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Type 3 Muscarinic Receptors Contribute to Clearance of *Citrobacter rodentium*

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Abstract

Background—The role of muscarinic receptors in mucosal homeostasis, response to enteric pathogens, and modulation of immune cell function is undefined.

Methods—The contribution of type 3 muscarinic receptors (M3R) to mucosal homeostasis within the colon and host defense against *Citrobacter rodentium* was determined in uninfected and *C. rodentium*-infected WT and M3R-deficient (*Chrm3*^{−/−}) mice. In addition, WT and *Chrm3*^{−/−} bone marrow-derived macrophages (BMDM) were studied to determine the ability of M3R to modulate macrophage phenotype and function.

Results—In *Chrm3*^{−/−} mice clearance of *C. rodentium* was delayed despite an amplified T_H1/T_H17 response. Delayed clearance of *C. rodentium* from *Chrm3*^{−/−} mice was associated with prolonged adherence of bacteria to colonic mucosa, decreased goblet cell number, and decreased *mucin 2* gene expression. Treatment of BMDM with bethanechol, a muscarinic-selective agonist, induced a classically activated macrophage phenotype, which was dependent on M3R expression. *Chrm3*^{−/−} BMDM retained their ability to attain a classically activated macrophage phenotype when treated with the T_H1 cytokine IFN-γ.

Conclusions—In *Chrm3*^{−/−} mice mucin production is attenuated and is associated with prolonged adherence of *C. rodentium* to colonic mucosa. The immune response, as characterized by production of T_H1/T_H17 cytokines, in *C. rodentium*-infected *Chrm3*^{−/−} mice is intact. In addition, M3R activity promotes the development of classically activated macrophages. Our data establish a role for M3R in host defense against *C. rodentium* through effects on goblet cell mucus production and in the modulation of macrophage phenotype and function.

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Keywords

Muscarinic receptor; *Citrobacter rodentium*; T_H1/T_H17 immunity; Colitis; Mucus

Introduction

Inflammatory bowel disease (IBD), comprised primarily of ulcerative colitis and Crohn's Disease, affects approximately 3.6 million people in the United States and Europe (1). Despite advances in understanding the roles of genetics, the immune system, and gut flora, the pathogenesis of IBD remains poorly understood. Nonetheless, IBD is more likely to occur in genetically-predisposed hosts that possess aberrant defensive responses to gut microbes, thereby triggering inappropriate activation of the immune system directed at autoantigens within the intestinal mucosa (2).

Although perturbations in mucosal immunity are linked to the development of IBD (3, 4), other factors such as genetics, environmental exposures, and alterations in the microbiome contribute. Infection with *Citrobacter rodentium*, a gram-negative attaching/effacing bacterium, evokes a highly polarized T_H1/T_H17 response (5–7) and serves as a model for Crohn's disease. Epithelial cell hyperplasia accompanied by an influx of CD4⁺ cells and mucosal damage occurs and closely mimics the features of Crohn's disease (5). Innate and adaptive immune responses, including upregulation of T_H1/T_H17 cytokines, as well as goblet cell mucus production and epithelial cell proliferation are required for *C. rodentium* clearance (8–10). The colonic mucus layer serves a variety of functions including lubrication, minimizing mechanical trauma to underlying mucosa, and acting as a nutrient source for commensal bacteria (11, 12) in addition to contributing to innate immunity by sequestering luminal antigens from gastrointestinal (GI) epithelium (13). Defects in the mucus layer allow increased contact between intestinal mucosa and enteric bacteria (12, 14), in turn promoting immune activation. Cholinergic activation of α7 nicotinic receptors (NR) attenuates TNF-α production (15). Activation of macrophage α7 NR is associated with attenuation of septic shock, post-operative ileus, arthritis, pancreatitis, and dextran sulfate sodium-induced colitis (16). Muscarinic receptors (MR), one of two types of cholinergic receptors along with NR, are G protein-coupled receptors that mediate cholinergic neurotransmission at effector cells. Five MR are identified (designated M1R – M5R, encoded by *Chrm1–5*). Altered muscarinic tone can adversely affect organ function as in chronic obstructive pulmonary disease and urinary retention (17). Within the intestine, M3R is implicated in intestinal epithelial proliferation, key to maintaining and regulating post-injury restitution of mucosal barrier function (18). In addition, MR are linked to mucus production and secretion within the GI tract (19, 20). Interestingly, MR activity has been associated with pro- and anti-inflammatory actions, depending on the tissue and MR subtype studied (21–23).

The current study was designed to investigate the role of M3R in colonic mucosal homeostasis, its contribution to host defense against *C. rodentium* infection, and its ability to modulate macrophage phenotype and function. Our results demonstrate that M3R activity does not impact the host's ability to mount an appropriate immune response to *C. rodentium*

as upregulation of T_H1/T_H17 cytokines is preserved in *C. rodentium*-infected *Chrm3*^{-/-} mice. Instead, M3R activity appears to modulate *C. rodentium* clearance by regulating mucin production by goblet cells, a function that contributes to innate immunity. Also, our findings indicate that muscarinic-specific cholinergic stimulation of macrophages induces a classically activated macrophage (CAM) phenotype through a M3R-dependent effect. However, macrophages that do not express M3R retain their ability to differentiate into CAM in the presence of an inflammatory stimulus such as IFN- γ . Collectively, these data suggest that muscarinic stimulation of macrophages is pro-inflammatory. M3R activation facilitates clearance of enteric pathogens by modulating goblet cell mucus production and also exerts pro-inflammatory effects on macrophages.

Methods

Animal Studies

Age and sex-matched wild-type (WT) and M3R-deficient (*Chrm3*^{-/-}) mice on a C57BL/6 or 129/SvEv $\times$ CF1 (50%:50%) background were purchased from Taconic Farms (Germantown, NY) or were a generous gift from Dr. Jürgen Wess, National Institutes of Health (Bethesda, MD). Mice were housed at the United States Department of Agriculture animal facility and provided food and water *ad libitum*. Animals were euthanized using cervical dislocation prior to performing physiologic studies and collecting and processing tissues. Tissue samples were collected for molecular analysis, histological evaluation, and assessment of bacterial invasion.

Citrobacter Infection

A naladixic acid-resistant mutant of *C. rodentium* strain DBS100 (American Type Culture Collection 51459; Manassas, VA) was used to inoculate WT and *Chrm3*^{-/-} mice. *Citrobacter rodentium* was grown overnight in Luria-Bertani (LB) medium. The bacteria were collected by centrifugation and re-suspended in LB medium. WT and *Chrm3*^{-/-} mice were infected by oral gavage with 0.2 mL of the bacterial suspension containing approximately 1.0E+10 colony forming units (CFU) of *C. rodentium*. The dose was confirmed by retrospective plating on LB agar plates containing 50 μ g/mL naladixic acid.

Citrobacter Clearance and Invasion

Stool was collected from WT and *Chrm3*^{-/-} mice throughout the course of infection to determine the fecal *C. rodentium* content. Fecal pellets were homogenized in LB medium, diluted serially, and plated onto LB agar plates containing 50 μ g/mL naladixic acid. Spleen and mesenteric lymph nodes (MLN) were collected from uninfected mice and mice 13 days post-infection (DPI), homogenized in LB medium, and diluted serially. The presence of *C. rodentium* in spleen and MLN was determined by plating on LB agar plates. *C. rodentium* clearance was based on a colony count of zero obtained from duplicate plates inoculated with samples of undiluted fecal or spleen homogenates. The limit of detection was calculated based on a colony count of 0.5 for statistical and graphical purposes and is denoted by a dashed line in Fig. 1A and 2B.

Microsnapwell Assay for Mucosal Transepithelial Electrical Resistance

The modified microsnapwell system is a miniaturized version of the standard Ussing chamber designed to measure mucosal transepithelial electrical resistance (TEER) (24). Decreased TEER indicates increased tissue permeability. Segments of muscle-free colon were harvested from uninfected or *C. rodentium*-infected WT and *Chrm3*^{-/-} mice, stripped of both muscularis externa and serosal layers, and mounted in the microsnapwell system. A total of 250 μ L DMEM containing 4.5 g/L glucose, 4 mM L-glutamine, and 1 mM nonessential amino acids was added to the mucosal side. Three milliliters of the same medium was added to the serosal side. The system was incubated at 37°C with 5% CO₂ for 30 min to permit stabilization of pH and TEER was measured every 30 min for 180 min.

Epithelial Proliferation

Bromodeoxyuridine (BrdU) dissolved in PBS (10 mg/0.2 mL) was injected intraperitoneally 1 h prior to euthanasia of uninfected and *C. rodentium*-infected WT and *Chrm3*^{-/-} mice. Immunohistochemistry was performed utilizing anti-BrdU antibody (Roche Diagnostics; Mannheim, Germany) diluted at 1:200 to assess epithelial proliferation. Bound antibodies were detected using a Mouse on Mouse (M.O.M.) kit (Vector Laboratories; Burlingame, CA) according to the manufacturer's instructions. The number of BrdU-positive cells per crypt was determined by counting 20 well-oriented crypts per stained section. For each mouse, BrdU-positive cells per crypt were averaged to yield a mean per animal.

RNA extraction, cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissue samples (distal colon) or bone-marrow derived macrophages (BMDM) with TRIzol reagent (Invitrogen; Carlsbad, CA) according to manufacturer's instructions. RNA integrity, quantity, and genomic DNA contamination were assessed using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies; Palo Alto, CA). Only those RNA samples with 28S/18S ratios between 1.5 and 2 and no DNA contamination were studied further. RNA samples (2 μ g) were reverse-transcribed to cDNA using the First Strand cDNA Synthesis Kit (MBI Fermentas; Hanover, MD) with random hexamer primer.

Amplification reactions were performed on an iCycler detection system (Bio-Rad Laboratories; Hercules, CA). Primer sequences were designed using Beacon Designer 5.0 (Premier Biosoft International; Palo Alto, CA), and synthesized by the Biopolymer Laboratory of the University of Maryland. PCR was performed in 25- μ L volume wells using SYBR Green Supermix (Bio-Rad Laboratories; Hercules, CA). Amplification conditions were: 95°C for 3 min, 60 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. The fold-change in mRNA expression for targeted genes was calculated relative to respective vehicle-treated groups of mice after normalization to 18s rRNA. 18s rRNA was selected as the internal standard based on preliminary studies demonstrating no significant differences in 18s rRNA level among different groups of samples studied.

Immunofluorescence

Colons were opened longitudinally along the mesenteric border, fixed for 2 h in 4% paraformaldehyde, and embedded in paraffin blocks. Five-micrometer sections affixed to glass slides were de-waxed in xylene and rehydrated in descending ethanol baths. Antigen retrieval was achieved by incubating slides in sodium citrate buffer. Nonspecific binding of antibodies was blocked with 5% normal goat serum in PBS containing 2% BSA and 0.05% Tween-20. Sections were incubated with anti-*C. rodentium* (25), a generous gift from Dr. Philip Sherman (The Hospital for Sick Children; Toronto, Canada), overnight at 4°C at a dilution of 1:300. After washing with PBS, slides were incubated with goat anti-rabbit at a dilution of 1:500 for 45 min and then washed with PBS. Slides were mounted using mounting medium containing DAPI (Sigma-Aldrich; St. Louis, MO). Images were acquired with an Axio Imager M2 microscope (Carl Zeiss Microscopy; Thornwood, NY) using ZEN Pro 2012 image acquisition software (Carl Zeiss Microscopy; Thornwood, NY).

Preparation and Treatment of Bone Marrow–Derived Macrophages

Macrophages were prepared from bone marrow mononuclear cells as previously described (26). For each experiment mononuclear cells were obtained by flushing bone marrow from femurs, tibiae, and humeri of 3–5 WT or *Chrm3*^{-/-} mice with HyClone alpha MEM medium (Thermo-Scientific; Chicago, IL) pre-equilibrated at 37°C. Cells were cultured overnight in alpha MEM medium containing 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂. Non-adherent cells were collected by centrifugation after lysis of red blood cells using red blood cell lysis buffer (Sigma-Aldrich; St. Louis, MO) and mononuclear cells were counted and plated. Mature macrophages were generated by differentiating isolated mononuclear cells with 20 ng/mL rM-CSF (R&D Systems; Minneapolis, MN) for 7 days. WT or *Chrm3*^{-/-} macrophages were then treated with IFN-γ, IL-4, bethanechol, atropine, or a combination of these agents for 24 h to determine their ability to attain a CAM or AAM phenotype.

Solutions and Drugs

All drugs used for physiological studies were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Data Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) with post hoc analysis for multiple comparisons. For two comparisons, statistical analyses were performed using a Student's *t*-test. Data analyses were performed using Graph Pad Prism software version 3.03. Differences between groups were considered statistically significant at *p*-values < 0.05.

Ethical Considerations

All studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Health and Human Services Publication (National Institutes of Health 85-23, revised 1996), and the Beltsville Area Animal Care and Use Committee (Protocol

#10-003). The protocol was also approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine (Protocol #0113014).

Results

M3R-Deficiency does not Alter Colonic Constitutive Cytokine Expression, Mucosal Histology, or Permeability

Increased expression of the T_H1/T_H17 cytokines IFN- γ , TNF- α , and IL-17 (27–30) or the T_H2 cytokines IL-4 and IL-13 (31, 32) are implicated in epithelial barrier dysfunction. Moreover, perturbations in intestinal permeability resulting from epithelial barrier dysfunction are associated with autoimmune diseases including IBD (33). We examined gene expression of T_H1/T_H17 and T_H2 cytokines in whole-tissue distal colon harvested from WT and *Chrm3*^{-/-} mice. Gene expression of IFN- γ , TNF- α , and IL-17 (Figure S1A) was similar in both strains, as was expression of the T_H2 cytokines IL-4 and IL-13 (Figure S1B). TEER, which serves as an inverse measurement of permeability, also did not differ between muscle-free colon from WT and *Chrm3*^{-/-} mice (Figures S1C & S1D). Finally, no morphological differences were identified in H&E-stained sections of proximal colon from WT and *Chrm3*^{-/-} mice (Figure S1E & S1F).

Clearance of *C. rodentium* from *Chrm3*^{-/-} Mice is Delayed

Stool was collected from WT and *Chrm3*^{-/-} mice and the fecal burden of *C. rodentium* was determined. Fecal *C. rodentium* content was greater in stool from *Chrm3*^{-/-} compared to WT mice at every time point studied (Figure 1A). To confirm increased *C. rodentium* mucosal adherence in *Chrm3*^{-/-} mice, we probed colonic mucosal tissue from uninfected and *C. rodentium*-infected WT and *Chrm3*^{-/-} mice with a polyclonal antibody directed against *C. rodentium* (25). At 13 DPI, *C. rodentium* was detected in colons from *Chrm3*^{-/-}, but not WT mice (Figure 1B), indicating prolonged adherence of *C. rodentium* in the former.

Increased Spleen Mass and Augmented Bacterial Invasion in *C. rodentium*-Infected *Chrm3*^{-/-} Mice

As an index of immune response, we determined spleen weight normalized to body weight of *C. rodentium*-infected WT and *Chrm3*^{-/-} mice. This was greater at 13 and 21 DPI in *Chrm3*^{-/-} mice compared to WT mice 13 DPI or uninfected *Chrm3*^{-/-} mice (Figure 2A). These data suggest an augmented immune response to *C. rodentium* in *Chrm3*^{-/-} compared to WT mice.

We also examined MLN and spleens for the presence of *C. rodentium*. Thirteen DPI *C. rodentium* was detected in spleens, but not MLN from WT and *Chrm3*^{-/-} mice (data not shown). Bacterial invasion was present in only one of four WT spleens (25%), but in three of four *Chrm3*^{-/-} spleens (75%) (Figure 2B), indicating increased susceptibility to bacterial translocation in *Chrm3*^{-/-} mice.

Amplified Cytokine Response in *C. rodentium*-Infected *Chrm3*^{-/-} Mice

In infected rodents, *C. rodentium* induces a stereotypic T_H1/T_H17 response (5–7). We determined cytokine expression in uninfected and *C. rodentium*-infected WT and *Chrm3*^{-/-}

whole-tissue colon at 13 DPI because this day represents the approximate time point at which maximum T_H1/T_H17 cytokines upregulation is observed in *C. rodentium*-infected WT mice (Figure S2). In addition, cytokine expression was examined at 21 DPI as resolution of T_H1/T_H17 cytokine upregulation is expected by this point (Figure S2). Expression of the T_H1/T_H17 cytokines *IFN- γ* , *TNF- α* , and *IL-17A* was up-regulated 13 DPI in *Chrm3*^{-/-} colon compared to 13 DPI in WT colon and to uninfected *Chrm3*^{-/-} colon, and then diminished in both strains of mice at 21 DPI (Figure 3). Similarly, upregulation of *IL-22*, linked to both clearance of *C. rodentium* and mucin production, was preserved in *Chrm3*^{-/-} colon (Figure 3C). Furthermore, *Nos-2* expression was up-regulated at 13 DPI in *Chrm3*^{-/-} colon compared to WT colon and to uninfected *Chrm3*^{-/-} colon and then diminished in both strains of mice at 21 DPI (Figure 4A), indicating that *in vivo* *Chrm3*^{-/-} macrophages appropriately attain a CAM phenotype when exposed to T_H1/T_H17 cytokines. The amplified T_H1/T_H17 response in *Chrm3*^{-/-} colon indicates that *Chrm3*^{-/-} mice maintain an intact cytokine response to *C. rodentium*. Furthermore, enhanced gene expression of the T cell marker *CD3* suggests that T cell recruitment is amplified in *C. rodentium*-infected *Chrm3*^{-/-} colon (Figure 4B).

Enhanced Epithelial Proliferation in *Chrm3*^{-/-} Mouse Colon

Mucosal proliferation is an important component of host immune defense in the setting of infectious colitis (34). We assessed the ability of colon mucosa from *Chrm3*^{-/-} mice to proliferate in response to *C. rodentium*. BrdU was injected into normal and infected mice prior to euthanasia to label proliferating cells. *Chrm3*^{-/-} mucosa exhibited more BrdU staining than WT mucosa at 13 DPI (Figure 5A) indicating enhanced epithelial proliferation in *Chrm3*^{-/-} colon. By 21 DPI, BrdU staining in WT and *Chrm3*^{-/-} mucosa was similar and approached basal levels (Figure 5A). The number of BrdU-positive cells per crypt was counted; significantly more BrdU-positive cells per crypt were present in WT mucosa 13 DPI compared to uninfected WT mucosa. Similarly, 13 DPI there were significantly more BrdU-positive cells per crypt in *Chrm3*^{-/-} mucosa compared to uninfected *Chrm3*^{-/-} mucosa as well as WT mucosa 13 DPI (Figures 5A & 5B). These data demonstrate that colon epithelial cell proliferation in *C. rodentium*-infected *Chrm3*^{-/-} mice is amplified at 13 DPI.

Mucus Production

The intestinal mucus layer, produced by epithelial goblet cells, contributes to host defense by inhibiting the ability of pathogens to adhere to intestinal and colonic mucosa (35). The number of goblet cells per crypt decreased in response to *C. rodentium* infection in *Chrm3*^{-/-} mucosa 13 DPI compared to uninfected *Chrm3*^{-/-} mucosa, but did not differ between WT mucosa 13 DPI and uninfected WT mucosa (Figures 6A & 6E). Gene expression of *CLCA3*, a calcium-activated chloride channel that is a marker of goblet cell hyperplasia (36), was significantly lower in *Chrm3*^{-/-} compared to uninfected *Chrm3*^{-/-} colon 13 DPI; however, gene expression of *CLCA3* in WT colon 13 DPI did not differ from uninfected WT colon (Figure 6B). Mucin-2, encoded by *Muc2*, is the primary mucin in the small intestine and colon (37, 38). Expression of *Muc2* was decreased significantly in *Chrm3*^{-/-} colon 13 DPI compared to uninfected *Chrm3*^{-/-} colon, but did not differ between WT colon 13 DPI and WT uninfected colon (Figure 6C). *Muc5AC* participates in host

defense against enteric pathogens such as nematodes (39). No compensatory upregulation of *Muc5AC* occurred in either WT or *Chrm3*^{-/-} *C. rodentium*-infected colon (Figure 6D). These data demonstrate differential expression of mucins in WT and *Chrm3*^{-/-} colon in response to *C. rodentium* infection.

WT Macrophages Express Cholinergic Receptors

Macrophages are a critical component of the innate immune system, contribute to tolerance to commensal organisms, and participate in host defense. To investigate the effects of M3R-deficiency on macrophage phenotype and function we generated and tested BMDM. To confirm that bone marrow cells differentiated in the presence of M-CSF were macrophages, we determined the proportion of cells expressing both CD11b and F4/80 by flow cytometry. Approximately 95% of cells expressed both CD11b and F4/80 (Figure S3), indicating that the cell population was highly enriched in macrophages.

Expression of *Chrm3* is Increased by Muscarinic Specific Stimulation of WT Macrophages as well as by IFN- γ Treatment of WT Macrophages

Treatment of WT BMDM with bethanechol, a muscarinic-specific agonist, upregulates expression of the gene for M3R (*Chrm3*) (Figure 7A). Expression of *Chrm3* was also increased by treatment with the T_H1 cytokine IFN- γ (Figure 7B). Treatment with the T_H2 cytokine IL-4 did not affect *Chrm3* expression. IFN- γ treatment did not affect expression of other muscarinic receptor genes expressed by macrophages, nor did it affect expression of the $\alpha 7$ nicotinic receptor gene (Figure 7C).

Muscarinic-Specific Stimulation of Macrophages Induces a Classically Activated Phenotype via M3R

Cholinergic tone exerts anti-inflammatory effects on macrophages via the $\alpha 7$ NR (40–42); however, the effect of muscarinic specific tone on macrophages is uncertain. Expression of *Nos-2*, a marker for CAM, was increased significantly in WT BMDM treated with bethanechol indicating that WT BMDM differentiate into CAM upon muscarinic stimulation (Figure 8A). *Nos-2* expression was unchanged in *Chrm3*^{-/-} BMDM treated with the same dose of bethanechol (Figure 8A). In addition, gene expression of arginase-1 (*Arg-1*), a marker of AAM, decreased significantly when WT BMDM were treated with bethanechol (Figure 8B), indicating that these macrophages have an impaired ability to attain an AAM phenotype. Finally, atropine treatment significantly decreased *Nos-2* gene expression in WT BMDM, but not in *Chrm3*^{-/-} BMDM (Figure 8C), further demonstrating that muscarinic-specific stimulation induces development of CAM.

Chrm3^{-/-} Macrophages Retain Their Ability to Attain a Classically Activated Phenotype in the Presence of Interferon- γ

Macrophages exposed to T_H1 cytokines such as IFN- γ attain a CAM phenotype (43). As expected, *Nos-2* gene expression increased significantly when WT BMDM were treated with IFN- γ (Figure 8D). Similarly, *Nos-2* gene expression increased significantly when *Chrm3*^{-/-} BMDM were treated with IFN- γ (Figure 8D), indicating that M3R is not required for BMDM to attain a CAM phenotype in the presence of IFN- γ . These data indicate that

Chrm3^{-/-} BMDM retain the ability to differentiate into CAM in the setting of a cytokine stimulus (IFN- γ), but their ability to differentiate into CAM in response to a muscarinic stimulus requires M3R expression.

Muscarinic Stimulation Acts Synergistically with Interferon- γ to Promote Development of a Classically Activated Macrophage Phenotype

We examined the ability of muscarinic stimulation to amplify the response of BMDM to IFN- γ . Gene expression of *Nos-2* in WT BMDM treated with bethanechol and IFN- γ increased when compared to treatment with IFN- γ alone (Figure 8E). This effect was not observed in *Chrm3*^{-/-} BMDM (Figure 8F). Treating BMDM with atropine, in addition to IFN- γ and bethanechol, abrogated the bethanechol-induced upregulation of *Nos-2* in WT BMDM (Figure 8E). Treatment of *Chrm3*^{-/-} BMDM with atropine in addition to IFN- γ and bethanechol did not alter *Nos-2* gene expression (Figure 8F). These data indicate that muscarinic-specific stimulation amplifies development of a CAM phenotype in IFN- γ -treated WT BMDM, an effect dependent on M3R expression.

Discussion

M3R are expressed on epithelial cells, including goblet cells, as well as on immune cells such as macrophages. In the present study we investigated the hypothesis that M3R affects *C. rodentium* clearance from *Chrm3*^{-/-} mice through direct effects on epithelial and/or immune cell function. We showed that clearance of *C. rodentium* was delayed in *Chrm3*^{-/-} mice, despite an intact cytokine response *in vivo*. Of interest is that genetic ablation of M3R affected goblet cell mucus production, thereby facilitating bacterial adherence to colonic mucosa. These data provide new insights into the importance of non-hematopoietic cells in host-pathogen interactions.

The contribution of the colonic mucus layer to host defense against *C. rodentium* is established (13, 44, 45). Delayed *C. rodentium* clearance from *Chrm3*^{-/-} mice, determined by fecal bacteria counts, was confirmed by demonstrating persistent adherence of *C. rodentium* to colonic mucosa as well as a greater crypt hyperproliferation 13 DPI when compared to *C. rodentium*-infected WT mice. Crypt hyperplasia is important to the clearance of *C. rodentium* and is attributed to the secretion of pathogen proteins that promote adherence to epithelial cells (46), epithelial growth factor receptor activation (47), and IFN- γ (48). Delayed clearance from *Chrm3*^{-/-} mice occurred despite an amplified upregulation of colonic IFN- γ as well as IL-17A, indicating that the mucosal immune system responds appropriately to *C. rodentium* infection in the absence of M3R. Thus, both the delay in clearance and amplified expression of T_H1/T_H17 cytokines are a consequence of prolonged adherence of *C. rodentium* to *Chrm3*^{-/-} colonic mucosa, rather than a defective immune response.

Goblet cells are an important source of mucins, such as Muc2, that form a physical barrier and contain protective factors such as RELM- β as well as antimicrobial peptides that are important to the maintenance of barrier function (49). Muc2 is part of the first line of defense against enteric pathogens and goblet cell depletion is a feature of *C. rodentium* infection. A proposed role of secreted mucus is to impede bacterial adherence, demonstrated

by *C. rodentium*-infected *Muc2*^{-/-} mice suffering up to 90% mortality (12, 45). Conversely, others suggested a temporal role of mucus production in *C. rodentium* infection with secretion early in infection followed by goblet cell depletion and decreased *Muc2* coupled with IFN- γ linked epithelial hyperproliferation that is protective (48). In the present study, clearance of *C. rodentium* from *Chrm3*^{-/-} mice at 13 DPI was associated with reduced goblet cell number and a 91% decrease in *Muc2* expression. In contrast, in *C. rodentium*-infected WT mice, goblet cell number was maintained and only a 41% decrease in *Muc2* expression from baseline occurred. These data are consistent with a protective role for mucus (*Muc2*) and demonstrate that an impaired mucus response to *C. rodentium* infection occurs even in the presence of intact IFN- γ and hyperproliferative responses.

Goblet cells secrete mucin in response to various stimuli by two distinct processes – simple and compound exocytosis (44). Simple exocytosis of mucus occurs in a constitutive, basal fashion whereas compound exocytosis is the result of exposure to various secretagogues, including neurotransmitters (such as acetylcholine), hormones, neuropeptides, and cytokines. In addition, the thickness of the intestinal mucus layer is directly proportional to goblet cell number and size (50), indicating that *Chrm3*^{-/-} mice are likely to have a thinner mucus layer than WT controls. It is known that cholinergic signaling triggers mucin release from goblet cells (20). Our data support a role for muscarinic signaling, specifically via M3R, in this process. The observation that *Chrm3*^{-/-} mice do not succumb to *C. rodentium*-infection indicates that M3R facilitates, but is not required for clearance of *C. rodentium*. Our studies link delayed *C. rodentium* clearance from *Chrm3*^{-/-} mice to impaired mucin production and/or secretion. This finding, which is likely a consequence of disrupted cholinergic signaling in M3R-deficient goblet cells, establishes an important role for M3R on non-hematopoietic cells in host defense. Our studies also demonstrate that M3R exerts pro-inflammatory effects on macrophage phenotype, an alternate pathway to the established anti-inflammatory role of $\alpha 7$ NR for modulating macrophage function via cholinergic signaling. Macrophage phenotype is influenced greatly by the local microenvironment. T_H1 cytokines or lipopolysaccharide stimulates macrophages to become CAM, which release cytokines such as TNF- α , IL-1, and IL-6 while T_H2 cytokines stimulate the formation of alternatively activated macrophages (AAM), which promote wound healing and tissue repair. Compared to $\alpha 7$ NR, few reports address the ability of MR to modulate inflammation. Prior studies suggest that muscarinic stimulation of alveolar macrophages may exert pro-inflammatory effects (51). Others examined the ability of MR to impact leukocyte infiltration and the response to tissue injury, concluding that MR act in opposition to NR (52). In the present study, we demonstrated that activation of M3R on macrophages promotes the expression of pro-inflammatory cytokines and CAM markers *in vitro* indicating that cholinergic stimulation has pro- or anti-inflammatory effects mediated by activation of M3R or $\alpha 7$ NR, respectively.

In isolated BMDM, we show that muscarinic stimulation is capable of inducing a CAM phenotype, an effect dependent on the presence of M3R. Interestingly, stimulation with IFN- γ increases M3R expression, suggesting a role for M3R in immune regulation of macrophage function. When stimulated with a muscarinic-specific agonist, macrophages attain a CAM phenotype (increased *Nos-2* expression). When macrophages were exposed to both inflammatory and muscarinic stimuli, upregulation of *Nos-2* was further amplified

compared to IFN- γ treatment alone, again supporting the ability of muscarinic stimulation to exert pro-inflammatory effects on macrophages. We observed an intact upregulation of T_H1/T_H17 cytokines in response to *C. rodentium* infection suggesting M3R activation during an immune response *in vivo* plays a modulatory role.

It is well established that cholinergic tone exerts a net anti-inflammatory effect on macrophages (Figure 9A). Our *in vitro* data indicate that IFN- γ upregulates M3R expression on macrophages. This may act to counter-balance anti-inflammatory effects of α 7 NR stimulation (Figure 9B), preventing or attenuating the development of AAM. These divergent actions of cholinergic signaling are likely to have important consequences for the organism's ability to mount an effective response to pathogens as α 7 NR stimulation ameliorates inflammatory mediator release *in vitro* (53), but is associated with more severe experimental colitis *in vivo* (54). The ability of vagus nerve stimulation to modulate various inflammatory states or conditions is an area of active investigation (55). At present, it appears that cholinergic tone exerts relatively greater effect via α 7 NR rather than M3R as activation of both MR and NR by acetylcholine exerts anti-inflammatory effects on macrophages (15); however, if *in vivo* studies confirm a pro-inflammatory role for M3R on immune cells, exploration of the ability to modulate inflammatory conditions via M3R would be warranted.

Overall, our study provides novel evidence that M3R contributes to host defense against the enteric bacterium *C. rodentium* through alterations in mucus production and exerts pro-inflammatory effects on macrophages. These studies highlight an expanding role for cholinergic receptors, specifically MR, as modulators of non-hematopoietic cell function in colonic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Fecal bacterial content of *C. rodentium* infected WT (black bars) and *Chrm3*^{-/-} (white bars) mice at 6, 9, 12, 14, 16, and 19 DPI. N = 8–10 mice per strain at 6, 9, 12 DPI. N = 3–5 mice per strain at 14, 16, 19 DPI. Student's *t*-test was used to determine differences in bacterial content between WT and *Chrm3*^{-/-} mice at each time point. The dashed line indicates the limit of detection for the assay.

CFU: colony forming unit. WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. * *p* < 0.05 vs. WT. CR: *C. rodentium*. DPI: days post-infection.

(B) Immunofluorescent staining demonstrating persistent adherence of *C. rodentium* in *Chrm3*^{-/-} colon mucosa 13 DPI. Uninfected WT and *Chrm3*^{-/-} mucosa serve as negative controls. Images are representative of two experiments.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. DAPI: 4',6-diamidino-2-phenylindole.

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Figure 2.

(A) Spleen weight per body weight in *C. rodentium*-infected *Chrm3*^{-/-} mice 13 DPI is increased compared to spleen weight per body weight in WT mice 13 DPI and uninfected *Chrm3*^{-/-} mice. Each symbol represents one mouse. A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. V: vehicle. 13: 13 DPI. 21: 21 DPI. ** $p < 0.01$ vs. WT 13. $\xi\xi$ $p < 0.01$ vs. WT V. # $p < 0.05$ vs. *Chrm3*^{-/-} V.

(B) At 13 DPI *C. rodentium* was detected in 1 of 4 WT spleens and 3 of 4 *Chrm3*^{-/-} spleens. Each symbol represents one mouse. The dashed line indicates the limit of detection for the assay.

CFU: colony forming unit. WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. V: vehicle.

Figure 3.

(A) Gene expression of IFN- γ in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

(B) Gene expression of TNF- α in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

(C) Gene expression of IL-17A in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

(D) Gene expression of IL-22 in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. V: vehicle. 13: 13 DPI. 21: 21 DPI. ** p < 0.01 vs. WT vehicle. * p < 0.05 vs. WT vehicle. ## p < 0.01 vs. WT 13 DPI. ξξ p < 0.01 vs. *Chrm3*^{-/-} V.

Figure 4.

(A) Gene expression of *Nos-2* in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

(B) Gene expression of *CD3* in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. V: vehicle. 13: 13 DPI. 21: 21 DPI. ** p < 0.01 vs. WT vehicle. * p < 0.05 vs. WT vehicle. ## p < 0.01 vs. WT 13 DPI. # p < 0.05 vs. WT 13 DPI. ξξ p < 0.01 vs. *Chrm3*^{-/-} vehicle. ξ p < 0.05 vs. *Chrm3*^{-/-} vehicle.

A **B** **Figure 5.**

(A) BrdU-labeled cells in uninfected and *C. rodentium*-infected WT and *Chrm3*^{-/-} colon. Relative number of BrdU-positive cells is increased in *Chrm3*^{-/-} colon 13 DPI. Images are representative of two experiments.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. BrdU: bromodeoxyuridine.

(B) Mean $\pm$ SEM of BrdU-positive cells per crypt in uninfected and *C. rodentium*-infected WT (black bars) and *Chrm3*^{-/-} (white bars) colon mucosa. Twenty crypts counted per genotype per time point.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. SEM: Standard error. V: vehicle. 13: 13 DPI. 21: 21 DPI. ** p < 0.01 vs. WT vehicle. * p < 0.05 vs. WT vehicle. ## p < 0.01 vs. WT 13 DPI. §§ p < 0.01 vs. *Chrm3*^{-/-} vehicle.

Figure 6.

(A) Goblet cells per crypt in uninfected and *C. rodentium*-infected WT (black bars) and *Chrm3*^{-/-} (white bars) mice. N = 3 mice per genotype per time point with 10 crypts counted per mouse.

(B) Gene expression of *CLCA3* in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice per genotype per time point.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

(C) Gene expression of *Muc2* in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice/genotype.

(D) Gene expression of *Muc5AC* in WT (black bars) and *Chrm3*^{-/-} (white bars) uninfected and *C. rodentium*-infected colon. N = 3–5 mice/genotype.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below.

(E) H&E stained sections of uninfected and *C. rodentium*-infected WT and *Chrm3*^{-/-} colon. WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. V: vehicle. 13: 13 DPI. 21: 21 DPI. ** p < 0.01 vs. WT vehicle. * p < 0.05 vs. WT vehicle. ## p < 0.01 vs. *Chrm3*^{-/-} vehicle. # p < 0.05 vs. *Chrm3*^{-/-} vehicle. H&E: hematoxylin and eosin.

Figure 7.

(A) Gene expression of *Chrm3* in WT BMDM treated with vehicle (black bar) or bethanechol 1–100 nM (white bars). Data are representative of three experiments.

(B) Gene expression of *Chrm3* in vehicle, IFN- γ (10 ng/mL), and IL-4 (20 ng/mL) treated WT BMDM. Data are representative of three experiments.

(C) Gene expression of *Chrm1*, *Chrm2*, *Chrm4*, and $\alpha 7$ in vehicle (black bars) and IFN- γ (10 ng/mL, white bars) treated WT BMDM. Data are representative of three experiments. A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below (A&B). Student's *t*-test was used to determine differences in cholinergic receptor expression between WT and *Chrm3*^{-/-} macrophages (C).

** $p < 0.01$ vs. WT vehicle.

Figure 8.

(A) Gene expression of *Nos-2* in WT and *Chrm3*^{-/-} BMDM treated with vehicle (black bars) or bethanechol 100 nM (white bars). Data are representative of three experiments.

(B) Gene expression of *Arg-1* in WT BMDM treated with vehicle (black bar) or bethanechol (100 nM, white bar). Data are representative of three experiments.

(C) Gene expression of *Nos-2* in WT and *Chrm3*^{-/-} BMDM treated with vehicle (black bars) or atropine 50 nM (white bars). Data are representative of three experiments.

(D) Gene expression of *Nos-2* in WT and *Chrm3*^{-/-} BMDM treated with vehicle or IFN- γ (10 ng/mL, white bars). Data are representative of three experiments.

Student's *t*-test was used to determine differences in *Nos-2*/*Arg-1* expression between WT and *Chrm3*^{-/-} macrophages (A, B, C).

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. * $p < 0.05$ vs. WT vehicle. ** $p < 0.01$ vs. WT vehicle.

(E) Gene expression of *Nos-2* in WT BMDM treated with IFN- γ 10 ng/mL (black bars), IFN- γ 10 ng/mL and bethanechol 100 nM (white bars), and IFN- γ 10 ng/mL, bethanechol 10 nM, and atropine 50 nM (hatched bars). Data are representative of three experiments.

(F) Gene expression of *Nos-2* in *Chrm3*^{-/-} BMDM treated with IFN- γ 10 ng/mL (black bars), IFN- γ 10 ng/mL and bethanechol 100 nM (white bars), and IFN- γ 10 ng/mL, bethanechol 10 nM, and atropine 50 nM (hatched bars). Data are representative of three experiments.

A one-way ANOVA with post hoc analysis for multiple comparisons was used to determine differences between comparisons as indicated below (E, F).

WT: Wild type. *Chrm3*^{-/-}: M3R-deficient. IFN: interferon- γ . BTH: bethanechol. ATR: atropine.

* $p < 0.05$ vs. IFN- γ .

Figure 9.

Proposed model of effects of M3R on macrophage phenotype.

(A) In the absence of an inflammatory stimulus, acetylcholine acts at both $\alpha 7$ and M3R with net effect driving the macrophage towards an AAM phenotype.

(B) In the presence of T_H1/T_H17 cytokines, M3R expression on macrophages is increased and acetylcholine acts at relatively greater number of M3R, causing the macrophage to attain a more classically activated phenotype (or a less alternatively activated phenotype).

$\alpha 7$: alpha 7 nicotinic receptor. M3: type 3 muscarinic receptor. *C. rod.*: *C. rodentium*