

Muscarinic M₃ Receptors Contribute to Allergen-Induced Airway Remodeling in Mice

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Abstract

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 muscarinic M₃ receptor. The objective of this work was to study the contribution of the muscarinic M₃ receptor to allergen-induced remodeling using muscarinic M₃ receptor subtype-deficient (M₃R^{-/-}) mice. Wild-type (WT), M₁R^{-/-}, and M₂R^{-/-} mice were used as controls. C57Bl/6 mice were sensitized and challenged with ovalbumin (twice weekly for 4 wk). Control animals were challenged with saline. 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 I (1.7-fold) and fibronectin (1.6-fold) in the airway wall of WT mice. These effects were absent or markedly lower in M₃R^{-/-} mice (30–100%), whereas M₁R^{-/-} and M₂R^{-/-} mice responded similarly to WT mice. In addition, airway smooth muscle and pulmonary vascular smooth muscle mass were 35–40% lower in saline-challenged M₃R^{-/-} mice compared with WT mice. Interestingly, allergen-induced airway inflammation, assessed as infiltrated eosinophils and T helper type 2 cytokine expression, was similar or even enhanced in M₃R^{-/-} mice. Our data indicate that acetylcholine contributes to allergen-induced

remodeling and smooth muscle mass via the muscarinic M₃ receptor, and not via M₁ or M₂ receptors. No stimulatory role for muscarinic 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.

Keywords: airway pharmacology; anticholinergics; asthma; nonneuronal acetylcholine

Clinical Relevance

Acetylcholine induces bronchoconstriction via the muscarinic M₃ receptor, and has also been shown to induce airway inflammation and remodeling via muscarinic receptors. The recent finding that bronchoconstriction induces remodeling suggests a specific role for muscarinic M₃ receptors in this process. Our data indicate that acetylcholine contributes to allergen-induced remodeling via the M₃ receptor, and not via M₁ or M₂ receptors. No stimulatory role for individual muscarinic receptors in inflammation was observed, suggesting that the role of acetylcholine in remodeling is independent of the allergic inflammatory response, and may involve bronchoconstriction.

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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 (Th) type 2 cytokines, 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 muscarinic M_3 receptor stimulation, and can thereby contribute to symptoms and loss of lung function in asthma (4). Parasympathetic nerve activity is increased in patients with asthma, and the airways are hyperresponsive to cholinergic stimuli (5, 6). Indeed, a recent randomized controlled trial demonstrated that the long-acting anticholinergic, tiotropium, induces bronchodilation in symptomatic patients with asthma when used on top of standard combination therapy of inhaled glucocorticosteroids and long-acting β_2 -agonists (7), indicating a role for muscarinic receptors in airflow obstruction in asthma (8–10).

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 (11–13). 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 and colleagues (14), who demonstrated that repeated methacholine challenges induced airway remodeling in patients with asthma, with no effect on inflammation.

The lung expresses multiple muscarinic receptor subtypes that have different roles in regulating bronchoconstriction, inflammation, and remodeling (4). This limits the interpretation of the effects of nonselective anticholinergics and muscarinic receptor agonists on these parameters. Because the muscarinic M_3 receptor is the muscarinic receptor subtype

that causes bronchoconstriction in the airways (15), we studied the specific role of the muscarinic M_3 receptor in allergen-induced inflammation and remodeling using muscarinic M_3 receptor subtype-deficient ($M_3R^{-/-}$) mice. We investigated the role of the muscarinic M_3 receptor on ovalbumin (OVA)-induced goblet cell metaplasia, airway smooth muscle thickening, pulmonary vascular remodeling, extracellular matrix deposition, airway eosinophilia, and cytokine expression. Muscarinic $M_1R^{-/-}$ and $M_2R^{-/-}$ mice were used to verify that the effects were specific for the muscarinic M_3 receptor. In this study, we demonstrate the specific involvement of the muscarinic M_3 receptor in allergen-induced airway remodeling *in vivo*.

Materials and Methods

Animals

Homozygous, inbred, specific pathogen-free breeding colonies of $M_1R^{-/-}$, $M_2R^{-/-}$, and $M_3R^{-/-}$ mice and C57Bl/6NTac wild-type (WT) mice with the same genetic background were obtained from Taconic (Cambridge City, IN). The $M_1R^{-/-}$, $M_2R^{-/-}$, and $M_3R^{-/-}$ mice used were on a 129 Sv/J background and backcrossed for at least 10 generations onto the C57Bl/6NTac background (16). Knockout animals did not differ from their WT littermates in overall health, fertility, and longevity (16–18). Animals were housed conventionally under a 12-hour light/dark cycle, and received food and water *ad libitum*. All experiments were performed in accordance with national guidelines and approved by the University of Groningen Committee for Animal Experimentation (no. 5463A).

Animal Model

Female mice ($n = 8–10$ per group, 10–12 wk old) were sensitized to OVA (Sigma-Aldrich, Zwijndrecht, The Netherlands) on

Days 1, 14, and 21 by an intraperitoneal injection of 10 μ g OVA emulsified in 1.5 mg aluminum hydroxide (Aluminject; Pierce Chemical, Etten-Leur, The Netherlands) and diluted to 200 μ l with 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 liter) 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 (19).

Tissue Collection

At 24 hours after the last challenge, animals were killed 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 online supplement.

Statistical Analysis

Data are presented as means ($\pm$ SEM). Statistical differences between means were calculated using two-way ANOVA, followed by Newman-Keuls multiple comparison tests. Differences were considered significant at P less than 0.05.

Results

Goblet Cell Metaplasia

To investigate the effect of OVA challenge on mucus-producing cells, sections were stained with periodic acid-Schiff to identify

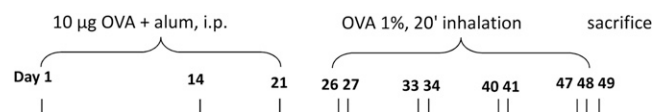


Figure 1. Experimental procedure. Female C57Bl/6NTac mice ($n = 8–10$ per group) were sensitized to ovalbumin (OVA) on Days 1, 14, and 21. Subsequently, mice were challenged with saline or OVA aerosols for 20 minutes twice weekly for 4 weeks. At 24 hours after the last challenge, mice were killed and lungs were harvested.

goblet cells, and MUC5AC gene expression was analyzed in lung homogenates. OVA challenge increased the number of goblet cells in the airways of WT mice, as well as in $M_3R^{-/-}$ mice (Figures 2A and 2B). However, the increase in goblet cells was 30% lower in $M_3R^{-/-}$ mice compared with that in WT mice ($P < 0.05$). This inhibitory effect was not seen in mice deficient in the muscarinic M_1 or M_2 receptors (*see* Figure E1 in the online supplement). In line with findings on goblet cell number, OVA 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 with 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 actin (α -SMA). OVA challenge induced a 1.7-fold increase in α -SMA-positive area around the airways of WT mice (Figure 3). This increase was fully absent in $M_3R^{-/-}$ mice (Figure 3). Interestingly, α -SMA-positive area was lower in $M_3R^{-/-}$ mice compared with WT

mice, irrespective of saline or OVA treatment ($P < 0.001$ by two-way ANOVA). Mice deficient in the muscarinic M_1 or M_2 receptor responded similarly to WT mice, although the increase in α -SMA deposition in response to OVA was not significant in $M_2R^{-/-}$ mice (Figure E2). This indicates that these effects are specific for the muscarinic M_3 receptor.

Vascular Remodeling

To evaluate vascular remodeling, the α -SMA-positive area in pulmonary arteries was determined. Arterial α -SMA-positive

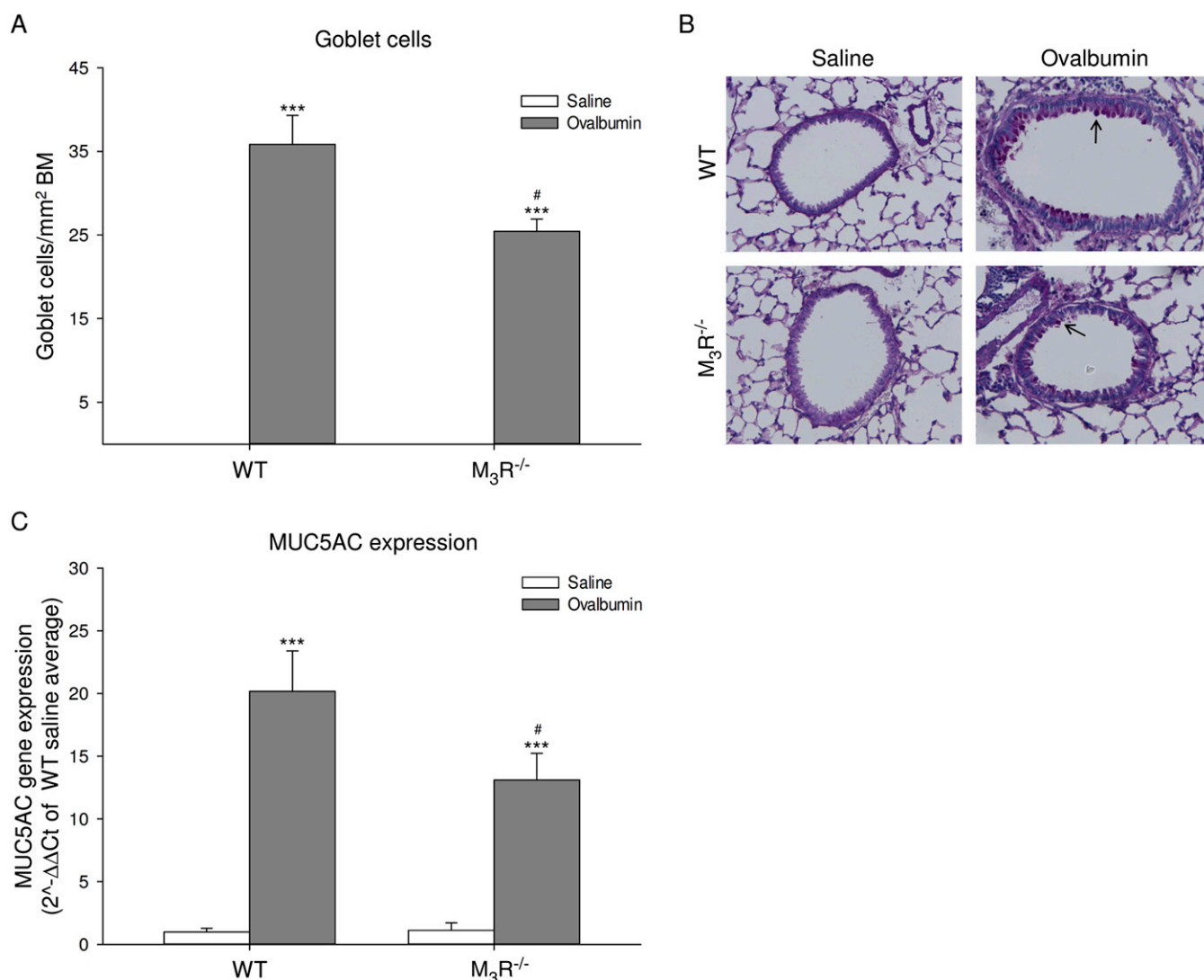


Figure 2. Effect of OVA challenge on goblet cell metaplasia in the airways of wild-type (WT) and M_3 receptor subtype-deficient ($M_3R^{-/-}$) mice. Mice were treated as described in Figure 1. Lungs were collected 24 hours after the last challenge, and goblet cell numbers were determined by periodic acid-Schiff 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 $\pm$ SEM. *** $P < 0.001$ compared with saline-challenged control mice; # $P < 0.05$ compared with OVA-challenged WT mice. Magnification, $\times 200$.

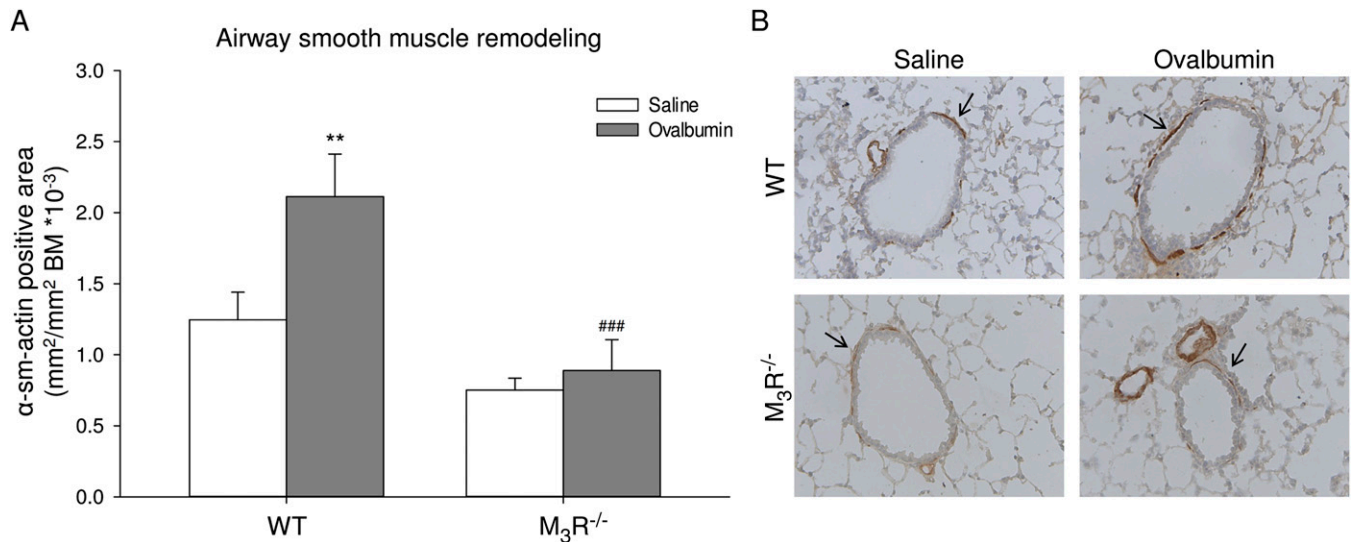


Figure 3. Effect of OVA challenge on airway smooth muscle thickening in WT and M₃R^{-/-} mice. Mice were treated as described in Figure 1. Lungs were collected 24 hours after the last challenge, and airway smooth muscle mass was determined by α -smooth muscle actin (α -sm-actin [α -SMA]) staining. Quantification (A) and representative images (B) are shown. Airway α -SMA-positive area is indicated by arrows. Data represent mean $\pm$ SEM. Five airways were analyzed for each animal ($n = 8$ –10 mice per group). ** $P < 0.01$ compared with saline-challenged control mice; ### $P < 0.001$ compared with OVA-challenged WT mice. Magnification, $\times 200$.

area was increased by 1.5-fold in WT mice after OVA challenge (Figures 4A and 4B). A similar 1.5-fold increase was observed in M₃R^{-/-} mice; however, these animals had significantly less arterial α -SMA compared with WT animals, regardless of saline or OVA treatment ($P < 0.001$ by two-way ANOVA; Figures 4A and 4B). Next, VEGF expression in lung homogenates was analyzed. OVA challenge induced a 1.4-fold increase in the expression of VEGF in WT mice, whereas only a small, nonsignificant increase in VEGF expression in M₃R^{-/-} mice was observed (Figure 4C). As for arterial α -SMA-positive area, basal levels of VEGF in lung homogenates were lower in M₃R^{-/-} mice than in WT mice (Figure 4C).

Extracellular Matrix Deposition

To assess the effects of OVA 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 pattern too diffuse to be adequately quantifiable (data not shown). OVA challenge induced a 1.7-fold increase in collagen type I deposition around the airways of WT mice (Figures 5A and 5B). This increase was fully absent in M₃R^{-/-} mice (Figures 5A and 5B). Fibronectin

protein level was enhanced by 1.6-fold in WT mice after OVA challenge (Figures 5C and 5D). Similar to the results for collagen deposition, OVA-induced fibronectin deposition was completely absent in M₃R^{-/-} mice (Figures 5C and 5D). Baseline levels of collagen I and fibronectin were similar in WT and M₃R^{-/-} mice.

Inflammation

To investigate whether the observed effects on remodeling could be related to reduced inflammation in M₃R^{-/-} mice after OVA challenge, the number of eosinophils and the expression of cytokines in the lungs of WT and M₃R^{-/-} mice were analyzed. In WT mice, OVA challenge induced a 10-fold increase in the number of eosinophils (Figures 6A and 6B). A similar 10-fold increase was observed in M₃R^{-/-} mice (Figures 6A and 6B). The number of eosinophils was also increased in M₁R^{-/-} and M₂R^{-/-} mice after OVA challenge (Figure E3). Subsequently, cytokine expression was analyzed. OVA 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 with WT mice (Figures 6C–6E). Similar findings were obtained at the gene expression level for IL-13 (Figure 6F).

The Nonneuronal Cholinergic System

It has been proposed that the nonneuronal cholinergic system (NNCS) might contribute to airway inflammation and remodeling (4, 20). To investigate effects of OVA 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, high-affinity choline transporter 1 and choline transporter-like protein 1, the acetylcholine-synthesizing enzyme, choline acetyltransferase, the acetylcholine-degrading enzyme, acetylcholinesterase, and the different muscarinic receptor subtypes. As shown in Figure E4, OVA 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 with WT mice after OVA challenge, whereas airway smooth muscle thickening and excessive extracellular matrix deposition are completely absent. These effects are specific for the muscarinic M₃ receptor, because M₁R^{-/-} and M₂R^{-/-} mice

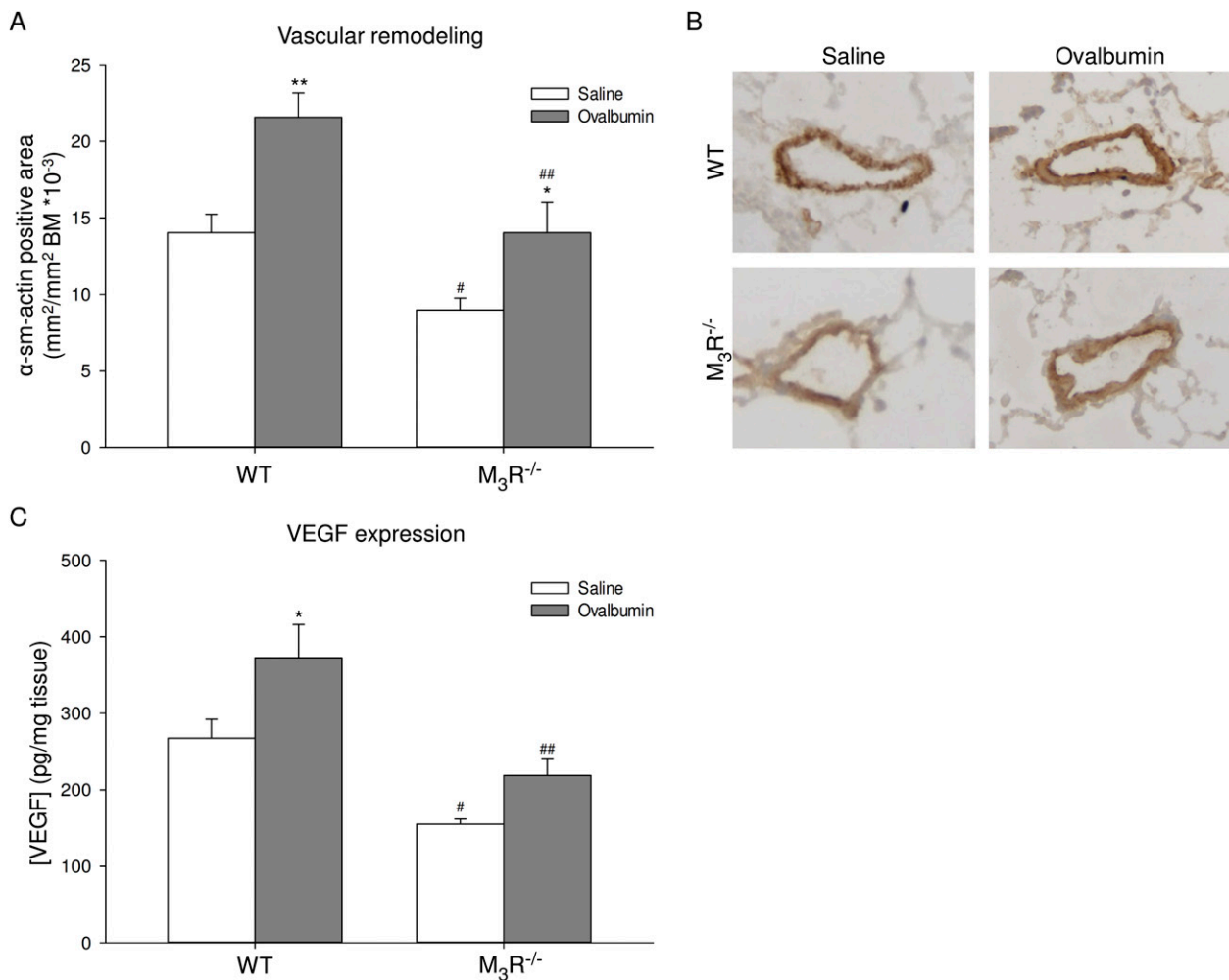


Figure 4. Effect of OVA challenge on vascular remodeling in the lungs of WT and M₃R^{-/-} mice. Mice were treated as described in Figure 1. Lungs were collected 24 hours after the last challenge, and vascular smooth muscle mass was determined by α -SMA staining. Quantification (A) and representative 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 $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$ compared with saline-challenged control mice; # $P < 0.05$; ## $P < 0.01$ compared with WT mice. Magnification, $\times 200$.

responded similarly to OVA as WT mice. Surprisingly, no stimulatory role for the muscarinic M₃ receptor in allergic inflammation was observed, indicating that the role of acetylcholine in remodeling does not involve an increased Th2-type inflammatory response. This study is the first to demonstrate the specific role of the muscarinic 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 (21), which can be inhibited by acclidinium, a novel long-acting anticholinergic (22). Furthermore, muscarinic receptor stimulation can enhance growth factor-induced proliferation of airway smooth muscle cells and fibroblasts (23, 24), and enhances extracellular matrix deposition (25, 26) and contractile protein expression in these cells (27). Moreover, fibroblast-to-myofibroblast transition appears to be regulated by muscarinic receptors (28). This is further supported by *in vivo* studies in mice and guinea pigs, in which OVA-induced structural changes, including mucus gland hypertrophy, airway smooth muscle remodeling, and

excessive extracellular matrix deposition, can be inhibited by the anticholinergic, tiotropium (11–13). Acetylcholine contributes to the asthmatic response in these models, as indicated by profound inhibitory effects of tiotropium (11, 29). Here, we demonstrate that the remodeling-promoting effects of acetylcholine *in vivo* in response to allergen exposure are solely mediated via muscarinic M₃ receptors, and not via M₁ or M₂ receptors.

Furthermore, our study also demonstrates that the muscarinic M₃ receptor is involved in smooth muscle mass development and/or maintenance, as basal levels of α -SMA are lower in M₃R^{-/-} mice compared with WT mice, both in the airways and arteries. For the

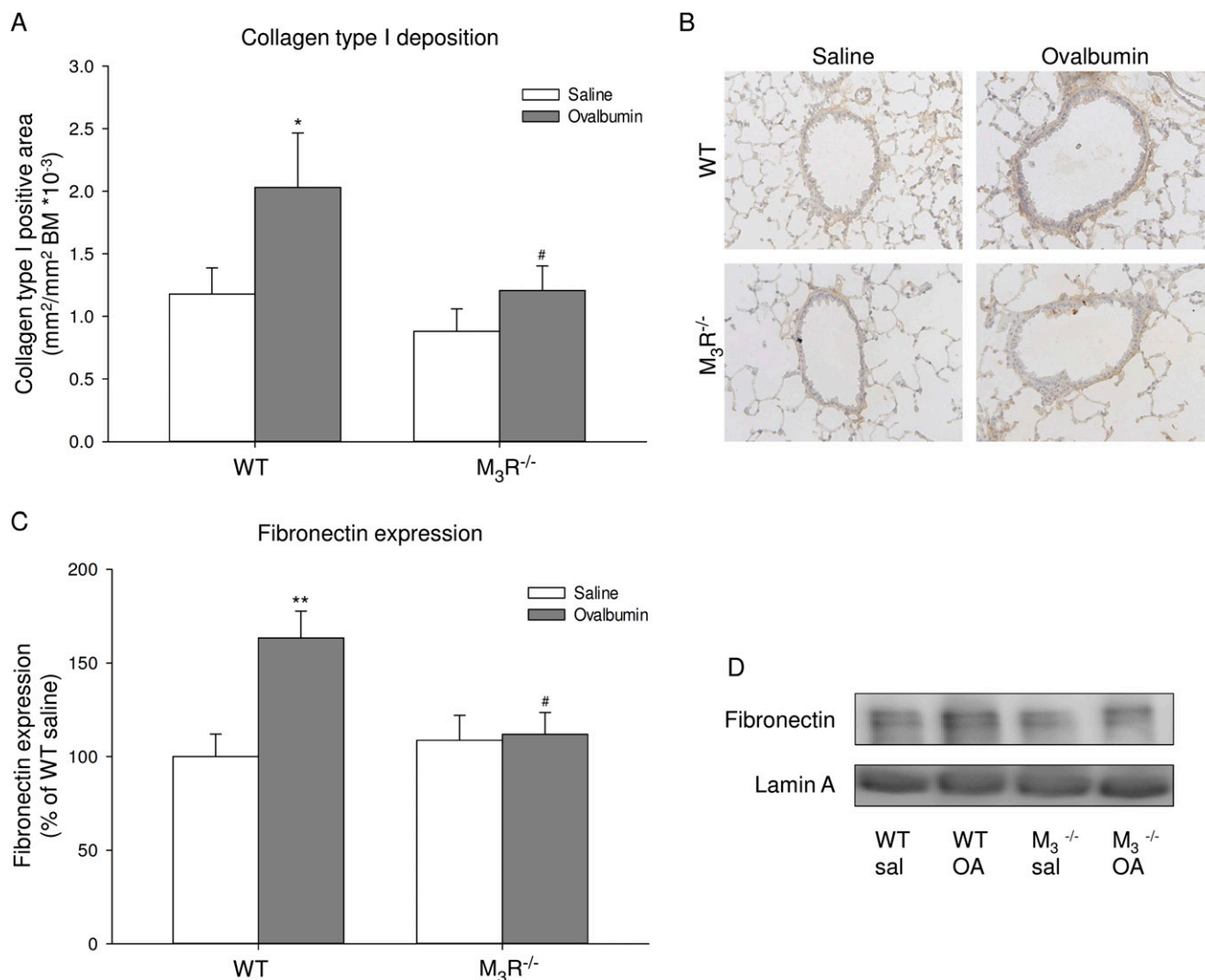


Figure 5. Effect of OVA challenge on extracellular matrix deposition in the airways of WT and M₃R^{-/-} mice. Mice were treated as described in Figure 1. Lungs were collected 24 hours after the last challenge, and collagen type I-positive area was determined by immunohistochemistry. Quantification (A) 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). OA, OVA; sal, saline. Data represent mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$ compared with saline-challenged control mice; # $P < 0.05$ compared with OVA-challenged WT mice. Magnification, $\times 200$.

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 proangiogenic 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 (30).

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 (11, 29). Furthermore, to study the contribution of the individual muscarinic receptor subtypes, we used muscarinic receptor knockout 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 muscarinic M₃ receptor antagonists that are available have significant affinity for muscarinic M₁ receptors. The same is true for tiotropium, which is known to be kinetically selective for muscarinic M₃ receptors, but also has a substantial dissociation half-life for the muscarinic M₁ receptor (31). Despite the limitations, the use of muscarinic receptor

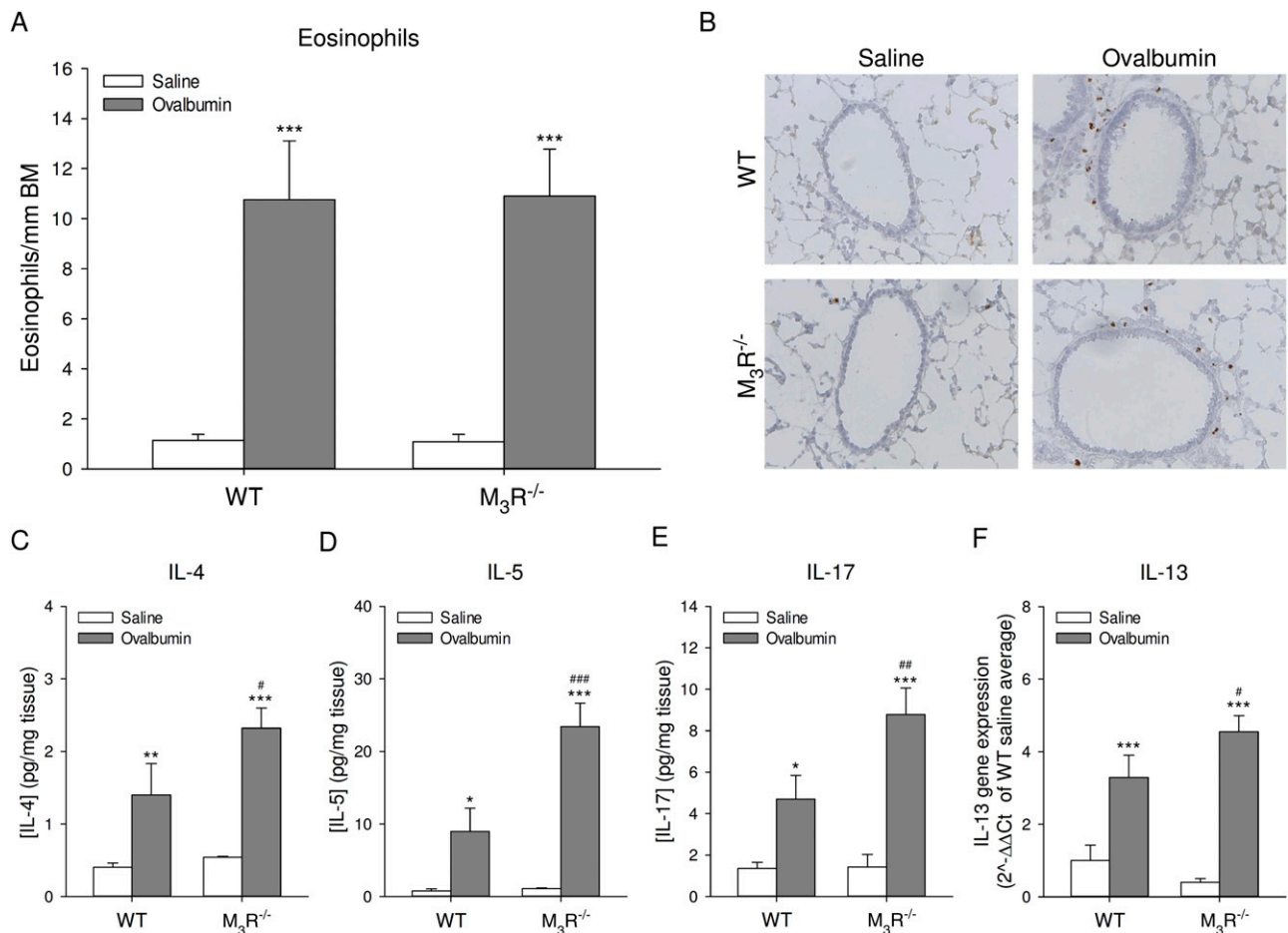


Figure 6. Effect of OVA challenge on inflammatory responses in the airways of WT and $M_3R^{-/-}$ mice. Mice were treated as described in Figure 1. Lungs were collected 24 hours after the last challenge, and eosinophil numbers were determined by diaminobenzidine staining. Quantification (A) and representative 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 $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with saline-challenged control mice; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ compared with OVA-challenged WT mice. Magnification, $\times 200$.

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 muscarinic M_3 receptors, and not muscarinic M_1 or M_2 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 $M_3R^{-/-}$ mice. Previously, it has been shown that acetylcholine exerts proinflammatory effects mediated by muscarinic receptors, both *in vitro* and *in vivo* (4). OVA-induced eosinophilia can be inhibited by tiotropium in guinea pigs and mice (11, 13). Similar inhibitory effects of tiotropium are found on OVA-induced

cytokine release in mice, including IL-4, IL-5, and IL-13 (13). The absence of an inhibitory effect on eosinophilia and increased cytokine production, as observed in $M_3R^{-/-}$ 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 muscarinic M_3 receptors, but also has a substantial dissociation half-life for the muscarinic M_1 receptor (31). As shown in Figure E3, there is a trend toward lower eosinophil numbers in $M_1R^{-/-}$ mice after OVA challenge compared with WT mice. It is therefore possible that the proinflammatory effect of acetylcholine depends on the simultaneous activation of

both muscarinic M_1 and M_3 receptors, and that a single muscarinic receptor is not sufficient to induce these effects. Anti-inflammatory effects of acetylcholine in certain experimental settings (4, 32), and relaxation of bronchi by acetylcholine-induced nitric oxide release (33), may also help to explain the differential outcomes of these studies. Furthermore, the antimuscarinic agents were administered after allergen sensitization in the studies cited previously here, whereas the mice used in our protocols lack specific muscarinic receptors throughout development. Moreover, because cytokines responsible for recruiting eosinophils and inducing goblet cell metaplasia are increased in $M_3R^{-/-}$ mice compared with WT mice, whereas eosinophil numbers are

similar, and goblet cell numbers are even reduced in $M_3R^{-/-}$ mice compared with WT mice, $M_3R^{-/-}$ mice may actually be less sensitive to inflammatory mediators.

The finding that OVA-induced remodeling is greatly inhibited in $M_3R^{-/-}$ mice, with no inhibitory effect on inflammation, has significant implications. Generally, airway structural changes after allergen challenge are attributed to eosinophilic inflammation (34). However, Grainge and colleagues (14) recently demonstrated that repeated methacholine challenges in patients with asthma, inducing bronchoconstriction, are sufficient to induce remodeling without causing an inflammatory response. Thus, methacholine challenge induced epithelial transforming growth factor (TGF)- β staining, collagen deposition, goblet cell metaplasia, and epithelial cell proliferation, with no effect on eosinophil numbers (14). *In vitro*, muscarinic receptor stimulation, in combination with mechanical strain, can induce α -SMA and smooth muscle myosin mRNA in intact bovine tracheal smooth muscle strips and myosin light-chain kinase expression in human airway smooth muscle cells (35, 36). Moreover, methacholine-induced bronchoconstriction induces epithelial epidermal growth factor receptor activation in mice (37), and promotes TGF- β release and airway smooth muscle remodeling in guinea pig lung slices (38). The muscarinic M_3 receptor is the muscarinic receptor subtype that mediates bronchoconstriction and, in $M_3R^{-/-}$ mice, bronchoconstriction is abolished (30). Therefore, our current findings are in line with the findings discussed previously here, and suggest that inhibition of bronchoconstriction may underlie the inhibition of allergen-induced remodeling after knockout of the muscarinic M_3 receptor. Our hypothesis is that acetylcholine-induced

bronchoconstriction via muscarinic M_3 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 and colleagues (14), who report that bronchoconstriction activates remodeling processes in the epithelial and submucosal compartment, presumably related to mechanical compression by the airway smooth muscle. 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 β_2 -agonists (7). Furthermore, tiotropium reduced severe exacerbations and episodes of worsening asthma (7). 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 FEV₁, and daily symptom scores (10). Together, these observations indicate that patients with asthma may benefit from anticholinergic therapy. Our data suggest that muscarinic M_3 receptor antagonism may also affect airway remodeling in patients with asthma; however, clinical studies are clearly required to confirm this interesting hypothesis.

In the last decade, it has become clear that nonneuronal 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 NNCS (4, 39, 40). Direct evidence for such a role is still lacking, however. In contrast to the previously reported decreased expression in components of the NNCS after OVA challenge (41), we did not find any regulation of these components after OVA challenge. Similarly, such a type of regulation was also not observed after cigarette smoke-induced inflammation (42). On the basis of 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 nonneuronal cells can produce acetylcholine (21, 40). 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 muscarinic M_3 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 muscarinic M_3 receptors. This study may, therefore, open new perspectives on the role of muscarinic M_3 -selective anticholinergics in the treatment of asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

- Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546–3556.
- Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:S28–S38.
- Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 2001;107:1034–1038.
- 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;91:1126–1133.
- Gross NJ. Anticholinergic agents in asthma and COPD. *Eur J Pharmacol* 2006;533:36–39.
- Minette PA, Lammers JW, Dixon CM, McCusker MT, Barnes PJ. A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J Appl Physiol* (1985) 1989;67:2461–2465.
- Kerstjens HA, Engel M, Dahl R, Paggiaro P, Beck E, Vandewalker M, Sigmund R, Seibold W, Moroni-Zentgraf P, Bateman ED. Tiotropium in asthma poorly controlled with standard combination therapy. *N Engl J Med* 2012;367:1198–1207.
- Bateman ED, Kornmann O, Schmidt P, Pivovarov A, Engel M, Fabbri LM. Tiotropium is noninferior to salmeterol in maintaining improved lung function in B16-Arg/Arg patients with asthma. *J Allergy Clin Immunol* 2011;128:315–322.

9. Kerstjens HA, Disse B, Schröder-Babo W, Bantje TA, Gahlemann M, Sigmund R, Engel M, van Noord JA. Tiotropium improves lung function in patients with severe uncontrolled asthma: a randomized controlled trial. *J Allergy Clin Immunol* 2011;128:308–314.
10. Peters SP, Kunselman SJ, Icitovic N, Moore WC, Pascual R, Ameredes BT, Boushey HA, Calhoun WJ, Castro M, Cherniack RM, et al.; National Heart, Lung, and Blood Institute Asthma Clinical Research Network. Tiotropium bromide step-up therapy for adults with uncontrolled asthma. *N Engl J Med* 2010;363:1715–1726.
11. 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.
12. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med* 2005;171:1096–1102.
13. Ohta S, Oda N, Yokoe T, Tanaka A, Yamamoto Y, Watanabe Y, Minoguchi K, Ohnishi T, Hirose T, Nagase H, et al. Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. *Clin Exp Allergy* 2010;40:1266–1275.
14. Grainge CL, Lau LC, Ward JA, Dulay V, Lahiff G, Wilson S, Holgate S, Davies DE, Howarth PH. Effect of bronchoconstriction on airway remodeling in asthma. *N Engl J Med* 2011;364:2006–2015.
15. 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.
16. 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.
17. Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodtkin J, Grinberg A, Sheng H, Wess J. Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci USA* 1999;96:1692–1697.
18. Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, et al. Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature* 2001;410:207–212.
19. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, van der Strate BW, Kerstjens HA, Timens W, Hylkema MN. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am J Respir Cell Mol Biol* 2004;30:880–885.
20. 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.
21. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* 2004;145:2498–2506.
22. Cortijo J, Mata M, Milara J, Donet E, Gavalda A, Miralpeix M, Morcillo EJ. Acridinium inhibits cholinergic and tobacco smoke-induced MUC5AC in human airways. *Eur Respir J* 2011;37:244–254.
23. Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H, Halayko AJ. Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1348–L1358.
24. Matthiesen S, Bahulayan A, Kempkens S, Haag S, Fuhrmann M, Stichnote C, Juergens UR, Racké K. Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. *Am J Respir Cell Mol Biol* 2006;35:621–627.
25. Oenema TA, Mensink G, Smedinga L, Halayko AJ, Zaagsma J, Meurs H, Gosens R, Dekkers BG. Cross-talk between TGF-beta1 and muscarinic M2 receptors augments airway smooth muscle proliferation. *Am J Respir Cell Mol Biol* 2013;49:18–27.
26. Haag S, Matthiesen S, Juergens UR, Racké K. Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. *Eur Respir J* 2008;32:555–562.
27. Oenema TA, Smit M, Smedinga L, Racké K, Halayko AJ, Meurs H, Gosens R. Muscarinic receptor stimulation augments TGF-beta1-induced contractile protein expression by airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L589–L597.
28. Milara J, Serrano A, Peiró T, Gavalda A, Miralpeix M, Morcillo EJ, Cortijo J. Acridinium inhibits human lung fibroblast to myofibroblast transition. *Thorax* 2012;67:229–237.
29. Raemdonck K, de Alba J, Birrell MA, Grace M, Maher SA, Irvin CG, Fozard JR, O'Byrne PM, Belvisi MG. A role for sensory nerves in the late asthmatic response. *Thorax* 2012;67:19–25.
30. Fisher JT, Vincent SG, Gomeza J, Yamada M, Wess J. Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M2 or M3 muscarinic acetylcholine receptors. *FASEB J* 2004;18:711–713.
31. Barnes PJ. The pharmacological properties of tiotropium. *Chest* 2000;117(2 Suppl):63S–66S.
32. Rosas-Ballina M, Tracey KJ. Cholinergic control of inflammation. *J Intern Med* 2009;265:663–679.
33. Matera MG, Calzetta L, Passeri D, Facciolo F, Rendina EA, Page C, Cazzola M, Orlandi A. Epithelium integrity is crucial for the relaxant activity of brain natriuretic peptide in human isolated bronchi. *Br J Pharmacol* 2011;163:1740–1754.
34. Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, Gauldie J, O'Byrne PM. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J Allergy Clin Immunol* 2010;125:349–356.
35. Fairbank NJ, Connolly SC, Mackinnon JD, Wehry K, Deng L, Maksym GN. Airway smooth muscle cell tone amplifies contractile function in the presence of chronic cyclic strain. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L479–L488.
36. Wahl M, Eddinger TJ, Hai CM. Sinusoidal length oscillation- and receptor-mediated mRNA expression of myosin isoforms and alpha-SM actin in airway smooth muscle. *Am J Physiol Cell Physiol* 2004;287:C1697–C1708.
37. Tschumperlin DJ, Dai G, Maly IV, Kikuchi T, Laiho LH, McVittie AK, Haley KJ, Lilly CM, So PT, Lauffenburger DA, et al. Mechanotransduction through growth-factor shedding into the extracellular space. *Nature* 2004;429:83–86.
38. Oenema TA, Maarsingh H, Smit M, Groothuis GMM, Meurs H, Gosens R. Precision cut lung slices: an *in vitro* model to study the role of TGF-beta1 and muscarinic receptors in airway remodeling [abstract]. *Am J Respir Crit Care Med* 2012;185:A2239.
39. Kummer W, Lips KS, Pfeil U. The epithelial cholinergic system of the airways. *Histochem Cell Biol* 2008;130:219–234.
40. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *Br J Pharmacol* 2008;154:1558–1571.
41. Lips KS, Lüthmann A, Tschernig T, Stoeger T, Alessandrini F, Grau V, Haberberger RV, Koepsell H, Pabst R, Kummer W. Down-regulation of the non-neuronal acetylcholine synthesis and release machinery in acute allergic airway inflammation of rat and mouse. *Life Sci* 2007;80:2263–2269.
42. Kistemaker LE, Bos IS, Hylkema MN, Nawijn MC, Hiemstra PS, Wess J, Meurs H, Kerstjens HA, Gosens R. Muscarinic receptor subtype-specific effects on cigarette smoke-induced inflammation in mice. *Eur Respir J* 2013;42:1677–1688.

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