CHAPTER 6

MUSCARINIC M₃ RECEPTORS CONTRIBUTE TO ALLERGEN-INDUCED AIRWAY REMODELING IN MICE

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Abstract

Rationale
Asthma is a chronic obstructive airway disease, characterized by inflammation and remodeling. Acetylcholine contributes to symptoms by inducing bronchoconstriction via the muscarinic M3 receptor. Recent evidence suggests that bronchoconstriction can regulate airway remodeling and therefore implies a role for the M3 receptor. In this study, the contribution of the M3 receptor to allergen-induced remodeling was investigated using M3 receptor subtype deficient (M3R-/-) mice. Wild-type, M1R-/- and M2R-/- mice were used as controls.

Methods
C57Bl/6 mice were sensitized and challenged with ovalbumin (twice weekly, for 4 weeks). Control animals were challenged with saline.

Results
Allergen exposure induced goblet cell metaplasia, airway smooth muscle thickening (1.7-fold), pulmonary vascular smooth muscle remodeling (1.5-fold) and deposition of collagen type I (1.7-fold) and fibronectin (1.6-fold) in the airway wall of wild-type mice. These effects were absent or markedly lower in M3R-/- mice (30-100%), whereas M1R-/- and M2R-/- mice responded similar to wild-type mice. In addition, airway smooth muscle and pulmonary vascular smooth muscle mass were 35-40% lower in saline-challenged M3R-/- mice compared to wild-type mice. Interestingly, allergen-induced airway inflammation, assessed as infiltrated eosinophils and Th2-cytokine expression, was similar or even enhanced in M3R-/- mice.

Conclusions
Our data indicate that acetylcholine contributes to allergen-induced remodeling and smooth muscle mass via the M3 receptor, and not via M1 or M2 receptors. No stimulatory role for M3 receptors in allergic inflammation was observed, suggesting that the role of acetylcholine in remodeling is independent of the allergic inflammatory response and may involve bronchoconstriction.

Introduction
Asthma is an obstructive lung disease, characterized by airway inflammation and remodeling. In the inflammatory process eosinophils, mast cells and T cells play an important role. Inflammation is mainly regulated by the T-helper type 2 (Th2) cytokines interleukin (IL)-4, IL-5 and IL-13 (1). The inflammatory response may contribute to remodeling of the airways, inducing structural changes such as goblet cell metaplasia, airway smooth muscle thickening and subepithelial fibrosis (2). Furthermore, pulmonary
vascular remodeling and increased angiogenesis are observed, associated with higher vascular endothelial growth factor (VEGF) release (2, 3).

The parasympathetic neurotransmitter acetylcholine induces bronchoconstriction via M3 receptor stimulation and can thereby contribute to symptoms and loss of lung function in asthma (chapter 2). Parasympathetic nerve activity is increased in patients with asthma and the airways are hyperresponsive to cholinergic stimuli (4, 5). Indeed, a recent randomized controlled trial demonstrated that the long-acting anticholinergic tiotropium induces bronchodilation in symptomatic asthma patients, when used on top of standard combination therapy of inhaled glucocorticosteroids and long-acting β2-agonists (6), indicating a role for muscarinic receptors in airflow obstruction in asthma (7-9).

Recent evidence indicates that acetylcholine also regulates airway inflammation and remodeling, as indicated by the protective effects of anticholinergics on these parameters in animal models of asthma (10-12). Inhibition of remodeling by anticholinergics may be a consequence of reduced inflammation, but might also be caused by direct inhibitory effects on bronchoconstriction. This latter hypothesis is supported by a recent study from Grainge et al., who demonstrated that repeated methacholine challenges induced airway remodeling in asthma patients, with no effect on inflammation (13).

The lung expresses multiple muscarinic receptor subtypes that have differential roles in regulating bronchoconstriction, inflammation and remodeling (chapter 2). This limits the interpretation of the effects of non-selective anticholinergics and muscarinic receptor agonists on these parameters. Since the M3 receptor is the muscarinic receptor subtype that causes bronchoconstriction in the airways (14), we studied the specific role of the M3 receptor in allergen-induced inflammation and remodeling using M3 receptor subtype deficient (M3R−/−) mice. We investigated the role of the M3 receptor on ovalbumin-induced goblet cell metaplasia, airway smooth muscle thickening, pulmonary vascular remodeling, extracellular matrix deposition, airway eosinophilia and cytokine expression. M1R−/− and M2R−/− mice were used to verify that the effects were specific for the M3 receptor. In this study we demonstrate the specific involvement of the M3 receptor in allergen-induced airway remodeling in vivo.
Methods

Animals
Homozygous, inbred, specific-pathogen-free breeding colonies of M1R+/+, M2R+/+ and M3R+/+ mice and C57Bl/6NTac wild-type (WT) mice with the same genetic background were obtained from Taconic (Cambridge City, Indiana, USA). The M1R+/+, M2R+/+ and M3R+/+ mice used were on a 129 Sv/J background and backcrossed for at least 10 generations onto the C57Bl/6NTac background (15). Knock-out animals did not differ from their WT littermates in overall health, fertility and longevity (15-17). 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 (number: 5463A).

Animal model
Female mice (n=8-10 per group, 10–12 weeks old) were sensitized to ovalbumin (OVA; Sigma-Aldrich, Zwijndrecht, The Netherlands) on days 1, 14 and 21 by an intraperitoneal injection of 10 µg OVA emulsified in 1.5 mg aluminium hydroxide (Aluminject; Pierce Chemical, Etten-Leur, The Netherlands) and diluted to 200 µl with phosphate buffered saline (PBS). Subsequently, mice were challenged with saline or OVA aerosols (1% in PBS) for 20 minutes twice weekly on consecutive days for 4 weeks (figure 1). The aerosol was delivered to a Perspex exposure chamber (9 l) by a De Vilbiss nebulizer (type 646; De Vilbiss, Somerset, PA) driven by an airflow of 40 l/min providing an aerosol with an output of 0.33 ml/min as described previously (18).

Tissue collection
Twenty-four hours after the last challenge, animals were euthanized by intraperitoneal pentobarbital injection (400 mg/kg, hospital pharmacy, University Medical Center Groningen) and lungs were harvested. The smallest lower left lobe was snap frozen for mRNA analysis, the upper left lobe was snap frozen for multiplex ELISA and protein
analysis, the two lower right lung lobes were snap frozen for immunohistochemistry and the upper right lobe was formalin fixed and embedded in paraffin for immunohistochemistry. A complete description of the methods for mRNA analysis, Western Blot analysis, cytokine analysis and immunohistochemistry can be found in the supplement.

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

Results
Goblet cell metaplasia
In order to investigate the effect of ovalbumin challenge on mucus-producing cells, sections were stained with PAS to identify goblet cells and MUC5AC gene expression was analyzed in lung homogenates. Ovalbumin challenge increased the number of goblet cells in the airways of WT mice, as well as in M_{3R}^−/− mice (figure 2A and B). However, the increase in goblet cells was 30% lower in M_{3R}^−/− mice compared to that in WT mice (p<0.05). This inhibitory effect was not seen in mice deficient in the M_{1} or M_{2} receptor (figure S1). In line with findings on goblet cell number, ovalbumin challenge increased MUC5AC expression in the lungs of WT mice and M_{3R}^−/− mice, but the increase was 35% lower in M_{3R}^−/− mice compared to WT mice (p<0.05; figure 2C).

Airway smooth muscle remodeling
To assess airway smooth muscle remodeling, lung sections were stained for α-smooth muscle (sm)-actin. Ovalbumin challenge induced a 1.7-fold increase in α-sm-actin positive area around the airways of WT mice (figure 3). This increase was fully absent in M_{3R}^−/− mice (figure 3). Interestingly, sm-α-actin positive area was lower in M_{3R}^−/− mice compared to WT mice, irrespective of saline or ovalbumin treatment (p<0.001 by two-way ANOVA). Mice deficient in the M_{1} or M_{2} receptor responded similar to WT mice, although the increase in α-sm-actin deposition in response to ovalbumin was not significant in M_{3R}^−/− mice (figure S2). This indicates that these effects are specific for the M_{3} receptor.
Figure 2. Effect of ovalbumin challenge on goblet cell metaplasia in the airways of WT and M3R\(^{-/-}\) mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and goblet cell numbers were determined by PAS staining. Quantification (A) and representative images (B) are shown. Five airways were analyzed for each animal, n=8-10 mice per group, goblet cells are indicated by arrows. MUC5AC gene expression was assessed in lung homogenates, n=8-10 mice per group (C). Data represent mean ± s.e. of the mean. *** p<0.001 compared to saline-challenged control mice, # p<0.05 compared to ovalbumin-challenged WT mice. Magnification 200x.
**Figure 3.** Effect of ovalbumin challenge on airway smooth muscle thickening in WT and M3R−/− mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and airway smooth muscle mass was determined by α-sm-actin staining. Quantification (A) and representative images (B) are shown. Airway α-sm-actin positive area is indicated by arrows. Data represent mean ± s.e. of the mean. Five airways were analyzed for each animal, n=8-10 mice per group. ** p<0.01 compared to saline-challenged control mice, ### p<0.001 compared to ovalbumin-challenged WT mice. Magnification 200x.

**Vascular remodeling**

To evaluate vascular remodeling, the α-sm-actin positive area in pulmonary arteries was determined. Arterial α-sm-actin positive area was increased by 1.5-fold in WT mice after ovalbumin challenge (figure 4A and B). A similar 1.5-fold increase was observed in M3R−/− mice; however, these animals had significantly less arterial α-sm-actin compared to WT animals, irrespective of saline or ovalbumin treatment (p<0.001 by two-way ANOVA; figure 4A and B). Next, VEGF expression in lung homogenates was analyzed. Ovalbumin challenge induced a 1.4-fold increase in the expression of VEGF in WT mice, whereas only a small increase in VEGF expression in M3R−/− mice was observed, which was not significant (figure 4C). As for arterial α-sm-actin positive area, basal levels of VEGF in lung homogenates were lower in M3R−/− mice than in WT mice (figure 4C).
Figure 4. Effect of ovalbumin challenge on vascular remodeling in the lungs of WT and M3R−/− mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and vascular smooth muscle mass was determined by α-sm-actin staining. Quantification (A) and representative images (B) are shown. Five airways were analyzed for each animal, n=8-10 mice per group. Vascular endothelial growth factor (VEGF) release was assessed in lung homogenates, n=6-8 mice per group (C). Data represent mean ± s.e. of the mean. * p<0.05; ** p<0.01 compared to saline-challenged control mice, # p<0.05; ## p<0.01 compared to WT mice. Magnification 200x.

Extracellular matrix deposition

To assess the effects of ovalbumin challenge on the deposition of extracellular matrix proteins, collagen type I positive area was determined by immunohistochemistry and fibronectin protein levels were determined by Western blot analysis as immunohistochemistry for fibronectin produced a too diffuse pattern to be adequately quantifiable (data not shown). Ovalbumin challenge induced a 1.7-fold increase in collagen type I deposition around the airways of WT mice (figure 5A and B). This increase was fully absent in M3R−/− mice (figure 5A and B). Fibronectin protein level was enhanced by 1.6-fold in WT mice after ovalbumin challenge (figure 5C and D). Similar to the results for collagen...
deposition, ovalbumin-induced fibronectin deposition was completely absent in M3R⁻/⁻ mice (figure 5C and D). Baseline levels of collagen I and fibronectin were similar in WT and M3R⁻/⁻ mice.

**Figure 5.** Effect of ovalbumin challenge on extracellular matrix deposition in the airways of WT and M3R⁻/⁻ mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and collagen type I positive area was determined by immunohistochemistry. Quantification (A) and representative images (B) are shown. Five airways were analyzed for each animal, n=8-10 mice per group. Fibronectin protein level was determined by Western Blot analysis on lung homogenates. Quantification (C) and a representative blot (D) is shown, n=8-10 mice per group. Sal = saline, OA = ovalbumin. Data represent mean ± s.e. of the mean. * p<0.05; ** p<0.01 compared to saline-challenged control mice, # p<0.05 compared to ovalbumin-challenged WT mice. Magnification 200x.
Inflammation

In order to investigate whether the observed effects on remodeling could be related to reduced inflammation in M3R−/− mice after ovalbumin challenge, the number of eosinophils and the expression of cytokines in the lungs of WT and M3R−/− mice were analyzed. In WT mice, ovalbumin challenge induced a 10-fold increase in the number of eosinophils (figure 6A and B). A similar 10-fold increase was observed in M3R−/− mice (figure 6A and B). The number of eosinophils was also increased in M1R−/− and M2R−/− mice after ovalbumin challenge (figure S3). Subsequently, cytokine expression was analyzed. Ovalbumin challenge induced an increase in IL-4 (figure 6C), IL-5 (figure 6D) and IL-17 (figure 6E) in WT mice at the protein level. Expression of these cytokines was further enhanced in M3R−/− mice compared to WT mice (figure 6C, D and E). Similar findings were obtained at the gene expression level for IL-13 (figure 6F).

Figure 6. Effect of ovalbumin challenge on inflammatory responses in the airways of WT and M3R−/− mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and eosinophil numbers were determined by DAB staining. Quantification (A) and representative images (B) are shown. Five airways were analyzed for each animal, n=8-10 mice per group. Cytokine expression was assessed in lung homogenates. Levels of IL-4 (C), IL-5 (D) and IL-17 (E) were assessed by ELISA and levels of IL-13 (F) by mRNA analysis, n=8-10 mice per group. Data represent mean ± s.e. of the mean. * p<0.05; ** p<0.01; *** p<0.001 compared to saline-challenged control mice, # p<0.05; ## p<0.01; ### p<0.001 compared to ovalbumin-challenged WT mice. Magnification 200x.
The non-neuronal cholinergic system

It has been proposed that the non-neuronal cholinergic system (NNCS) might contribute to airway inflammation and remodeling (chapter 2, 19). In order to investigate effects of ovalbumin challenge on the NNCS, gene expression of different components of the NNCS 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 shown in figure S4, ovalbumin challenge did not influence expression levels of these components of the NNCS.

Discussion

In this study we demonstrate that the muscarinic M3 receptor plays a profound role in allergen-induced airway remodeling. In M3R−/− mice, goblet cell metaplasia is partly prevented compared to WT mice after ovalbumin challenge, whereas airway smooth muscle thickening and excessive extracellular matrix deposition are completely absent. These effects are specific for the M3 receptor, since M2R−/− and M1R−/− mice responded similar to ovalbumin as WT mice. Surprisingly, no stimulatory role for the M2 receptor in allergic inflammation was observed, indicating that the role of acetylcholine in remodeling does not involve an increased Tpα2 type inflammatory response. This study is the first to demonstrate the specific role of the M3 receptor in regulating allergen-induced remodeling in vivo and implies that alternative mechanisms to induce remodeling, independent of eosinophilic inflammation, play an important role.

Remodeling of the airways, including goblet cell metaplasia, airway smooth muscle thickening and excessive extracellular matrix deposition, is an important feature of asthma. It is known from in vitro studies that acetylcholine can induce proliferation of and mucus secretion from epithelial cells (20), which can be inhibited by aclidinium, a novel long-acting anticholinergic (21). Furthermore, muscarinic receptor stimulation can enhance growth factor-induced proliferation of airway smooth muscle cells and fibroblasts (22, 23) and enhances extracellular matrix deposition (24, 25) and contractile protein expression in these cells (26). Moreover, fibroblast to myofibroblast transition appears to be regulated by muscarinic receptors (27). This is further supported by in vivo studies in mice and guinea pigs, in which ovalbumin-induced structural changes, including mucus gland hypertrophy, airway smooth muscle remodeling and excessive extracellular matrix deposition, can be inhibited by the anticholinergic tiotropium (10-12). Acetylcholine contributes to the asthmatic response in these models as indicated by profound inhibitory effects of tiotropium (10, 28). Here, we demonstrate that the remodeling-promoting
effects of acetylcholine *in vivo* in response to allergen exposure are solely mediated via M₃ receptors, and not via M₁ or M₂ receptors.

Furthermore, our study also demonstrates that the M₃ receptor is involved in smooth muscle mass development and/or maintenance, since basal levels of α-sm-actin are lower in M₃R⁻/⁻ mice compared to WT mice, both in the airways and arteries. For the airways, this may imply that parasympathetic regulation of for example bronchoconstriction is needed to fully develop and/or maintain the smooth muscle, as this is lacking in M₃R⁻/⁻ mice. For the arteries this is less easily explained. Possibly, the lower levels of pro-angiogenic factors such as VEGF, or cardiovascular effects secondary to M₃ deficiency might account for the decreased actin levels. M₃R⁻/⁻ mice show intact bradycardia in response to vagal stimulation but have reduced peripheral vasodilation in response to methacholine stimulation (29).

There are some limitations to our study. Using an animal model we recapitulated different features of human asthma, including eosinophilia, goblet cell metaplasia, airway smooth muscle thickening and enhanced extracellular matrix deposition. However, some features of asthma, including cough and mucus gland hypertrophy, are not observed in mice, whereas these may be sensitive to cholinergic inhibition as demonstrated recently (10, 28). Furthermore, to study the contribution of the individual muscarinic receptor subtypes, we used muscarinic receptor knock-out mice. Although these mice do not differ from WT mice in overall health, fertility and longevity, they do have some known, and maybe also unknown, developmental differences. There is no clear alternative however, as subtype-selective agonists are not available and the subtype selective M₁ receptor antagonists that are available have significant affinity for M₂ receptors. The same accounts for tiotropium, which is known to be kinetically selective for M₃ receptors, but also has a substantial dissociation half-life for the M₁ receptor (30). Despite the limitations, the use of muscarinic receptor knockout mice does provide a unique strength and novel view on the role of muscarinic receptor subtypes in allergic airways disease, as we now present strong evidence that M₃ receptors, and not M₁ or M₂ receptors, are involved in allergen-induced airway remodeling *in vivo*.

We did not observe a stimulatory role for individual muscarinic receptor subtypes on inflammation in our study. If anything, the inflammatory response was enhanced in M₃R⁻/⁻ mice. Previously, it has been shown that acetylcholine exerts pro-inflammatory effects mediated by muscarinic receptors, both *in vitro* and *in vivo* (Chapter 2). Ovalbumin-induced eosinophilia can be inhibited by tiotropium in guinea pigs and mice (10, 12). Similar inhibitory effects of tiotropium are found on ovalbumin-induced cytokine release.
in mice, including IL-4, IL-5 and IL-13 (12). The absence of an inhibitory effect on
eosinophilia and increased cytokine production as observed in M3R<sup>−/−</sup> mice in our study
were therefore unexpected. A possible explanation for this observation is that multiple
subtypes may be involved in the inflammatory response induced by acetylcholine.
Tiotropium is kinetically selective for M<sub>3</sub> receptors; however, also has a substantial
dissociation half-life for the M<sub>1</sub> receptor (30). As shown in figure S3, there is a trend
towards lower eosinophil numbers in M3R<sup>−/−</sup> mice after ovalbumin challenge compared to
WT mice. It is therefore possible that the pro-inflammatory effect of acetylcholine
depends on the simultaneous activation of both M<sub>1</sub> and M<sub>3</sub> receptors, and that a single
muscarinic receptor is not sufficient to induce these effects. Anti-inflammatory effects of
acetylcholine in certain experimental settings (chapter 2, 31) and relaxation of bronchi by
acetylcholine-induced NO release (32), may also help to explain the differential outcomes
of these studies. Furthermore, the antimuscarinic agents were administered after allergen
sensitization in the above mentioned studies, whereas the mice used in our protocols lack
specific muscarinic receptors throughout development. Moreover, since cytokines
responsible for recruiting eosinophils and inducing goblet cell metaplasia are increased in
M3R<sup>−/−</sup> mice compared to WT mice, whereas eosinophil numbers are similar and goblet cell
numbers are even reduced in M3R<sup>−/−</sup> mice compared to WT mice, M3R<sup>−/−</sup> mice may actually
be less sensitive to inflammatory mediators.

The finding that ovalbumin-induced remodeling is greatly inhibited in M3R<sup>−/−</sup> mice, with no
inhibitory effect on inflammation, has significant implications. Generally, airway structural
changes after allergen challenge are attributed to eosinophilic inflammation (33).
However, Grainge et al. recently demonstrated that repeated methacholine challenges in
asthma patients, inducing bronchoconstriction, are sufficient to induce remodeling
without causing an inflammatory response (13). Thus, methacholine challenge induced
epithelial TGF-β staining, collagen deposition, goblet cell metaplasia and epithelial cell
proliferation, with no effect on eosinophil numbers (13). In vitro, muscarinic receptor
stimulation in combination with mechanical strain can induce α-sm-actin and sm-myosin
mRNA in intact bovine tracheal smooth muscle strips and myosin light-chain kinase
expression in human airway smooth muscle cells (34, 35). Moreover, methacholine-
induced bronchoconstriction induces epithelial epidermal growth factor receptor
activation in mice (36) and promotes TGF-β release and airway smooth muscle remodeling
in guinea pig lung slices (37). The M<sub>3</sub> receptor is the muscarinic receptor subtype that
mediates bronchoconstriction and in M3R<sup>−/−</sup> mice bronchoconstriction is abolished (29).
Therefore our current findings are in line with the above mentioned findings and suggest
that inhibition of bronchoconstriction may underlie the inhibition of allergen-induced
remodeling after knock-out of the M<sub>3</sub> receptor. Our hypothesis is that acetylcholine-
induced bronchoconstriction via M₃ receptors results in mechanical stress and the subsequent release of growth factors (e.g. TGF-β) and activation of mechanosensitive transcription factors (e.g. myocardin). These events drive remodeling of the airways, both in the airway smooth muscle and the airway submucosal compartment, suggesting activation of both airway smooth muscle cells and fibroblasts by acetylcholine. This is supported by the findings from Grainge et al., who report that bronchoconstriction activates remodeling processes in the epithelial and submucosal compartment, presumably related to mechanical compression by the airway smooth muscle (13). This hypothesis implies that prevention of bronchoconstriction in patients with asthma may be an important means of preventing remodeling later on.

Currently, anticholinergic controller therapy is not included in the guidelines for the treatment of asthma. However, recently it has been shown that tiotropium can induce bronchodilation in patients with severe asthma on top of the use of inhaled glucocorticoids and long-acting β₂-agonists (6). Furthermore, tiotropium reduced severe exacerbations and episodes of worsening of asthma (6). Moreover, adding tiotropium to glucocorticoid therapy is more effective than doubling the glucocorticoid dose by means of morning peak expiratory flow, the proportion of asthma-control days, the forced expiratory volume in 1 second and daily symptom scores (9). Together, these observations indicate that asthma patients may benefit from anticholinergic therapy. Our data suggests that M₃ receptor antagonism may also affect airway remodeling in asthma patients, however, clinical studies are clearly required to confirm this interesting hypothesis.

In the last decade, it has become clear that non-neuronal cells can also produce and release acetylcholine and it has been suggested that the observed effects of acetylcholine on inflammation and remodeling might be, at least in part, attributed to this non-neuronal cholinergic system (NNCS) (chapter 2, 38, 39). Direct evidence for such a role is still lacking however. In contrast to the previously reported decreased expression in components of the NNCS after ovalbumin challenge (40), we did not find any regulation of these components after ovalbumin challenge. Similarly, such type of regulation was also not observed after cigarette smoke-induced inflammation (chapter 3). Based on our studies, which are limited to measurement of mRNA expression levels, we cannot confirm or exclude a role for the NNCS. Until now, evidence for a role of the NNCS is limited to the fact that non-neuronal cells can produce acetylcholine (20, 39). The functional contribution of the NNCS to airway inflammation and remodeling therefore warrants further research.
In conclusion, the results of the present study demonstrate that the M₃ receptor contributes to allergen-induced airway remodeling in mice. These findings provide novel insight into the previously established role of acetylcholine in airway diseases and demonstrate that the remodeling-promoting effect of acetylcholine is solely mediated via M₃ receptors. This study may therefore open new perspectives on the role of M₃ selective anticholinergics in the treatment of asthma.

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Supplement
Methods

Immunohistochemistry

Transverse cross-sections of 5 µm thick were used for morphometric analyses.

Goblet cells were stained with Periodic Aced Schiff’s (PAS, Sigma-Aldrich, Zwijndrecht, The Netherlands) in paraffin-embedded sections. PAS-positive cells were counted and expressed per mm² basement membrane.

Collagen type I and α-sm-actin were stained on cryo-sections with a goat anti-type-I collagen antibody (SBA, Birmingham, AL, USA) and a rabbit anti-alpha smooth muscle actin antibody (Abcam, Cambridge, UK), respectively. Primary antibodies were visualised using horseradish-peroxidase-linked secondary antibodies and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands). Airways within sections were digitally photographed after which collagen I and α-sm-actin presence around the airway was quantified using Image J (National Institute of Health). The surface of positively stained tissue was expressed as mm² per mm² basement membrane.

Eosinophils were determined by staining of cryo-sections for cyanide resistant endogenous peroxidase activity with diaminobenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands). The number of eosinophils around the airways was counted and expressed as number of cells per mm basement membrane.

mRNA analysis

Lung homogenates were prepared by pulverizing the tissue under liquid nitrogen and total RNA was extracted using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. 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. The specific forward and reverse primers used are listed in table S1.
Table S1. Primers used for qRT-PCR analysis. CHT1: high-affinity choline transporter 1; CTL: choline transporter-like protein 1; ChAT: choline acetyltransferase; AChE: acetylcholinesterase.

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<th>Gene</th>
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<td>Reverse – GCAGAAGCAGGAGTGGTAG</td>
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Western analysis
Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonication in SDS lysis buffer supplemented with protease inhibitors. Homogenates were stored at –80 °C until further use. Protein content was determined by a BCA assay according to Pierce. Protein homogenates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, followed by standard immunoblotting techniques. Rabbit anti-fibronectin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and mouse anti-lamin AC (Santa Cruz biotechnology, CA, USA) were used as first antibodies. Expression levels are expressed relative to Lamin AC.
**Cytokine analysis**

Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonication in Tris buffer (pH=7.4). Homogenates were stored at –80 °C until further use. Protein content was determined by a BCA assay according to Pierce. Cytokine concentrations (IL-4, IL-5, IL-17 and VEGF) were determined by a MILLIPLEX assay (Millipore, Billerica, USA) on a Luminex 100 system using Starstation software (Applied Cytometry Systems, Sheffield, UK) according to the manufacturer’s instructions.

**Figures**

**Figure S1.** Effect of ovalbumin challenge on goblet cell metaplasia in the airways of WT, M1R⁺/‐, M2R⁺/‐ and M3R⁺/‐ mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and goblet cell numbers were determined by PAS staining. Data represent mean ± s.e. of the mean, five airways were analyzed for each animal. *** p<0.001 compared to saline-challenged control mice, # p<0.05 compared to ovalbumin-challenged WT mice, n=8-10 mice per group.
**Figure S2.** Effect of ovalbumin challenge on airway smooth muscle thickening in WT, M1R−/−, M2R−/−, and M3R−/− mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and airway smooth muscle mass was determined by α-sm-actin staining. Data represent mean ± s.e. of the mean. Five airways were analyzed for each animal. * p<0.05 compared to saline-challenged control mice, ## p<0.01 compared to ovalbumin-challenged WT mice, n=8-10 mice per group.

**Figure S3.** Effect of ovalbumin challenge on eosinophilic inflammation in the airways of WT, M1R−/−, M2R−/−, and M3R−/− mice. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and eosinophil numbers were determined by DAB staining. Data represent mean ± s.e. of the mean. Five airways were analyzed for each animal. * p<0.05; *** p<0.001 compared to saline-challenged control mice, n=8-10 mice per group.
Figure S4. Effect of ovalbumin challenge on the expression of the non-neuronal cholinergic system. Mice were treated as described in figure 1. Lungs were collected 24 hours after the last challenge and gene expression in lung tissue homogenates was analyzed. Ct values corrected for 18S are depicted, expressed as mean ± s.e. of the mean, n=4 mice per group. Note that low values mean high expression levels. High-affinity choline transporter-1 (CHT1), choline transporter like protein-1 (CTL1), choline acetyl transferase (ChAT), acetylcholine-esterase (AChE), M1 receptor (M1R), M2 receptor (M2R) and M3 receptor (M3R).
References


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