoc test; * p < 0.05; *** p < 0.001 compared to air exposed control mice, # p < 0.05; ### p < 0.001 compared to WT CS-exposed mice, n=8-9 mice per group.

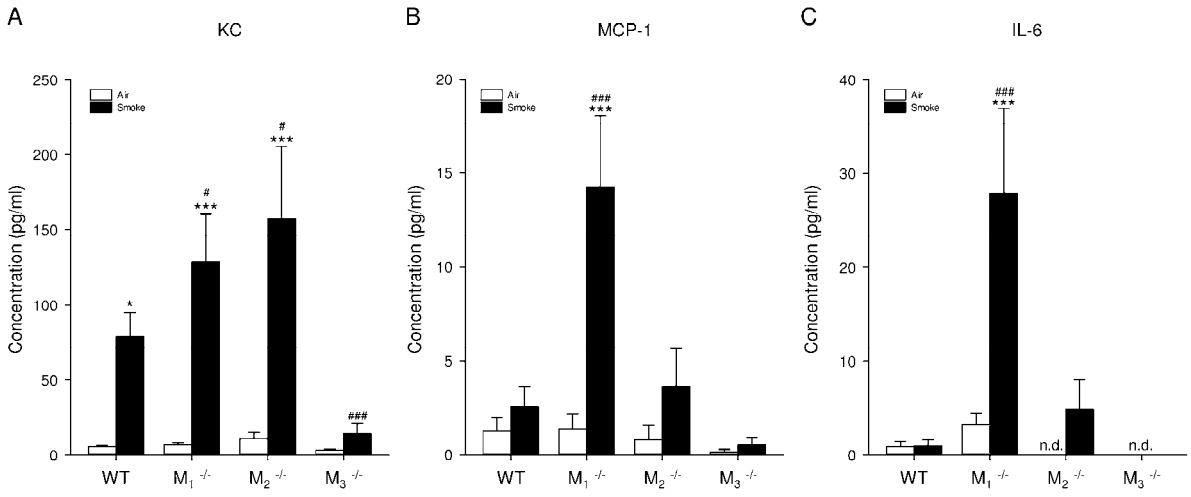

Figure 6. Parameters of remodeling after cigarette smoke exposure. Mice were treated as described in Figure 1. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed and lungs were collected. Release of transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) in BALF was determined (A). In lung tissue homogenates, gene expression of TGF-β1 (B), collagen Iα1 and fibronectin (C) and MUC5AC (D) was determined. Results are expressed as mean ± s.e. of the mean. Data were analyzed using two-way ANOVA; TGF-β1 protein: F[1,56]=4.72, p=0.034 for sham vs. smoke treatment.

F[3,56]=3.10, p=0.034 for the muscarinic receptor subtypes. F[3,56]=0.18, p=0.908 for the interaction. VEGF protein: F[1,56]=2.15, p=0.148 for sham vs. smoke treatment. F[3,56]=3.84, p=0.014 for the muscarinic receptor subtypes. F[3,56]=1.13, p=0.347 for the interaction. TGF-β1 mRNA: F[1,56]=3.16, p=0.081 for sham vs. smoke treatment. F[3,56]=18.38, p<0.001 for the muscarinic receptor subtypes. F[3,56]=5.66, p=0.002 for the interaction. Collagen Iα1: F[1,56]=6.91, p=0.011 for sham vs. smoke treatment. F[3,56]=43.02, p<0.001 for the muscarinic receptor subtypes. F[3,56]=5.97, p=0.001 for the interaction. Fibronectin: F[1,56]=0.03, p=0.862 for sham vs. smoke treatment. F[3,56]=36.10, p<0.001 for the muscarinic receptor subtypes. F[3,56]=1.37, p=0.262 for the interaction. MUC5AC: F[1,56]=$4.95*10^{-4}$, p=0.982 for sham vs. smoke treatment. F[3,56]=3.19, p=0.030 for the muscarinic receptor subtypes. F[3,56]=1.04, p=0.381 for the interaction. Individual comparisons were made using a Student-Newman-Keuls multiple comparisons post-hoc test; * $p < 0.05$; *** $p < 0.001$ compared to air exposed control mice, # $p < 0.05$; ### $p < 0.001$ compared to WT control mice, n=8-9 mice per group. Statistics were performed on log-transformed data.

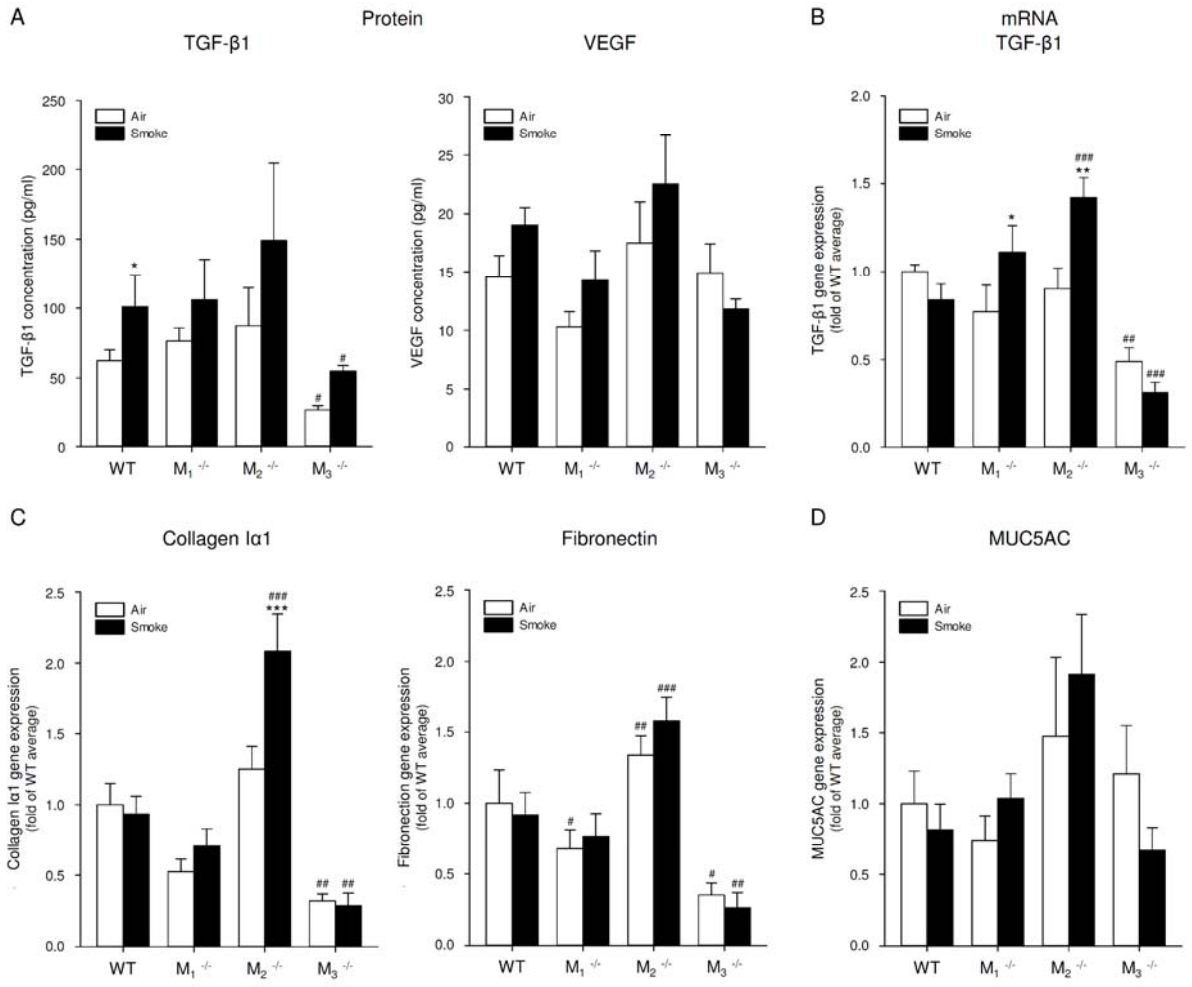

Figure 7. Cigarette smoke-induced inflammation after pretreatment with 4-DAMP. The same experimental model was used as described in Figure 1; however prior to each smoke exposure mice were treated with the selective muscarinic M_3 antagonist 4-DAMP. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed and total cells (A), macrophages (B), lymphocytes (C), neutrophils (D) and KC release (E) were determined in the BALF. Results are expressed as mean ± s.e. of the mean. Data were analyzed using one-way ANOVA; total cells: $F[2,30]=12.98$, $p<0.001$ between groups. Macrophages: $F[2,30]=11.92$, $p<0.001$ between groups. Lymphocytes: $F[2,30]=5.87$, $p=0.007$ between groups. Neutrophils: $F[2,30]=8.23$, $p=0.001$ between groups. KC: $F[2,30]=9.53$, $p<0.001$ between groups. Individual comparisons

were made using a Student-Newman-Keuls multiple comparisons post-hoc test; * $p < 0.05$; *** $p < 0.001$ compared to air exposed control mice, # $p < 0.05$ compared to CS-exposed mice, n=7-13 mice per group.