CHAPTER 4

MUSCARINIC M3 RECEPTORS ON STRUCTURAL CELLS REGULATE CIGARETTE SMOKE-INDUCED NEUTROPHILIC AIRWAY INFLAMMATION IN MICE

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Abstract

Rationale
Anticholinergics, blocking the muscarinic M₁ receptor, are effective bronchodilators for patients with COPD. Recent evidence from M₃ receptor deficient mice (M₃R⁻/⁻) indicates that M₃ receptors also regulate neutrophilic inflammation in response to cigarette smoke (CS). M₃ receptors are present on almost all cell types and in this study we investigated the relative contribution of M₃ receptors on structural cells versus inflammatory cells to CS-induced inflammation using bone-marrow chimeric mice.

Methods
Bone-marrow chimeras (C56Bl/6 mice) were generated and engraftment was confirmed after 10 weeks. Thereafter, irradiated and non-irradiated control animals were exposed to CS or fresh air for four consecutive days.

Results
CS induced a significant increase in neutrophil numbers in non-irradiated and irradiated control animals (4-35 fold). Interestingly, wild-type animals receiving M₃R⁻/⁻ bone marrow showed a similar increase in neutrophil number (15-fold). In contrast, no increase in the number of neutrophils was observed in M₃R⁻/⁻ animals receiving wild-type bone marrow. The increase in KC levels was similar in all smoke-exposed groups (2.5-5.0 fold). Micro-array analysis revealed that fibrinogen α and CD177, both involved in neutrophil migration, were downregulated in CS-exposed M₃R⁻/⁻ animals receiving wild-type bone marrow compared to CS-exposed wild-type animals, which was confirmed by RT-qPCR (1.6-2.5 fold).

Conclusions
These findings indicate that the M₃ receptor on structural cells plays a pro-inflammatory role in CS-induced neutrophilic inflammation, whereas the M₃ receptor on inflammatory cells does not. This effect is probably not mediated via KC release, but may involve altered adhesion and transmigration of neutrophils via fibrinogen α and CD177.

Introduction
Acetylcholine is the primary parasympathetic neurotransmitter in the airways. It induces bronchoconstriction and mucus secretion via M₁ receptors (chapter 2). Cholinergic tone is increased in patients with chronic obstructive pulmonary diseases (COPD), and this is the major reversible component of airflow obstruction in COPD (1, 2). Therefore, anticholinergics, which block M₁ receptors, are effective bronchodilators for patients with COPD (2).
In addition to inducing bronchoconstriction and mucus secretion, acetylcholine has been shown to affect airway inflammation (chapter 2), which is a hallmark feature of COPD (3). In animal models of COPD, pretreatment with anticholinergics inhibits cigarette smoke-induced and LPS-induced neutrophilic inflammation (4, 5). More recently, we demonstrated that these pro-inflammatory effects of acetylcholine are mediated via M₃ receptors. Total knock-out of the M₃ receptor, or inhibition of the M₃ receptor by a pharmacological approach using the muscarinic antagonist 4-DAMP, which is selective for M₃ receptors over M₂ receptors, prevented cigarette smoke-induced neutrophilic inflammation in an early smoke induced-inflammation model in mice (chapter 3). Together, these data suggest a pro-inflammatory role for acetylcholine via M₃ receptors.

M₃ receptors are expressed abundantly throughout the airways and almost all cell types express M₃ receptors. This includes structural cells, such as airway smooth muscle, epithelial and endothelial cells, but also inflammatory cells, such as macrophages and neutrophils (6). From in vitro studies it is known that muscarinic receptor stimulation of both structural and inflammatory cells might contribute to the pro-inflammatory effects of acetylcholine. For instance, stimulation of airway smooth muscle cells and epithelial cells with methacholine or acetylcholine induces the release of IL-8 (7, 8). Furthermore, acetylcholine activates human monocytes and alveolar macrophages to promote neutrophil migration, which is also observed when using macrophages from COPD patients (9, 10). It is not known whether the pro-inflammatory effects of acetylcholine observed in vivo are primarily mediated via M₃ receptors on structural cells or via M₃ receptors on inflammatory cells.

Therefore, in this study, we investigated the relative contribution of the M₃ receptor on structural cells versus inflammatory cells to cigarette smoke-induced inflammation. In order to distinguish between structural cells and inflammatory cells, bone marrow chimeric mice were generated. Here, we demonstrate that the pro-inflammatory effects of acetylcholine on neutrophilic inflammation are primarily mediated via M₃ receptors on structural cells, which may involve altered adhesion and transmigration of neutrophils.

Methods

Animals

Homozygous, inbred, specific-pathogen-free breeding colonies of muscarinic M₃ receptor deficient (M₃R⁻/⁻) mice and C57Bl/6NTac wild-type mice with the same genetic background (CD45.2) were obtained from Taconic (Cambridge City, Indiana, USA). The M₃R⁻/⁻ mice were on a 129 Sv/J background and had been backcrossed for at least 10 generations onto
the C57Bl/6NTac background (11). CD45.1 congenic C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in our own facility. 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: 5463G).

**Generation of bone marrow chimeras**

Bone marrow cells were collected from femurs and tibia of M3R+/− mice and wild-type CD45.2 or CD45.1 mice, and retro-orbitally injected into irradiated male mice (9 Gy), within 24 hours after irradiation. Per mouse, $8 \times 10^6$ bone marrow cells were transplanted. Non-irradiated wild-type animals were used as additional controls; see table 1 for an overview of the groups. After 10 weeks, engraftment was analyzed by flow cytometry of peripheral blood. Seventy microliter of heparinized peripheral blood was collected 10 weeks after bone marrow transplantation and at the end of the study when the mice were sacrificed. Red blood cells were lysed and the remaining cells were stained with antibodies directed against the following markers: CD45.2-PE (CD45.2), CD45.1-Pacific Blue (CD45.1), CD3-APC (T cells), B220-FITC (B cells), GR-1-PE/Cy7 and Mac1-PE/Cy7 (monocytes and granulocytes, respectively) (Biolegend, San Diego, CA, USA). The stained cells were analyzed with a LSR-II flow cytometer (BD Biosciences, San Diego, CA, USA). Engraftment was calculated as a percentage of CD45.1 versus CD45.2 cells. Engraftment was high in all animals (>90% donor derived cells in all lineages), and mice were included in the study 3 months after bone marrow reconstitution (figure 1).

**Table 1.** Overview of the experimental groups included in this study.

<table>
<thead>
<tr>
<th>Background</th>
<th>Recipient</th>
<th>Irradiation</th>
<th>Donor</th>
<th>Groups</th>
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</tr>
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<td>CD45.2</td>
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<tr>
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<td>M3R inflammatory cells</td>
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<td></td>
<td>M3R+/−</td>
<td>+</td>
<td>CD45.1</td>
<td>M3R structural cells</td>
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</table>
Cigarette smoke exposure
Mice (n=5-12 per group) were exposed to cigarette smoke from Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, USA) on 4 consecutive days by whole body exposure in an early smoke induced-inflammation model, as described previously (chapter 3). Mice were exposed to mainstream smoke of one cigarette in the morning and three cigarettes in the afternoon on the first day. On day 2 to 4, mice were exposed to five cigarettes in the morning and five in the afternoon (figure 1). 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). Control animals were handled in the same way but instead exposed to fresh air only. 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. Bronchoalveolar lavage (BAL) fluid was stored at -20°C and lung tissue was stored at -80°C.

Analysis of bronchoalveolar lavage cells and cytokines
A BAL was performed as described previously (chapter 3). Briefly, lungs were lavaged 5 times with 1 ml PBS. The first fraction was used for measurement of cytokines by ELISA. Levels of KC, IL-6 and MCP-1 were determined by a multiplex assay (R&D Systems, Minneapolis, USA). Total cell numbers were determined and cytospins were prepared and stained with May–Grünwald and Giemsa (both Sigma, St. Louis) after which a differential cell count was performed by counting at least 400 cells in duplicate in a blinded fashion.

![Experimental protocol](image)

**Figure 1.** Experimental protocol. Chimeric animals were generated by sublethal irradiation (9 Gy) of recipient male C57Bl/6 mice (n=5-12) and subsequent transplantation of 8 x 10^6 donor bone marrow cells via retro-orbital injection within 24h after irradiation. To allow for complete engraftment, animals were included in the protocol three months after bone marrow reconstitution. Animals 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 was performed and lungs were harvested for lung tissue homogenates.
Analysis of gene expression in lung tissue

Total RNA was extracted from lung tissue (right superior lobe) using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), as described previously (chapter 3). Equal amounts of total mRNA were then reverse transcribed and cDNA was subjected to real-time qPCR (Westburg, Leusden, The Netherlands) or to micro-array analysis using an Illumina chip and GeneSpring software. The specific forward and reverse primers used for real-time qPCR are listed in table 2.

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, or by a Student’s t-test with two-tailed distribution where appropriate. Differences were considered significant at p<0.05.

Table 2. Primers used for qRT-PCR analysis. KC: keratinocyte-derived chemokine, the mouse orthologue of IL-8, also known as CXCL1 in mice. MIP-2 is also known as CXCL2, LIX is also known as CXCL5.

<table>
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<th>Gene</th>
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<td>Reverse – AGGTGCATCACAGACGTCT</td>
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<td>IL-6</td>
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<td>NM_031168.1</td>
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<td></td>
<td>Reverse – TCCAGATTTCCAGACGAAC</td>
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<tr>
<td>MIP-2</td>
<td>Forward – AAGTTTGCCTTGAACCTGAA</td>
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</tr>
<tr>
<td></td>
<td>Reverse – AGGCACATCAGTGACGATCC</td>
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<tr>
<td>LIX</td>
<td>Forward – GAAAGCTAAGCGGAATGCAC</td>
<td>NM_009141.3</td>
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<td></td>
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<td>Fibrinogen α</td>
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<td>Reverse – GGTTTCAGCAGAGGACGAG</td>
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<td>18S</td>
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<tr>
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<td>Reverse – CCTCCAATGGATCCCTGTTA</td>
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Results

Analysis of engraftment

To confirm that the bone marrow transplantation was successful, engraftment was analyzed after 10 weeks and at the day of sacrifice of the mice. As depicted in figure 2, after 10 weeks engraftment was high and this was not different between the experimental groups (not shown). Therefore, all animals were included in the study 3 months after bone marrow reconstitution. Engraftment was even further enhanced at the day of sacrifice. Engraftment was as follows: total cells 97.1% (ranging from 94.2 to 99.0%), monocytes 99.4% (97.5 to 100%), granulocytes 99.8% (97.8 to 100%), B cells 99.9% (99.3 to 100%), and T cells 90.6% (81.7 to 97.4%) (figure 2).

Figure 2. Engraftment of bone marrow cells. Engraftment was analyzed in peripheral blood of mice, 10 weeks after the bone marrow transplantation and at the day of sacrifice. Average engraftment levels (A) and representative FACS images for total single cells (B), monocytes (C), granulocytes (D), B cells (E) and T cells (F) at the day of sacrifice are shown. N=68 animals.
**Contribution of the M₃ receptor on inflammatory cells**

To study the contribution of the M₃ receptor on inflammatory cells to early cigarette smoke-induced airway inflammation, inflammatory cells in the lavage fluid of CD45.1 animals were determined after exposure to cigarette smoke. The following groups were compared: CD45.1 non-irradiated control animals, irradiated CD45.1 control animals receiving wild-type (CD45.2) bone marrow cells and irradiated CD45.1 animals receiving M₃R⁻/⁻ (CD45.2) bone marrow cells (table 1). Cigarette smoke exposure had no significant effect on macrophage and lymphocyte numbers in all groups (figure 3). Cigarette smoke induced a significant increase in neutrophil numbers in non-irradiated (figure 3A; 28-fold) and irradiated control animals receiving wild-type bone marrow cells (figure 3B; 18-fold). Interestingly, wild-type animals receiving M₃R⁻/⁻ bone marrow cells showed a similar increase in neutrophil number (figure 3C; 15-fold), suggesting that the M₃ receptor on inflammatory cells is not involved in cigarette smoke-induced neutrophilic inflammation.

**Contribution of the M₃ receptor on structural cells**

To study the contribution of the M₃ receptor on structural cells to early cigarette smoke-induced airway inflammation, inflammatory cells in the lavage fluid of CD45.2 animals were determined after exposure to cigarette smoke (figure 4). The following groups were compared: CD45.2 non-irradiated control animals, irradiated CD45.2 control animals receiving CD45.1 bone marrow cells and irradiated M₃R⁻/⁻ (CD45.2) animals receiving CD45.1 bone marrow cells (table 1). Cigarette smoke induced a small increase in macrophage numbers in the lavage fluid in all groups (1.3-1.4 fold). No increase in lymphocyte numbers was observed. Cigarette smoke induced a significant increase in neutrophil numbers in non-irradiated (figure 4A; 8-fold) and irradiated control animals receiving wild-type bone marrow cells (figure 4B; 4-fold). In contrast, no increase in the number of neutrophils was observed in M₃R⁻/⁻ animals receiving wild-type bone marrow (figure 4C), suggesting that the M₃ receptor on structural cells is involved in cigarette smoke-induced neutrophilic inflammation.
M3 receptors on structural cells regulate inflammation

Figure 3. Inflammatory cells in the lavage fluid of CD45.1 animals. Mice were treated as described in figure 1. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed and macrophages, lymphocytes and neutrophils were determined in the lavage fluid of non-irradiated wild-type animals (A), wild-type animals receiving wild-type bone marrow cells (B) and wild-type animals receiving M3R−/− bone marrow cells (C). * p<0.05, ** p<0.01, n=6-12 animals.
Figure 4. Inflammatory cells in the lavage fluid of CD45.2 animals. Mice were treated as described in figure 1. Sixteen hours after the last smoke exposure a bronchoalveolar lavage was performed and macrophages, lymphocytes and neutrophils were determined in the lavage fluid of non-irradiated wild-type animals (A), wild-type animals receiving wild-type bone marrow cells (B) and M3R<sup>−/−</sup> animals receiving wild-type bone marrow cells (C). * p<0.05, n=5-8 animals.
Cigarette smoke-induced cytokine release
Subsequently, gene expression of KC (CXCL1), the mouse orthologue of IL-8 (CXCL8), was determined in lung homogenates. Cigarette smoke induced an increase in KC. Remarkably, this increase was similar in all bone marrow transplanted groups (2.8-5.0 fold), as depicted in figure 5. Similar findings were observed at the protein level in the lavage fluid. Of note, other neutrophil chemoattractants, including MIP-2 (CXCL2) and LIX (CXCL5) were found increased in response to cigarette smoke in the CD45.1 groups only, with no interaction of genotype, whereas no increase in IL-6 or MCP-1 expression in response to cigarette smoke was observed in all strains (data not shown).

Neutrophil adhesion
These observations suggest that M₃ receptors on structural cells do not mediate their effect via altered KC, MIP-2 or LIX production. Therefore, we considered the possibility that neutrophil adhesion might be altered after knock-out of the M₃ receptor on structural cells. We analyzed the expression of genes known to play a role in this process. In the airways, P-selectin, E-selectin and L-selectin are involved in rolling of neutrophils, whereas intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, β2 integrin and integrin α4β1 (very late antigen-4, VLA4) play a role in adhesion and

Figure 5. KC expression after cigarette smoke exposure. Mice were treated as described in figure 1. Sixteen hours after the last smoke exposure lungs were collected. Gene expression of keratinocyte-derived chemokine (KC) in lung homogenates was determined. Results are expressed as mean ± s.e. of the mean, * p<0.05; ** p<0.01; *** p<0.001, n=5-12 animals.
transmigration of neutrophils (12). However, we were not able to demonstrate regulation of these markers at the gene expression level in whole lung homogenates. Thus, no increase was observed in wild-type animals receiving wild-type bone marrow cells, nor were there any differences compared to M3R\(^{-/-}\) animals receiving wild-type bone marrow cells (data not shown). To be able to explain the observed effects, we proceeded with a micro-array gene expression analysis on whole lung homogenates of wild-type bone marrow cells and of M3R\(^{-/-}\) animals receiving wild-type bone marrow cells. We selected genes which were up regulated or down regulated by more than 1.5-fold in cigarette smoke-exposed M3R\(^{-/-}\) animals receiving wild-type bone marrow cells compared to cigarette smoke-exposed wild-type animals receiving wild-type bone marrow cells (table 3). Three genes were upregulated and twelve were downregulated. Genes which were shown to have a link with neutrophil inflammation according to literature were analyzed by real-time qPCR. Down regulation of fibrinogen\(\alpha\) and CD177 was confirmed, which was lower in M3R\(^{-/-}\) animals compared to wild-type animals, and not influenced by smoke exposure (figure 6). Interestingly, both genes are known to play a role in neutrophil adhesion and transmigration.

Table 3. Genes which were up regulated or down regulated by more than 1.5-fold in cigarette smoke-exposed M3R\(^{-/-}\) animals receiving wild-type bone marrow cells compared to cigarette smoke-exposed wild-type animals receiving wild-type bone marrow cells. Genes in bold were analyzed by real-time qPCR.

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<thead>
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<th>Gene</th>
<th>Definition</th>
<th>Regulation</th>
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<td>AKR1C19</td>
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</tr>
<tr>
<td>TNXB</td>
<td>Tenascin XB</td>
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</tr>
<tr>
<td>ERAF</td>
<td>Erythroid associated factor</td>
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<tr>
<td>FGG</td>
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<td>FGA</td>
<td>Fibrinogen alpha chain</td>
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<td>RETNLA</td>
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M<sub>3</sub> receptors on structural cells regulate inflammation

Figure 6. Fibrinogen α and CD177 expression after cigarette smoke exposure. Mice were treated as described in figure 1. Sixteen hours after the last smoke exposure lungs were collected. Micro-array analysis on lung homogenates revealed that fibrinogen α and CD177 were down regulated after smoke exposure in M<sub>3</sub>R<sup>−/−</sup> animals receiving wild-type bone marrow cells compared to wild-type animals. Therefore, gene expression of fibrinogen α (A) and CD177 (B) was determined by real-time qPCR. Results are expressed as mean ± s.e. of the mean, *p<0.05, n=5-8 animals.

Discussion

In this study, we demonstrated that the M<sub>3</sub> receptor on structural cells plays a pro-inflammatory role in cigarette smoke-induced inflammation. In M<sub>3</sub>R<sup>−/−</sup> mice receiving wild-type bone marrow cells, neutrophilic inflammation in response to cigarette smoke exposure is prevented. In contrast, cigarette smoke-induced neutrophilic inflammation is still observed after knock-out of the M<sub>3</sub> receptor on inflammatory cells, suggesting that the pro-inflammatory effects of acetylcholine are primarily mediated via M<sub>3</sub> receptors on structural cells. This was not regulated via inhibition of cytokine release, which was comparable in all groups, but might involve altered adhesion and transmigration of neutrophils via fibrinogen α and CD177.

Neutrophils play a central role in COPD, and previous studies have shown that acetylcholine regulates neutrophilic inflammation via M<sub>3</sub> receptors. Inhibition of muscarinic receptors by anticholinergics, including tiotropium, glycopyrrolate and aclidinium, prevented neutrophilic inflammation in response to cigarette smoke exposure.
or LPS exposure in guinea pigs and mice (4, 5, 13, 14). Since long-acting anticholinergics are kinetically selective for M3 receptors, these findings suggest the involvement of this specific muscarinic receptor subtype. A recent study from our lab using M3R−/− mice showed that the pro-inflammatory effects of acetylcholine are indeed mediated via the M3 receptor. We demonstrated that cigarette smoke-induced neutrophilic inflammation was prevented in M3R−/− mice, whereas opposite effects were observed in M1R−/− and M2R−/− mice (chapter 3). In the present study we have extended these findings by demonstrating that the pro-inflammatory effects of acetylcholine are primarily mediated via M3 receptors on structural cells in an early smoke induced-inflammation model in mice. The involvement of the M3 receptor on structural cells in the inflammatory response is also supported by evidence from in vitro studies. Methacholine and cigarette smoke-induced IL-8 release from airway smooth muscle cells can be inhibited by the M3 antagonists 4-DAMP and DAUS884 (7). Moreover, acetylcholine-induced IL-8 release from bronchial epithelial cells can be inhibited by 4-DAMP (8). Strikingly, acetylcholine-induced effects on inflammatory cells were also shown to be mediated via M3 receptors. 4-DAMP inhibited acetylcholine-induced neutrophil chemotactic activity from macrophages (15), as well as neutrophil chemotaxis from LPS-activated macrophages from COPD patients (10). These in vitro studies suggest a direct effect of acetylcholine on both structural cells and inflammatory cells. Apparently, the observed effects of acetylcholine on inflammatory cells in vitro are not relevant to the in vivo situation, as we demonstrate that cigarette smoke-induced inflammation is still observed after knock-out of the M3 receptor on inflammatory cells.

Although there was no increase in neutrophil numbers in M3R−/− animals receiving wild-type bone marrow cells after exposure to cigarette smoke compared to wild-type animals, the increase in the neutrophil chemoattractant KC was similar to the increase observed in wild-type animals. This suggests that neutrophil recruitment is altered after knock-out of the M3 receptor. Neutrophil recruitment is a complex cellular process which occurs through a cascade of multiple steps, including tethering, rolling, adhesion, crawling and subsequent transmigration of neutrophils (12). An initial screen on genes known to be involved in the process of neutrophil recruitment, including selectins, ICAM-1, VCAM-1, β2 integrin and VLA4, could not explain the observed increase in KC in M3R−/− animals receiving wild-type bone marrow cells, without an increase in neutrophil numbers. However, using a micro-array analysis, two genes which were recently linked to the process of neutrophil recruitment, fibrinogen α and CD177 (16, 17), were shown to be down regulated in M3R−/− animals compared to wild-type animals.
Fibrinogen α, besides its role in blood hemostasis, regulates the adhesive behavior of neutrophils. Fibrinogen can bind to β2 integrins and thereby alter the recruitment of neutrophils (16, 18). Neutrophil recruitment in response to platelet activating factor (PAF) is altered in fibrinogen α knock-out mice. Specifically, the number of rolling neutrophils and the number of adhering neutrophils was decreased in fibrinogen α knock-out mice compared to wild-type mice (16). Therefore, a reduction in fibrinogen α levels in M3R+/− mice might affect neutrophil recruitment in response to cigarette smoke exposure, by reducing the rolling and adhesion of neutrophils.

CD177 is a neutrophil specific antigen, expressed by a subpopulation of neutrophils. CD177 can bind to platelet endothelial cell adhesion molecule (PECAM)-1, and thereby alter the recruitment of neutrophils (17, 19). PECAM-1 is known to be involved in neutrophil transmigration (12). In vitro, transmigration of neutrophils through endothelial cells in response to IL-8 or fMLP was inhibited after inhibition of the CD177-PECAM-1 interaction (17). Interestingly, the same study showed that CD177-positive neutrophils transmigrated more efficiently than CD177-negative neutrophils, indicating the potential relevance of CD177 for transmigration. These findings were confirmed by Kuckleburg et al., who demonstrated that inhibition of CD177 or inhibition of the interaction between CD177 and PECAM-1 inhibits neutrophil transmigration under both static and flow conditions (20). Therefore, a reduction in CD177 levels in M3R+/− mice might affect neutrophil recruitment in response to cigarette smoke exposure, by reducing the transmigration of neutrophils.

The gene expression of neither fibrinogen α nor CD177 was regulated by cigarette smoke, indicating that this is a constitutive difference between the wild-type and M3R+/− mice. Expression levels of both genes were lower in the M3R+/− strain compared to wild-type mice, which further supports the observation that neutrophilic inflammation is altered in M3R+/− mice receiving wild-type bone marrow, and not in wild-type animals receiving M3R+/− bone marrow. Interestingly, both genes were recently identified as potential inflammatory biomarkers for COPD. Fibrinogen was identified as a biomarker in the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study (21), and an elevated level of fibrinogen was independently and significantly associated with mortality in patients with COPD (22). CD177 levels were increased in the airways of Cynomolgus monkeys after ozon challenge (23), indicating the potential relevance of both genes for COPD pathophysiology. It is beyond the scope of the current manuscript to investigate the functional roles of CD177 and fibrinogen α in detail; however, our results and the above mentioned clinical data certainly provide strong rationale for future studies in this area.
The fact that KC levels were increased in M3R−/− mice receiving wild-type bone marrow cells in our study also has other implications. Previously, we have shown that increased KC release in response to cigarette smoke exposure is prevented after total knock-out of the M3 receptor (chapter 3). KC can be released from airway structural cells, including airway smooth muscle cells and epithelial cells, and from inflammatory cells, including macrophages. The finding that KC release is prevented after total knock-out of the M3 receptor, but not after knock-out of the M3 receptor on structural cells only, suggests a role for inflammatory cells in this response. The suggestion that acetylcholine induces the release of KC from inflammatory cells is supported by in vitro studies showing enhanced neutrophil recruitment after stimulation of macrophages with acetylcholine (10, 15). However, knock-out of the M3 receptor only on inflammatory cells does not prevent neutrophilic inflammation, whereas knock-out of the M3 receptor on structural cells does. Therefore, the contribution of the M3 receptor on inflammatory cells to the pro-inflammatory effect of acetylcholine is only small, and this is mainly dependent on M3 receptors on structural cells.

Acetylcholine is not only released as a neurotransmitter, but can also be released from non-neuronal cells. Almost all cell types in the airways have been shown to express synthesizing enzymes for acetylcholine, suggesting acetylcholine production (24). It has been proposed that this non-neuronal acetylcholine might contribute to inflammation, although direct evidence is still limited (chapter 2, 25). Results from our study indicate that non-neuronal acetylcholine acting on inflammatory cells does not significantly contribute to the pro-inflammatory effects of acetylcholine. There might be a role for non-neuronal acetylcholine released from airway structural cells including epithelial cells, however, future studies are needed to investigate this in more detail.

In conclusion, we demonstrate that acetylcholine regulates inflammation via M3 receptors on structural cells. This emphasizes the important role of airway structural cells in neutrophilic inflammation and the pathophysiology of COPD, and the contribution of acetylcholine to this response. Whether anticholinergic therapy also affects inflammation in patients with COPD still needs to be elucidated, but our results indicate that this might be an additional beneficial effect of M3 selective anticholinergics.

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References


