

Chlamydia muridarum Infection Impacts Murine Models of Intestinal Inflammation and Cancer

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Chlamydia muridarum has reemerged as a prevalent infectious agent in research mouse colonies. Despite its prevalence and ability to persistently colonize the murine gastrointestinal tract, few studies have evaluated the potential impact of *C. muridarum* on experimental models of gastrointestinal disease. Studies were conducted to evaluate the impact of *C. muridarum* on the *Citrobacter rodentium*, *Trichuris muris*, and *Il10*^{-/-} mouse models of intestinal inflammation, as well as on tumorigenesis in the Apc^{Min/+} mouse following administration of dextran sodium sulfate (DSS). Naïve C57BL/6J (B6), B6.129P2-*Il10*^{tm1Cgn/J} (*Il10*^{-/-}), and C57BL/6J-Apc^{Min/J} (Apc^{Min/+}) mice were infected with *C. muridarum* by cohousing with chronic *C. muridarum*-shedding BALB/cJ mice for 2 weeks; controls were cohoused with *C. muridarum*-free mice. After cohousing, B6 mice (*n* = 8 *C. muridarum* infected and free) were infected with *C. rodentium* (10⁹ CFU orally) or *T. muris* (200 ova orally). *Il10*^{-/-} mice (*n* = 8/group with and without *Helicobacter hepaticus* [10⁸ CFU/mouse] and with and without *C. muridarum*) and Apc^{Min/+} mice (*n* = 8/group) that received 2% DSS for 7 days in drinking water after cohousing. Mice were euthanized 14 days post-*C. rodentium* infection, 18 days post-*T. muris* infection, 60 days post-*H. hepaticus* infection, or control with *Il10*^{-/-} mice, and 28 days post-DSS administration to Apc^{Min/+} mice. The severity of the cecal and colonic lesions was evaluated and graded using a tiered, semiquantitative scoring system. *C. muridarum* infection attenuated colitis associated with *C. rodentium* (*P* = 0.03), had no effect on *T. muris*-associated pathology (*P* = 0.22), worsened colitis in *Il10*^{-/-} mice in the absence of *H. hepaticus* (*P* = 0.007), and reduced chemically induced colonic tumorigenesis in Apc^{Min/+} mice (*P* = 0.004). Thus, *C. muridarum* colonization differentially impacts several models of intestinal inflammation and tumorigenesis, and the presence of this bacterium in mouse colonies should be considered as a variable in these experimental readouts.

Abbreviations and Acronyms: Apc, adenomatous polyposis coli; B6, C57BL/6J; BCS, body condition score; C, BALB/cJ; DSS, dextran sodium sulfate; IHC, immunohistochemistry; ISH, in situ hybridization; GIT, gastrointestinal tract; MOMP, *Chlamydia* major outer membrane permeabilization

DOI: 10.30802/AALAS-JAALAS-25-078

Introduction

Chlamydia muridarum, a Gram-negative obligate intracellular bacterium, was initially described in the 1940s as mouse pneumonitis virus in mice used to investigate the causes of human respiratory infections.¹ Approximately a decade later, the organism was identified as *Chlamydia trachomatis* and in the 1990s was reclassified as *C. muridarum*, the only *Chlamydia* species known to naturally infect the family Muridae. *C. muridarum* had not been detected in research mice since these original descriptions,

although it has been used extensively to model genital tract infections in humans caused by *C. trachomatis*.²

We recently reported on the unexpected reemergence of *C. muridarum* in research mouse colonies. About a third of the mice imported to our institution, principally from other US and European academic institutions, were infected with *C. muridarum*.¹ Surveys of approximately 900 and 11,000 murine diagnostic and environmental samples submitted to research animal diagnostic laboratories from 120 institutions demonstrated that 16% and 14%, respectively, were quantitative PCR (qPCR) positive for *C. muridarum*.¹ This prevalence is markedly higher than most excluded murine adventitious agents including mouse hepatitis virus and mouse parvovirus, which were recently reported to have less than a 0.3% prevalence.³

C. muridarum has a 2-stage life cycle consisting of the infectious, nonreplicating elementary body and a replicating, noninfectious reticulate body.² Natural transmission is primarily fecal-oral.⁴ Natural infection of immunocompetent mouse strains is not associated with clinical signs, while immunocompromised strains develop respiratory signs, weight loss, and pulmonary pathology following natural infection.^{4–6} In immunocompetent mice, *C. muridarum* elicits IgG secretion and transient lymphocyte proliferation in draining mesenteric lymph nodes as well as induction of monocytes and activation

Submitted: 13 May 2025. Revision requested: 12 Jun 2025. Accepted: 16 Jul 2025.

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of T-cell subsets resulting in a significant reduction of *C. muridarum* within the gut, but the bacterium persists in the cecum and large intestine while being shed chronically in the feces.^{4,6}

Despite the high prevalence, little is known about the potential confounding effects that *C. muridarum* infection may have on research models. Since most mouse strains are persistently infected with *C. muridarum*, which has been shown to elicit both innate and adaptive immune responses, we speculated that *C. muridarum* may impact murine research models used to study intestinal inflammation and neoplasia.⁶ Wang et al⁷ recently showed that oral inoculation of *C. muridarum* protects against dextran sodium sulfate (DSS)-induced colitis, likely by promoting IL-22 secretion, a cytokine with strong antimicrobial properties. The DSS-induced model of colitis is only one of many commonly used murine models of intestinal inflammation that could potentially be impacted by *C. muridarum*. Based on the pathways involved in the pathophysiology of the associated disease, we investigated the impact of *C. muridarum* on the *Citrobacter rodentium*, *Trichuris muris*, and IL-10 knockout models of intestinal inflammation as well as in the *Apc*^{Min/+} mouse model of tumorigenesis.

Materials and Methods

Experimental models. To investigate the impact of *C. muridarum* infection on various murine gastrointestinal models, we first infected C57BL/6J, B6.129P2-Il10^{tm1Cgn}/J (Il10^{-/-}), and C57BL/6J-Apc^{Min}/J (Apc^{Min/+}) mice with *C. muridarum* by cohousing with chronically shedding *C. muridarum*-infected BALB/cJ (C) mice for 2 weeks (1 infected C mouse/1-4 naïve experimental mice). Naïve *C. muridarum*-free C mice were cohoused with the same strains as controls. The experimental design and timeline are depicted graphically in Figure 1.

C. muridarum-positive and -negative B6 mice, confirmed by qPCR, were inoculated with either *C. rodentium* (*n* = 8/*C. muridarum*+ or *C. muridarum*-; 10⁹/CFUs) or *T. muris* (*n* = 8/*C. muridarum*+ or *C. muridarum*-; 200 ova) via orogastric gavage 14 days after cohousing with *C. muridarum*-shedding or *C. muridarum*-free C mice. Fourteen or 18 days post-*C. rodentium* or post-*T. muris* infection, respectively, mice were euthanized, feces were collected for *C. muridarum* and *C. rodentium* qPCR (*C. rodentium*-infected mice only), a complete necropsy was

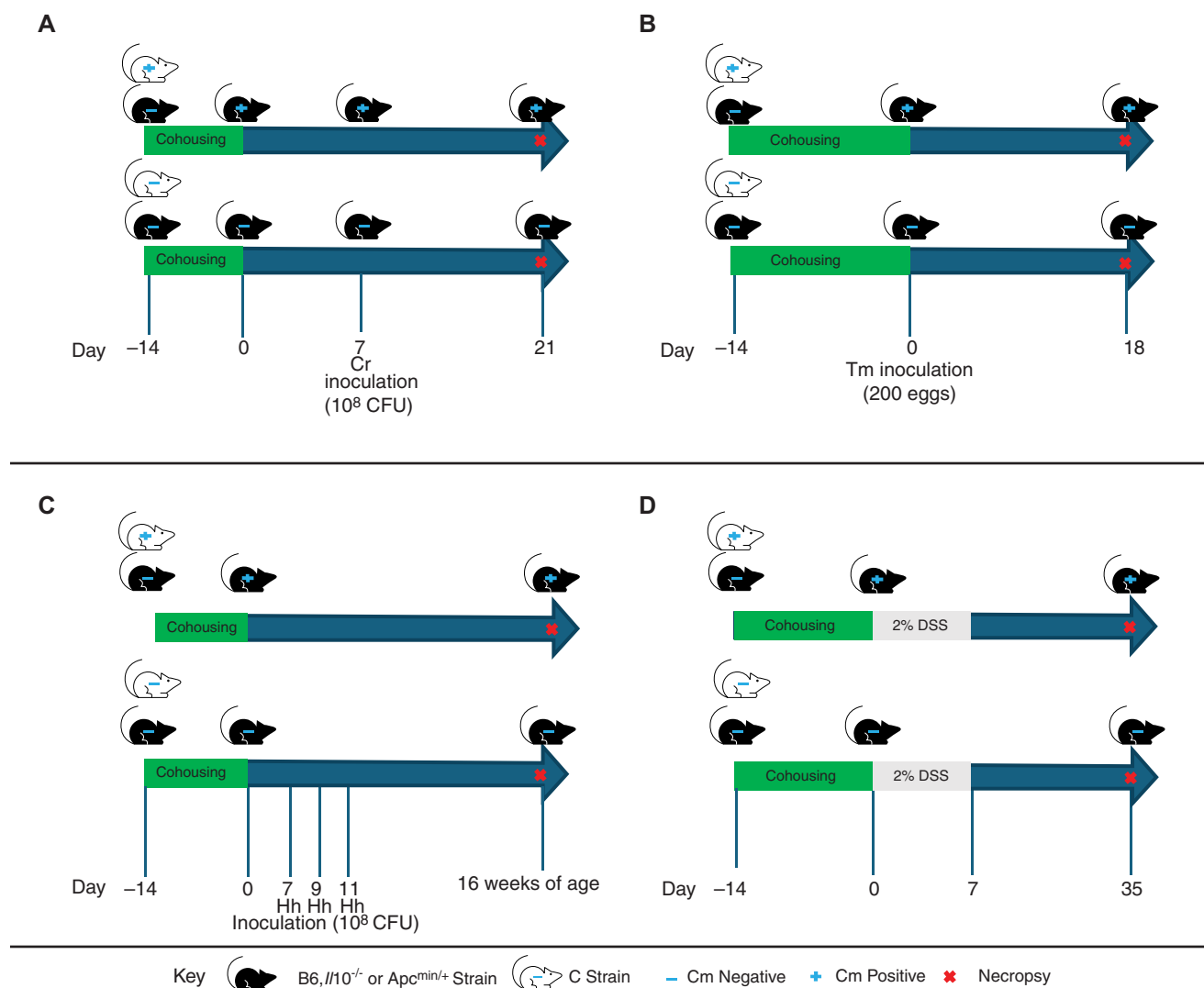


Figure 1. Graphical Representation of the Study Design and Timeline. Experimental design and timeline for (A) *Citrobacter rodentium* (Cr), (B) *Trichuris muris* (Tm), (C) Il10^{-/-}, and (D) Apc^{Min/+} experiments. *Citrobacter rodentium*, *T. muris*, and *Helicobacter hepaticus* (Hh) were administered via gavage. DSS was provided in the drinking water.

performed, and the intestinal lesions were scored using a modified published system.^{8,9}

II10^{-/-} mice ($n = 32$), 16 of which had been cohoused with *C. muridarum*-shedding and the remaining with *C. muridarum*-free C mice, were inoculated with *Helicobacter hepaticus* (10^8 CFU; $n = 16$) or saline ($n = 16$) via orogastric gavage every 2 days for a total of 3 doses at 7 weeks of age. At 16 weeks of age, mice were euthanized, feces were collected for *C. muridarum* and *H. hepaticus* qPCR, a complete necropsy was performed, and the intestinal lesions were scored using a modified scoring system.¹⁰

Apc^{Min/+} mice ($n = 8/8$; *C. muridarum* infected/*C. muridarum*-free) were provided 2% DSS in drinking water for 7 days immediately after cohousing with *C. muridarum*-infected or *C. muridarum*-free C mice for 2 weeks. Four weeks post-DSS treatment, mice were euthanized, and the number and location of gross tumors in the gastrointestinal tract (GIT) were counted under a dissecting microscope. Select tumors from each animal were analyzed histologically to confirm malignancy.

Animals. Female C57BL/6J (B6; $n = 32$; 6-week-old; The Jackson Laboratory, Bar Harbor, ME) mice were used for *C. rodentium* and *T. muris* experiments ($n = 16$ /experiment); female B6.129P2-II10^{tm1Cgn}/J (II10^{-/-}; $n = 32$; 4-week-old; The Jackson Laboratory) were used to assess inflammatory bowel disease; and female C57BL/6J-ApcMin/J (Apc^{Min/+}; $n = 16$; 4 week old; The Jackson Laboratory) were used to assess tumorigenesis. Female *C. muridarum*-infected and *C. muridarum*-free BALB/cJ (C; $n = 4$ and $n = 3$, respectively; greater than 16 week old; The Jackson Laboratory) mice were used for cohousing. Group sizes were selected following review of published studies using each model and based on recommendations of veterinary pathologists and researchers who use these models. In addition, using the resource equation method, a sample size of 8 with 2 groups yields an ANOVA degree of freedom of 14, which is sufficient to demonstrate statistical significance. Experiments were conducted once.

On study initiation all mice were free of ectromelia virus, Theiler meningoencephalitis virus, Hantaan virus, K virus, LDH elevating virus, lymphocytic choriomeningitis virus, mouse adenovirus, murine cytomegalovirus, murine chapparvovirus, mouse hepatitis virus, minute virus of mice, murine norovirus, mouse parvovirus, mouse thymic virus, pneumonia virus of mice, polyoma virus, reovirus type 3, epizootic diarrhea of infant mice (mouse rotavirus), Sendai virus, murine astrovirus-2, *Bordetella* spp., *C. rodentium*, *C. muridarum*, *Clostridium piliforme*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Filobacterium rodentium* (CAR bacillus), *Mycoplasma pulmonis* and other *Mycoplasma* spp., *Salmonella* spp., *Streptobacillus moniliformis*, *Helicobacter* spp., *Klebsiella pneumoniae* and *K. oxytoca*, *Pasteurella multocida*, *Rodentibacter pneumotropicus/heylii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and Beta-hemolytic *Streptococcus* spp., *Yersinia enterocolitica* and *Y. pseudotuberculosis*, *Proteus mirabilis*, *Pneumocystis murina*, *Encephalitozoon cuniculi*, ectoparasites (fleas, lice, and mites), endoparasites (tapeworms, pinworms, and other helminths), protozoa (including *Giardia* spp. and *Spironucleus* spp.), *Toxoplasma gondii*, trichomonads, and dematophytes.

Husbandry and housing. Mice were maintained in autoclaved, individually ventilated, polysulfone shoebox cages with stainless-steel wire-bar lids and filter tops (IVC; number 19; Thoren Caging Systems, Hazleton, PA) on autoclaved aspen chip bedding (PWI Industries, St. Hyacinthe, Quebec, Canada) at a density of no greater than 5 mice per cage. Each cage was provided with a Glatfelter paper bag containing 6 g of crinkled paper strips (EnviroPak; WF Fisher and Son, Somerville, NJ) and a

2-in. square of pulped virgin cotton fiber (Cotton square; Ancare, Bellmore, NY) for enrichment. Mice were fed a natural ingredient, closed-source, γ -irradiated, autoclaved diet (LabDiet 5KA1; PMI, Richmond, IN) ad libitum.¹¹ All animals were provided autoclaved reverse osmosis acidified (pH 2.5 to 2.8 with hydrochloric acid) water in polyphenylsulfone bottles with stainless-steel caps and sipper tubes (Tecniplast USA, West Chester, PA). Cages were changed every 7 days within a class II, type A2 biologic safety cabinet (LabGard S602-500; NuAire, Plymouth, MN). All cages were housed within a dedicated, restricted-access cubicle, which was maintained on a 12:12-h light:dark cycle (on 6 am:off 6 pm), relative humidity of 30% to 70%, and room temperature of 72 ± 2 °F (22.2 ± 1.1 °C). All cage changing and animal handling were performed by the author (GL). The animal care and use program at Memorial Sloan Kettering Cancer Center (MSK) is accredited by AAALAC International, and all animals are maintained in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals*.¹² All animal use described in this investigation was approved by MSK's IACUC in agreement with AALAS' position statements on the Humane Care and Use of Laboratory Animals and Alleviating Pain and Distress in Laboratory Animals.^{13,14}

Infection of experimental mice with *C. muridarum* via cohousing. The generation of chronically *C. muridarum*-infected C mice was previously described.^{1,6} Briefly, *C. muridarum* qPCR-negative C mice were inoculated with 2.72×10^3 IFU of a previously isolated *C. muridarum* field strain in 100 μ L sucrose-phosphate-glutamic acid buffer (pH 7.2) via orogastric gavage. A new, sterilized gavage needle (22 g $\times$ 38.1 mm; Cadence Science, Cranston, RI) was used for each cage.

***C. rodentium*, *T. muris*, and *H. hepaticus* culture and inoculation.** *C. rodentium* (cat. no. 51549; ATCC, Manassas, VA) was cultured in Luria-Bertani broth overnight (cat. no. 3002121; MP Biomedicals, Irvine, CA) at 37 °C with constant shaking. The administered inoculum was confirmed by plating bacteria on MacConkey agar (cat. no. 212123; BD Diagnostics, Franklin Lakes, NJ) and counting the colonies after 14 to 18 hours of incubation. B6 mice were inoculated with 1×10^9 CFU in 200 μ L of PBS via orogastric gavage. A new, sterilized gavage needle (24 g $\times$ 38.1 mm; Cadence Science, Cranston RI) was used for each cage.

Embryonated *T. muris* eggs (Artis Laboratory; Weill Cornell Medicine) were cultivated and maintained in the dark at 4 °C, and the inoculum prepared as previously described.¹⁵ Briefly, *T. muris* was maintained in Rag1^{-/-} animals. Following euthanize, adult nematodes were isolated from the cecum and proximal colon and cultured at 37 °C in serum-free Roswell Park Memorial Institute Medium 1640 (Corning, Herndon, VA) containing 500 U/mL penicillin and 500 μ g/mL streptomycin. Eggs were collected from the culture by filtering through a 70- or 100- μ m cell strainer, washed thrice in sterile water, and incubated at room temperature in the dark in a ventilated T75 cell culture flask (Corning, Herndon, VA) for 6 to 8 weeks. Embryonation was confirmed under a dissection microscope, and the eggs were stored at 4 °C in the dark after adjusting the concentration to 200 embryonated eggs per 200 μ L in water. B6 mice were inoculated with 200 embryonated *T. muris* eggs in 200 μ L water via oral gavage using a new, sterilized gavage needle (24 g $\times$ 38.1 mm; Cadence Science, Cranston, RI).

H. hepaticus (51449; ATCC, Manassas, VA) was cultured on blood agar plates (TSA with 5% sheep blood; Thermo Fisher Scientific, Waltham, MA) as previously described.¹⁶ Inoculated plates were placed into a Billups-Rothenberg hypoxia chamber (Embrient, San Diego, CA), and an anaerobic gas mixture

consisting of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide (Airgas, Radnor, PA) was added to create a microaerophilic atmosphere in which the oxygen concentration was 3% to 5%. The concentration of bacterial inoculation dose was determined using a spectrophotometric OD analysis at 600 nm and adjusted to OD at 600 nm readings between 1 and 1.5. Aliquots were then frozen at -80°C in *Brucella* broth with 20% glycerol. Frozen aliquots were thawed on ice before oral inoculation. *I/10⁻⁷* mice were inoculated with 10^8 CFU of *H. hepaticus* in 0.2 mL of culture broth via orogastric gavage every 2 days for a total of 3 doses. A new, sterilized gavage needle (24 g $\times$ 38.1 mm, Cadence Science, Cranston, RI) was used for each cage.

Clinical monitoring. All mice were monitored daily to assess activity, respiratory rate and effort, coat condition and posture, signs of rectal prolapse or diarrhea, and body condition score (BCS). Weights were recorded at the start of each experiment and as needed thereafter based on the BCS. Animals displaying weight loss (20% reduction from baseline) and/or reduced body condition (BCS $< 2/5$), presenting with dyspnea and/or cyanosis, and/or failing to respond to stimulation were euthanized.

Fecal collection. Fecal pellets for PCR were collected ante- or postmortem and pooled by cage for submission. When collected antemortem, the mouse was first lifted by the base of the tail and allowed to grasp onto the wire-bar lid while a 1.5-mL microcentrifuge tube was placed underneath the anus for fecal collection directly into the tube. If the animal did not defecate within 30 s, a fecal sample from the cage floor was collected. Postmortem fecal collection was collected directly from the rectum, if available, or the cage floor.

***C. muridarum*, *C. rodentium*, and *Helicobacter* spp. qPCR assays.** DNA and RNA were copurified from fecal samples using the Qiagen DNeasy 96 blood and tissue kit (Qiagen, Santa Clarita, CA). Nucleic acid extraction was performed using the manufacturer's recommended protocol, "Purification of Total DNA from Animal Tissues," with the following buffer volume modifications: 300 μL of Buffer ATL + Proteinase K, 600 μL of Buffer AL + EtOH, and 600 μL of lysate were added to individual wells of the extraction plate. Washes were performed with 600 μL of Buffers AW1 and AW2. Final elution volume was 150 μL of Buffer AE.

A probe-based PCR assay for *C. muridarum* was designed using IDT's PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA) based on the 16S rRNA sequence of *C. muridarum*, Nigg strain (Accession NR_074982.1, located in the National Center for Biotechnology Information database). Primer and Probe sequences generated from the PrimerQuest Tool were checked for specificity using NCBI's BLAST (Basic Local Alignment Search Tool). The probe was labeled with FAM and quenched with ZEN and Iowa Black FQ (Integrated DNA Technologies, Coralville, IA). Primer names, followed by associated sequences, were as follows: *C. muridarum*_For (GTGATGAAGGCTCTAGGGTTG); *C. muridarum*_Rev (GAGTTAGCCGGTGCTTCTTA); and *C. muridarum*_Probe (TACCCGTTGGATTGAGCGTACCA).

A probe-based PCR assay for *C. rodentium* was designed using IDT's PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA) based on *C. rodentium*'s espB gene sequence of *C. rodentium* (Accession AF_177537.1, located in the National Center for Biotechnology Information database). Primer and probe sequences generated from the PrimerQuest Tool were checked for specificity using NCBI's BLAST (Basic Local Alignment Search Tool). The probe was labeled with FAM and quenched with ZEN and Iowa Black FQ (Integrated DNA Technologies, Coralville, IA). Primer names, followed by associated sequences, are as

follows: *C. rodentium*_For (CAGGTATCGCTGATGATGTTACT); *C. rodentium*_Rev (CAGATTTGCCCTCCGTGTTAAAT); and *C. rodentium*_Probe (TGCTCAGAAAGCTTCTCAGGTAGCTG).

A proprietary probe-based PCR assay for *Helicobacter* spp. was designed by aligning 16S sequences from the following *Helicobacter* spp.: *H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastromyrmus*, *H. muridarum*, *H. rappini*, *H. rodentium*, *H. trogonum*, and *H. typhlonius*. Sequences were aligned using Sequencher 5.4.6 software (Gene Codes). Primers were designed to conserved regions between all species and checked for specificity using NCBI's BLAST (Basic Local Alignment Search Tool). The probe was labeled with HEX and quenched with ZEN and Iowa Black FQ (Integrated DNA Technologies, Coralville, IA).

Real-time qPCR assays were carried out using a real-time PCR system (BioRad CFX machine; Bio-Rad, Hercules, CA). Reactions were run using Qiagen's QuantiNova Probe PCR Kit (Qiagen, Santa Clarita, CA) using the kit's recommended concentrations and cycling conditions. The final concentration was $1\times$ QuantiNova Master mix, 0.4 μM Primers, and 0.2 μM FAM or HEX-labeled probe with cycling of 95°C 2 min, followed by 40 cycles of 95°C 5 s, 60°C 30 s.

All reactions were run in duplicate by loading 5 μL of template DNA into 15 μL of the reaction mixture. A positive and negative, no-template control were included in each run. The positive control was a purified PCR amplicon diluted to produce consistent values of Ct 28. A 16S universal bacterial PCR assay using primers 27_Forward and 1492_Reverse was run on all samples to check for DNA extraction and inhibitors. Samples were considered positive if both replicates had similar values and produced a cycle threshold value of less than 40. A sample was called negative if no amplification from the qPCR assay was detected, and a positive amplification from the 16S assay was detected.

Anatomic pathology, lesion scoring, and *T. muris* infection confirmation. Following euthanasia by carbon dioxide overdose, a complete necropsy was performed, and gross lesions were recorded. For all mice except for the *Apc^{Min/+}* mice, the GIT from the cecum to rectum was removed and placed in cassettes in a Swiss roll configuration and fixed in 10% neutral buffered formalin for at least 72 hours. Tissues were then processed in ethanol and xylene and embedded in paraffin in a tissue processor (Leica ASP6025; Leica Biosystems, Buffalo Grove, IL). Paraffin blocks were sectioned at 5 μm , stained with hematoxylin and eosin, and evaluated blindly by a board-certified veterinary pathologist (SC). The cecum and colon of *C. rodentium*- and *T. muris*-inoculated mice, and *I/10⁻⁷* mice were graded as described in Tables 1 and 2 using a modified scoring system for each model.⁸⁻¹⁰ A composite pathology score was calculated for each mouse by combining all subcategory scores. *T. muris* infection was confirmed by detecting nematodes histologically.

Tumorigenesis was assessed in the *Apc^{Min/+}* mice following euthanasia after which the GIT from the duodenum to the rectum was removed and placed in a culture dish containing 10% buffered formalin. The entire GIT was then flushed with formalin and opened longitudinally with scissors to expose the lumen for tumor enumeration under a dissecting microscope (Olympus SZX16; Olympus Corp. of the Americas, Center Valley, PA). Select tumors ($n = 16$) were subsequently submitted for histologic assessment as described above to assess for malignancy.

Immunohistochemistry. The entire cecum and colon from each *C. muridarum*-infected colitis model were processed and stained for *Chlamydia* major outer membrane permeabilization (MOMP) using a technique optimized and validated by MSK's

Table 1. Histopathologic Scoring Scheme for *Citrobacter rodentium* and *Trichuris muris* (*T. muris*) Infection in the Colon and Cecum Adapted from Bouadoux et al⁸ and Kopper et al⁹

Criterion	0 (absence)	1 (minimal)	2 (mild)	3 (moderate)	4 (severe)
Hyperplasia	None	1.5× normal crypt height	2× normal crypt height	3× normal crypt height	>4× normal crypt height
Goblet cell depletion (<i>Citrobacter rodentium</i>) or hyperplasia (<i>T. muris</i>)	None	5%	5% to 25%	25% to 60%	>60%
Inflammation	None to multifocal rare individual leukocytes scattered in the lamina propria	Multifocal, small discrete clusters of leukocytes in the lamina propria with or without transepithelial leukocyte infiltration	Multifocal, coalescing lamina propria inflammation with or without early submucosal extension	Multifocal, coalescing lamina propria inflammation with multifocal submucosal extension	Multifocal, diffuse transmural inflammation affecting mucosa, submucosa, and deeper layers (muscularis or serosa)
Epithelial defects	None	Occasional dilated glands with luminal cell debris (crypt abscesses)	Multifocal crypt abscesses and surface epithelial tattering and/or attenuation (intact)	Multifocal erosions or focal ulceration	Multifocal ulcerations
Fibrosis*	None	None or focal, rare in lamina propria	Occasional foci in small segments of the LI, fibroplasia (immature fibroblast proliferation) associated with submucosal inflammation	Multifocal, fibroplasia associated with submucosal inflammation	Multifocal, mature fibrosis with submucosal inflammation and replacement of normal tissue architecture

*Minimal, mild, and moderate fibrosis for the *Citrobacter rodentium* model was defined as 1, 1 to 2, and >2 foci, respectively. Minimal, mild, and moderate fibrosis for the *T. muris* model was defined as 1 to 2, 2 to 5, and >5 foci, respectively.

Table 2. Histopathologic Grading Scheme for the Colon and Cecum from *Il10^{-/-}* Mice (Adapted from Rogers and Houghton¹⁰)

Criterion	0 (absence)	1 (minimal)	2 (mild)	3 (moderate)	4 (severe)
Inflammation	None to multifocal rare individual leukocytes scattered in the lamina propria	Multifocal, small discrete clusters of leukocytes in the lamina propria	Multifocal, coalescing lamina propria inflammation with or without early submucosal extension	Multifocal, coalescing lamina propria inflammation with multifocal submucosal extension	Multifocal, diffuse transmural inflammation affecting mucosa, submucosa, and deeper layers (muscularis or serosa)
Hyperplasia	None	1.5× normal crypt height	2× normal crypt height	3× normal crypt height	>4× normal crypt height
Goblet cell depletion	None	5%	5% to 25%	25% to 60%	>60%
Epithelial defects	None	Occasional dilated glands with luminal cell debris (crypt abscesses), and surface epithelial tattering and/or attenuation (intact)	Multifocal crypt abscesses, herniation, and surface epithelial tattering and/or attenuation (intact)	Same changes as 2 plus multifocal erosions or focal ulceration	Same as 3 plus multifocal ulcerations
Crypt Atrophy	None	5% to 25%	25% to 50%	50% to 75%	>75%
Edema	None	Mild segmental expansion of submucosa	Moderate diffuse submucosal +/- mucosal lamina propria expansion	Severe edema of mucosa and submucosa	Transmural; very severe
Dysplasia	None	Mild dysplasia characterized by epithelial cell pleomorphism, plump and attenuated forms, aberrant crypt foci, gland malformation with mild splitting, branching, and infolding, rare cystic dilation	Moderate dysplasia characterized by pleomorphism, early cellular and nuclear atypia, mild piling and infolding, occasional cystic dilation, bulging toward muscularis mucosae and projection into lumen, loss of normal glandular, mucous, or goblet cells, = atypical hyperplasia	Gastrointestinal intraepithelial neoplasia (GIN), severe dysplasia confined to mucosa, features as earlier but greater severity, frequent, and sometimes bizarre mitoses, add 0.5: intramucosal carcinoma (extension of severely dysplastic regions into muscularis mucosae)	Invasive carcinoma: submucosal invasion, or any demonstrated invasion into blood or lymphatic vessels, regional nodes, or other metastasis

Laboratory of Comparative Pathology as a secondary confirmatory method.¹ Briefly, formalin-fixed, paraffin-embedded sections were stained using an automated staining platform (Leica Bond RX; Leica Biosystems, Buffalo Grove, IL). Following deparaffinization and heat-induced epitope retrieval in a citrate buffer at pH 6.0, the primary antibody against *Chlamydia* MOMP (NB100-65054; Novus Biologicals, Centennial, CO) was applied at a dilution of 1:2,000. A rabbit anti-goat secondary antibody (cat. no. BA-5000; Vector Laboratories, Newark, CA) and a polymer detection system (DS9800; Novocastra Bond Polymer Refine Detection; Leica Biosystems, Buffalo Grove, IL) were then applied to the tissues. The DAB was used as the chromogen, and the sections were counterstained with hematoxylin and examined by light microscopy. Small intestine from an NSG mouse infected with *C. muridarum* strain Nigg was used as a positive control.⁵ Positive MOMP antigen was identified as chromogenic brown immunolabeling under bright field microscopy.

In situ hybridization (ISH) was conducted on the large intestine of a subset of *C. muridarum*-positive *Il10*^{-/-} animals (*n* = 8) in which the MOMP immunohistochemistry (IHC) signal was absent. Briefly, the target probe was designed to detect region 581 to 617 of *C. muridarum* str. Nigg complete sequence, NCBI Reference Sequence NC_002620.2 (1039538-C1; Advanced Cell Diagnostics, Newark, CA). The target probe was validated on reproductive tracts from mice experimentally inoculated with *C. muridarum* strain Nigg.⁵ Slides were stained on an automated stainer (Leica Bond RX; Leica Biosystems, Buffalo Grove, IL) with RNAscope 2.5 LS Assay Reagent Kit-Red (322150; Advanced Cell Diagnostics, Newark, CA) and Bond Polymer Refine Red Detection (DS9390; Leica Biosystems, Buffalo Grove, IL). Control probes detecting a validated positive housekeeping gene (mouse peptidylprolyl isomerase B, *Ppib* to confirm adequate RNA preservation and detection; 313918; Advanced Cell Diagnostics, Newark, CA) and a negative control, *Bacillus subtilis* dihydrodipicolinate reductase gene (*dapB* to confirm absence of nonspecific staining; 312038; Advanced Cell Diagnostics, Newark, CA) were used. Positive RNA hybridization was identified as discrete, punctate chromogenic red dots under bright field microscopy.

Statistical analysis. All data sets were determined to be normally distributed using the Kolmogorov-Smirnov test. Mann-Whitney *U* (Wilcoxon rank sum) tests were used to compare colitis scores between *C. muridarum*-infected and *C. muridarum*-free control mice infected with *C. rodentium* and *T. muris*. The Kruskal-Wallis test for multiple comparisons was used to compare pathology scores between *C. muridarum*-infected, *H. hepaticus*-infected, coinfecting, and control *Il10*^{-/-} mice. An unpaired *t* test was used to compare gross tumor numbers between *C. muridarum*-infected and *C. muridarum*-free *Apc*^{Min/+} mice. For all tests, statistical significance was set as a *P* ≤ 0.05. Analysis and graphical representations were performed and created in Prism 9 (version 9.3.0; GraphPad Software, San Diego, CA) for Windows.

Results

***C. rodentium* colitis model.** Mice were confirmed to be infected with *C. rodentium* only or *C. rodentium* and *C. muridarum* as expected at the experimental endpoint via qPCR and IHC. A single *C. muridarum*- and *C. rodentium*-infected mouse was found dead 10 days postinoculation. Histopathology revealed a moderate to marked, multifocal necroulcerative and suppurative colitis. This mouse was excluded from analysis as the lesions may represent an atypical presentation of *C. rodentium* infection

or may have been caused by an unidentified etiology. All remaining animals remained clinically normal for the duration of the study. The colons of mice infected with both *C. rodentium* and *C. muridarum* had statistically significantly lower composite and individual scores for inflammation and goblet cell depletion when compared with colons from *C. rodentium*-inoculated, *C. muridarum*-free mice (Figure 2). Colitis was characterized in the *C. muridarum*-free mice as diffuse and moderate to marked, while *C. muridarum*-positive mice had mild to moderate colitis. Both groups had infiltration of the lamina propria by lymphocytes, plasma cells, and histiocytes with submucosal extension (Figure 2B and C).

***T. muris* colitis model.** All mice in each group were confirmed to be infected with only *T. muris* or both *T. muris* and *C. muridarum* at the end of the experiment by histology and qPCR. No mice developed signs of illness for the duration of the experiment. Mice coinfecting with *T. muris* and *C. muridarum* showed no significant score differences in the cecum and colon for any individual component or the composite score when compared with mice infected only with *T. muris* (Table 3). Colitis in both groups was characterized as multifocal and moderate with infiltration of the lamina propria by lymphocytes, plasma cells, eosinophils, and histiocytes with submucosal extension. *T. muris* larvae were readily appreciated in the lumen of all mice examined (Figure 3).

***Il10*^{-/-} colitis model (*H. hepaticus*-infected and -free).** Mice were confirmed *C. muridarum*-free or *C. muridarum*-infected at euthanasia by qPCR as expected except 2 animals representing a single mouse in the *C. muridarum*-only group and another in the *C. muridarum* + *H. hepaticus* coinfecting group despite having been qPCR positive after cohousing. Given the initial positive qPCR results after cohousing and similar intestinal pathology to other mice in the group, these mice were included in the analysis. None of the mice developed signs of illness for the duration of the experiment. MOMP staining of the cecum and colon was negative for *Il10*^{-/-} mice cohoused with a *C. muridarum*-shedding mouse for 2 weeks despite the fact that most of these mice were qPCR positive. ISH was also performed on cecum and colon sections to confirm the presence of *C. muridarum*. Staining was detected in the colon of all *C. muridarum*-infected animals, except for 1 of the 2 aforementioned qPCR-negative mice representing the *C. muridarum*+ *H. hepaticus* coinfecting group (Figure 4).

The individual pathology component scores as well as the composite scores for both the cecum and colon are provided in Figure 5; representative images demonstrating select pathologic changes are provided in Figure 4. While infecting *Il10*^{-/-} mice with *C. muridarum* did not impact the composite pathology score in the cecum compared to controls, it did result in significant colonic pathology equivalent in magnitude to that resulting from *H. hepaticus* infection in this model. *H. hepaticus* caused significant pathology in both the cecum and colon. Infection of *Il10*^{-/-} mice with both bacterial species did not increase the composite colonic pathology score more than the changes resulting from infection with either species alone.

The composite score difference between *C. muridarum*-free and -infected mice in the colon was a result of a significant increase in inflammation, epithelial defects, crypt atrophy, and goblet cell depletion following *C. muridarum* infection. While epithelial hyperplasia was observed in the *Il10*^{-/-} mice following *C. muridarum* infection, the change was not significant. Crypt atrophy was also a principal component of the pathologic changes resulting from *C. muridarum* infection, regardless as to whether the *Il10*^{-/-} were infected with *H. hepaticus*. Crypt

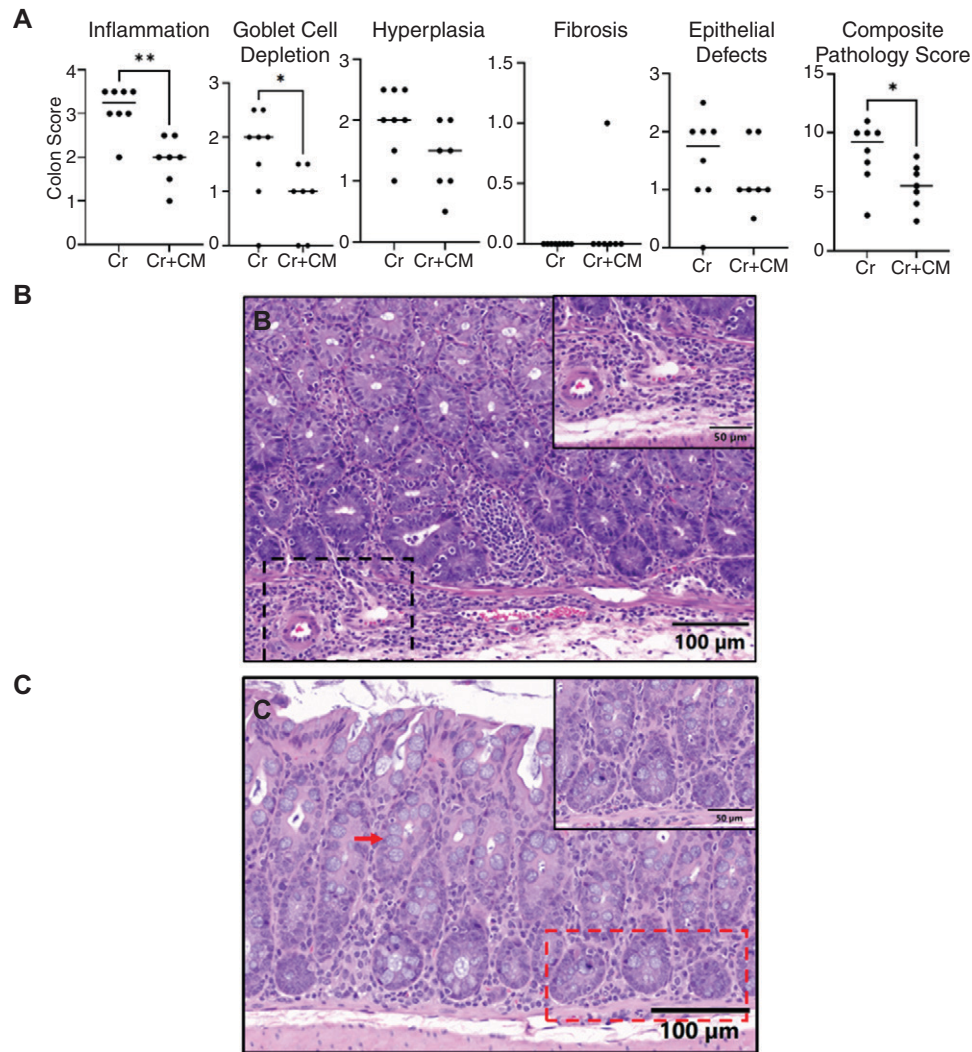


Figure 2. The Effects of *Chlamydia muridarum* (Cm) on *Citrobacter rodentium* (Cr)-Induced Colitis. (A) Individual and composite colitis scores of female C57BL/6J mice infected with only *Citrobacter rodentium* or infected with both *Citrobacter rodentium* and *C. muridarum* ($n = 8$ mice/group) 14 d post-*Citrobacter rodentium* infection. Composite pathology scores were calculated by combining scores from all subcategories. The crossbar represents the median lesion score for each parameter. (B and C) Representative H&E-stained images of colons from mice infected with only *Citrobacter rodentium* (B) or with both *Citrobacter rodentium* and *C. muridarum* (C) 14 d post-*Citrobacter rodentium* infection. (B) Moderate to marked mucosal and submucosal inflammation with infiltration of the lamina propria (inset), moderate crypt hyperplasia, and mild goblet cell depletion in affected crypts in the colon of a *Citrobacter rodentium*-infected, *C. muridarum*-free mice with a composite pathology score of 10 (10× magnification; inset, 40× magnification). (C) Less severe mucosal inflammation (inset), reduced crypt hyperplasia, and less goblet cell depletion (red arrow) in the colon of a *Citrobacter rodentium*-infected, *C. muridarum*-positive mouse with a composite pathology score of 7 (10× magnification; inset, 40× magnification). *, $P \leq 0.05$, **, $P \leq 0.01$.

Table 3. Pathology Scores Assigned to the Cecum and Colon of Mice Infected with *Trichuris muris* with and without *Chlamydia muridarum*

Category	Cecum		Colon	
	<i>T. muris</i>	<i>C. muridarum</i> + <i>T. muris</i>	<i>T. muris</i>	<i>C. muridarum</i> + <i>T. muris</i>
Inflammation	2.56 ± 0.50	2.31 ± 0.46	2.25 ± 0.46	2.56 ± 0.32
Epithelial hyperplasia	1.81 ± 0.26	1.75 ± 0.46	1.5 ± 0.38	1.75 ± 0.38
Goblet cell hyperplasia	1.25 ± 0.27	1.0 ± 0.53	1.38 ± 0.35	1.19 ± 0.26
Epithelial defects	1.81 ± 0.46	1.5 ± 0.80	1.31 ± 0.46	1.69 ± 0.37
Fibrosis	0.25 ± 0.46	0.44 ± 0.62	0.25 ± 0.46	0.13 ± 0.35
Composite pathology score	7.69 ± 1.56	7.0 ± 2.55	6.69 ± 1.89	7.31 ± 0.88

Pathology scores (mean ± SD) of all criteria of the cecum and colon of female C57BL/6J mice infected with *T. muris* or coinfectd with *T. muris* and *C. muridarum* ($n = 8$ mice/group) 18 d post-*T. muris* infection following cohousing with a *C. muridarum*-shedding or *C. muridarum*-free mouse for 2 wk. None of the score differences in any category or the composite scores in either the cecum or colon were significantly different.

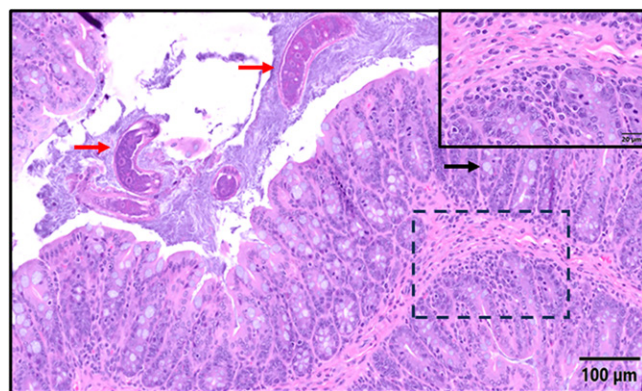


Figure 3. Histopathology of the Colon from a *Chlamydia muridarum*-Free Mouse Infected with *Trichuris muris* 18 d Post-*T. muris* Infection. Representative hematoxylin and eosin-stained image of the colon from a mouse infected with *T. muris* 18 d following cohousing with a *C. muridarum*-free mouse for 2 wk (composite pathology score = 7.5). A multifocal infiltrate of lymphocytes, eosinophils, and plasma cells is present in the lamina propria with submucosal extension (inflammation score = 3; inset) in association with mild to moderate hyperplasia and mild goblet cell hypertrophy (goblet cell hypertrophy score = 1; black arrow) in affected crypts and intraluminal *T. muris* larvae (red arrows). (10× magnification; inset: 60× magnification).

atrophy was inconsistently observed in *Il10*^{-/-} mice infected with only *H. hepaticus*. While *H. hepaticus* infection of *Il10*^{-/-} mice resulted in edema, edema was not a component of the pathology associated with *C. muridarum* infection in this model.

While *C. muridarum* infection of the *Il10*^{-/-} mouse led to a minimal increase in the cecal composite pathology score, these increases were principally a result of inflammation and epithelial defects; however, none of these differences were statistically significant. Cecal edema, epithelial hyperplasia, and goblet cell hyperplasia were the major pathologic features that were more severe in the *H. hepaticus*-infected as compared with the *C. muridarum*-infected *Il10*^{-/-} mice.

Apc^{Min/+} DSS model of intestinal neoplasia. Mice were confirmed *C. muridarum*-free or *C. muridarum*-infected at euthanasia by qPCR as expected. *C. muridarum* MOMP immunolabeling was also detected in tumors and normal intestinal mucosa (data not shown). None of the mice developed signs of illness for the duration of the experiment. As expected, DSS exposure resulted in increased tumorigenesis in the colon. Tumors were identified grossly as raised mucosal nodules (Figure 6B). *C. muridarum*-infected Apc^{Min/+} mice had statistically fewer identifiable colonic tumors as compared with *C. muridarum*-free Apc^{Min/+} mice (Figure 6A). The number of small intestinal and cecal tumors were not significantly different between *C. muridarum*-free and *C. muridarum*-infected Apc^{Min/+} mice. Overall tumor burden per mouse was reduced in the presence of *C. muridarum*. All *C. muridarum*-free Apc^{Min/+} mice exposed to 2% DSS developed gross intestinal tumors; however, one of the *C. muridarum*-infected mice failed to develop a colonic tumor. Two and 4 *C. muridarum*-free mice failed to develop small intestinal and cecal tumors, respectively, as compared with the *C. muridarum*-infected mice where one mouse failed to develop any small intestinal tumors, and none of the 8 mice developed cecal neoplasia. All tumors evaluated histologically were classified as adenomas (Figure 6C).

Discussion

Biosecurity is an integral and essential component of contemporary research mouse colony management as it is well

recognized that there is a plethora of infectious agents that, while they may not cause clinical disease and/or pathology, can nevertheless confound research in which infected mice are used. It is for these reasons that most institutions maintain their rodent colonies, especially mice, in barrier facilities implementing specific husbandry and operational processes focused on keeping out a host of excluded infectious agents that may adversely impact the research for which the animals are used. Ascertaining which agents should be excluded is becoming ever more challenging as novel agents are identified, and the impact of the microbiota and agents that had historically been considered commensals is continuing to evolve.

Our group recently discovered and reported on the reemergence and surprisingly high prevalence of the bacterium *C. muridarum* in research mouse colonies and showed that *C. muridarum* causes silent, but persistent infection of the large intestine of various commonly used murine strains and stocks.^{1,6} We further confirmed alterations of innate and adaptive immune responses following exposure by its natural route of infection.⁶ These findings lead to the question as to whether *C. muridarum* can have a significant impact on the research in which infected mice are used, particularly those analyzing the intestine. A recent study⁷ revealed that *C. muridarum* altered responses in the commonly used murine DSS-induced colitis model of inflammatory bowel disease. *C. muridarum*-infected DSS-treated mice had less pathology through an IL-22-dependent pathway. Therefore, we speculated that *C. muridarum* would have a significant impact on other mouse models used to study gastrointestinal disease. We elected to assess *C. muridarum*'s impact on 4 commonly used murine models, 3 of which are used to interrogate intestinal inflammation and infection with the bacterium *C. rodentium* and the parasite *T. muris*, as well as the genetically engineered IL-10 knockout mouse model in which gut inflammation develops spontaneously in association with specific intestinal consortia. The DSS-treated Apc^{Min/+} mouse model of intestinal neoplasia was also assessed. While the study's aim was not to determine the mechanism(s) by which *C. muridarum*, if shown to influence any or all of these models, altered the models, but rather to aid us and others in determining the significance of its presence and assess whether the impact is sufficient to consider exclusion from some or all murine mouse colonies. These models were selected on their frequency of use as well as the likelihood, based on *C. muridarum*'s pathophysiology, to alter the model phenotype.

The *C. rodentium* mouse model of bacterial infection is commonly used to enhance the understanding of bacterial pathogenesis and mucosal immunity, especially as it relates to enterohemorrhagic and enteropathogenic *Escherichia coli* infection.⁸ Infection of B6 mice with 10⁸ to 10⁹ CFU of *C. rodentium* results in transient colonic crypt hyperplasia typically lasting 2 to 3 weeks.⁸ Initially, *C. rodentium* colonizes the colon and rapidly expands after infection. By day 7 postinfection, the bacterial load plateaus, and by day 10, the infection starts to clear with complete clearance observed by 2 to 3 weeks.⁸ Hallmark histologic changes associated with the developing colitis is the colonic crypt hyperplasia.⁸ We demonstrated that coinfecting *C. muridarum*-infected mice with *C. rodentium* significantly decreased the severity of colitis when compared with *C. muridarum*-free mice inoculated with the same *C. rodentium* dose. *C. muridarum* and *C. rodentium*-coinfected mice had significantly less inflammation and goblet cell depletion, and while the magnitude of the epithelial hyperplasia and cell defects was not statistically different, both these parameters were also reduced in coinfecting mice. We speculate that local and systemic upregulation of IFN γ , IL-17, and IL-22 following *C. muridarum* infection

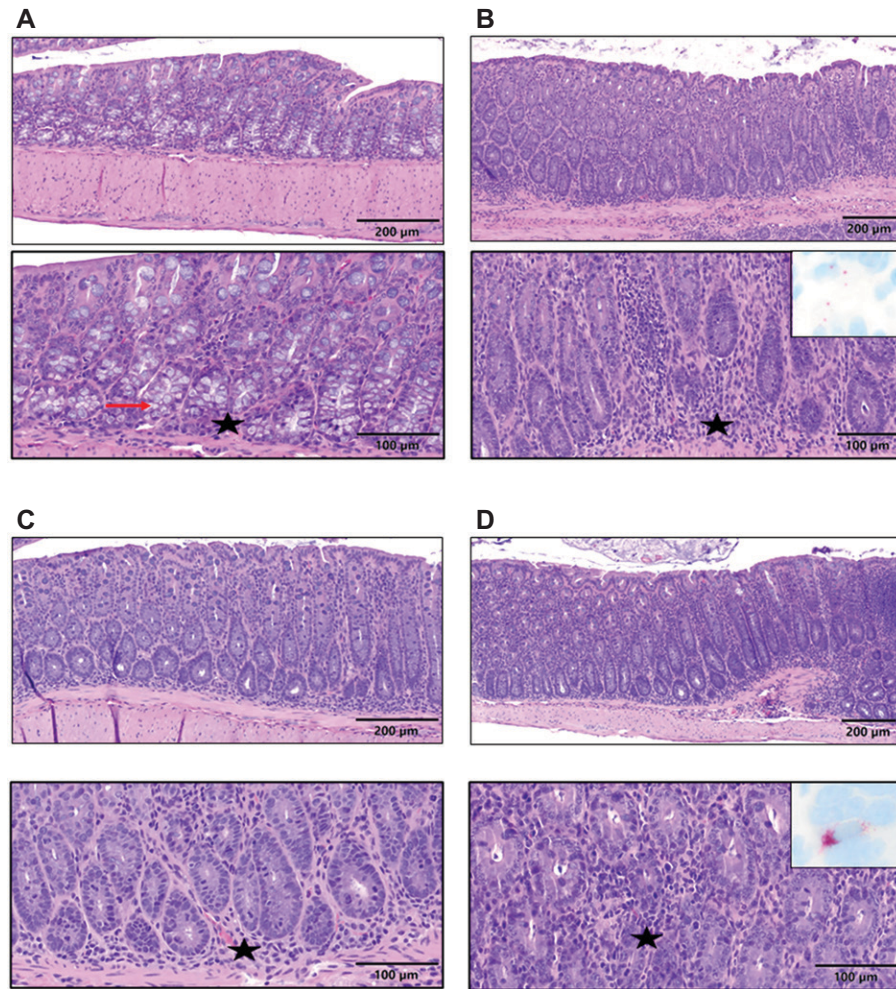


Figure 4. Colonic Lesions in *Helicobacter hepaticus*-Infected and -Free *Il10*^{-/-} Mice Coinfecting with *Chlamydia muridarum*. Representative images from are from (A) Colon of a *C. muridarum*- and *H. hepaticus*-free *Il10*^{-/-} mouse showing occasional lymphocytes in the lamina propria (black star) and abundant goblet cells (red arrow; composite score=2). (B) Colon from a *C. muridarum*-infected *Il10*^{-/-} mouse with moderate colitis (black star), mild mucosal hyperplasia and moderate goblet cell atrophy (composite score = 11). Inset: *C. muridarum* ISH signal (red) in mucosal epithelium. (C) Colon from an *H. hepaticus*-infected, *C. muridarum*-free *Il10*^{-/-} mouse showing moderate to marked colitis (black star) with mild to moderate mucosal hyperplasia and moderate goblet cell atrophy (composite score = 11.5). (D) Colon from a *C. muridarum* and *H. hepaticus* coinfecting *Il10*^{-/-} mouse with epithelial hyperplasia and colitis (black star) and moderate goblet cell atrophy (composite score = 10). Inset: *C. muridarum* ISH signal (red) in mucosal epithelium. H&E stain (10× and 20× magnification; insets: 40× magnification).

resulted in the reduction of *C. rodentium*-induced colitis.⁶ Th1, Th17 and Th22 and their respective cytokines, IFN γ , IL-17A, and IL-22, respectively, mediate host response to *C. rodentium* infection.¹⁷ IFN γ -deficient mice fail to clear *C. rodentium* as a consequence of reduced activation of *C. rodentium*-antigen-specific T cells and the subsequent reduction in antibacterial IgG production and macrophage phagocytosis.¹⁸ IL-17 and IL-22, in particular, are essential for protection against *C. rodentium*.⁸ The latter is protective by upregulating epithelial repair and production of antimicrobial peptides.¹⁷ We excluded a single *C. muridarum* and *C. rodentium* coinfecting mouse from the study, which was unexpectedly found dead 10 days after *C. rodentium* inoculation with unusual and unexpected lesions.

We also examined the impact of *C. muridarum* on the *T. muris*-induced colitis model in B6 mice. This model of human whipworm infection is useful for dissecting host-nematode interactions.¹⁹ A low dose of 50 ova elicits a Th1 response in which B6 mice become persistently infected and develop a chronic colitis, while mice gavaged with a high dose of 200 ova develop a Th2 response resulting in worm expulsion within 21 days.^{19,20} We elected to evaluate *C. muridarum*'s impact on the

latter model as we hypothesized the Th1 response and increase in IFN γ elicited by a preexisting *C. muridarum* infection would increase susceptibility to *T. muris*.⁶ However, there were no differences detected. All pathology criteria scored as well as the composite score were similar in *C. muridarum*-free as compared with *C. muridarum*-infected mice. The Th1 cytokines IL-12, IL-18, and IFN γ induce susceptibility to *T. muris* infection.¹⁹ IFN γ is thought to be the principal mediator as depletion results in expulsion of *T. muris* in susceptible strains.²¹ IL-18 and IL-12 induce IFN γ production.²² *Il12b*^{-/-} and *Il18*^{-/-} animals are resistant to infection.²³ Resistance in *Il12*^{-/-} mice is primarily mediated by reducing IFN γ production; however, resistance in *Il18*^{-/-} is a result of IL-13 suppression inhibiting the Th2 response.²³ Administration of recombinant IL-12 or IL-18 to a resistant strain results in susceptibility to infection, further implicating the Th1-cell response is key to susceptibility to chronic *T. muris* infection.²³ The observed lack of differences in our study may reflect *C. muridarum*'s other effects. Although *C. muridarum* infection elicits an increase in IFN γ , it also increases IL-22 and IL-17.⁶ IL-22 deficiency leads to susceptibility to *T. muris* infection in normally resistant mice.²⁴ Loss of IL-22 decreases goblet

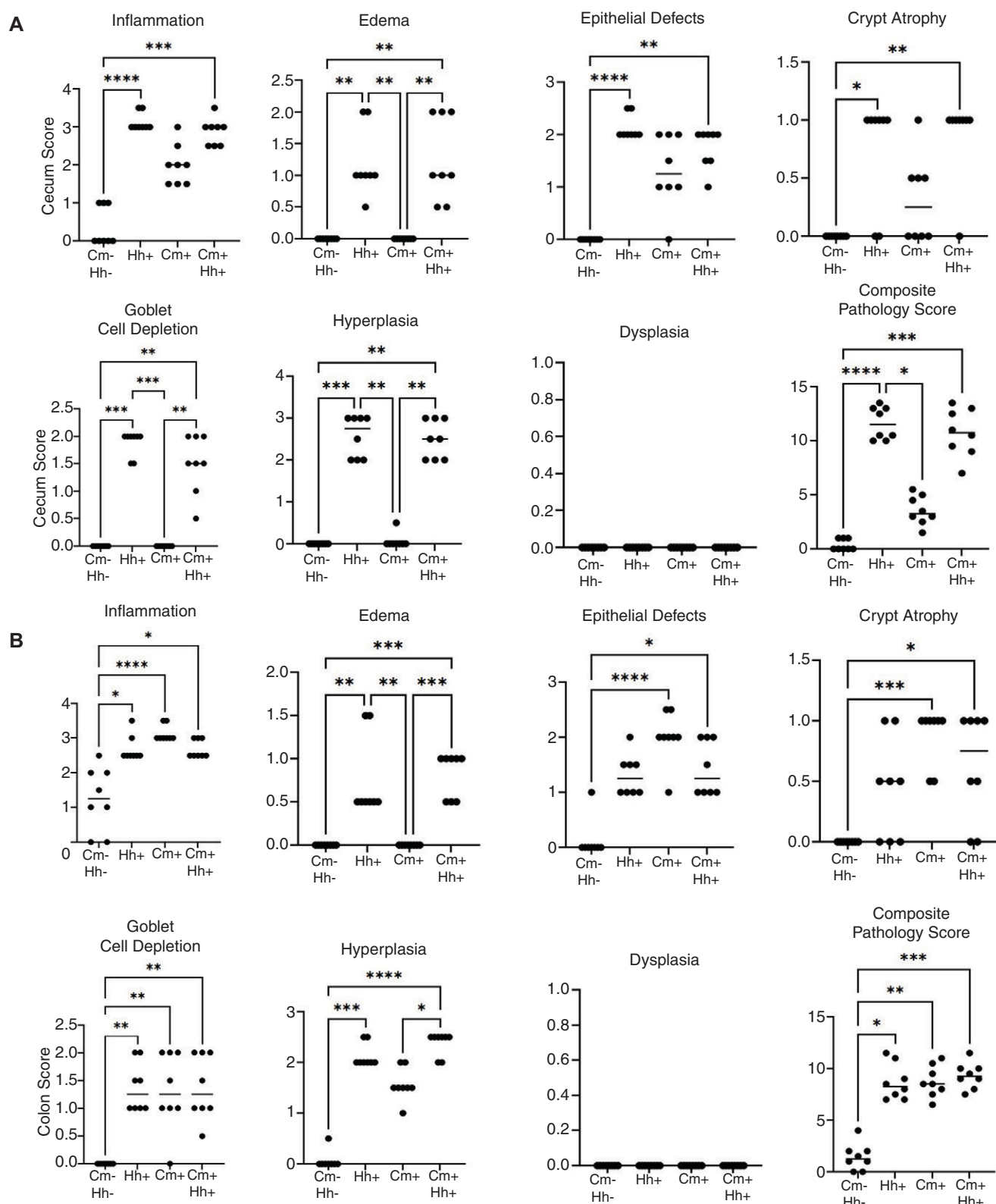


Figure 5. The Effects of *Chlamydia muridarum* (Cm) on Colitis in *Helicobacter hepaticus* (Hh)-Infected and -Free *Il10*^{-/-} Mice. Pathology scores of the (A) cecum and (B) colon of female *Il10*^{-/-} mice (control), infected with *H. hepaticus*, infected with *C. muridarum*, or coinfecting with *C. muridarum* and *H. hepaticus* ($n = 8$ mice/group). Scores for inflammation, edema, epithelial defects, crypt atrophy, goblet cell depletion, hyperplasia, dysplasia, and composite scores of all score parameters. The crossbars on the graphs indicate the median lesion scores for each parameter. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

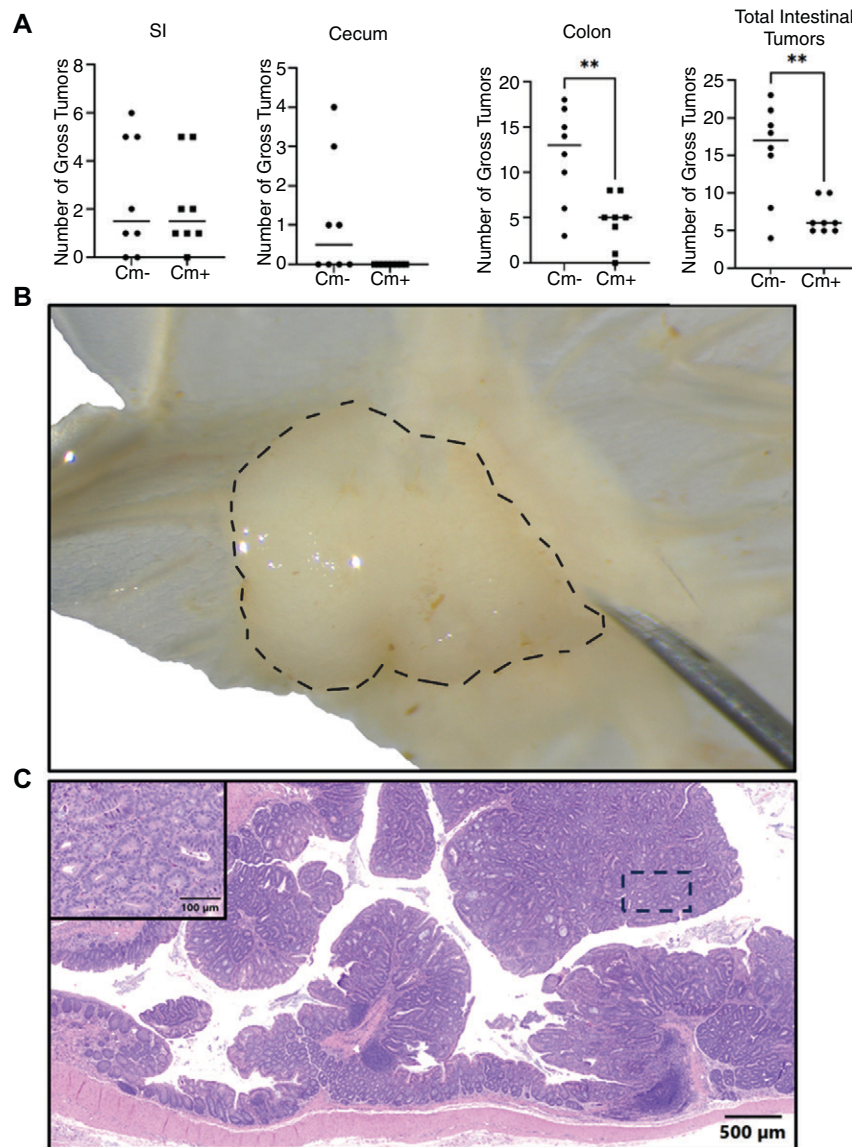


Figure 6. Colonic Tumors in the Small Intestine, Cecum and Colon of *C. muridarum*-Infected (Cm+) and -Free (Cm-) DSS Treated, *Apc^{Min/+}* mice. (A) Gross tumor count in the small intestine, cecum, and total tumor count per mouse at necropsy of female control *C. muridarum*-free *Apc^{Min/+}* mice or *C. muridarum*-infected *Apc^{Min/+}* mice at 4-wk post 2% DSS treatment ($n = 8$ /group). The crossbar represents the median lesion score for each parameter. **, $P < 0.005$. (B) Representative photo of a gross tumor (outlined by a dashed line) in the colon of *Apc^{Min/+}* mice 4-wk post 2% DSS treatment at necropsy. (C) Representative H&E-stained images of a colonic tumor from *Apc^{Min/+}* mice 4-wk post-2% DSS treatment. Tumors were characterized as adenomas (2× magnification; inset: 20× magnification).

cell proliferation and mucous production impairing worm expulsion.²⁴ Therefore, even though *C. muridarum* infection leads to an increase in production of IFN γ , the increase in IL-22 may have counteracted its impact. Given these results, it would be interesting to evaluate *C. muridarum*'s impact on the low-dose B6 model as the heightened Th1-cell response could increase pathology and worm burden.

C. muridarum's impact on the *Il10*^{-/-} model of gut inflammation was also examined as it has a clinicopathologic phenotype influenced by the microbiota.^{25–27} The loss of IL-10 leads to colitis as a result of an aberrant immune response in the presence of specific agents such as *H. hepaticus*.²⁵ We demonstrated that *C. muridarum* induced colitis of similar magnitude to that caused by *H. hepaticus*. Therefore, *C. muridarum* can be added to the list of agents capable of inducing colitis in the model. Interestingly, coinfection with both agents did not lead to colitis of greater severity than either bacterium caused alone. This finding may reflect that both bacteria induce colitis by the same mechanism.

IFN γ , IL-17, and IL-22 are upregulated in the absence of IL-10.^{25,28} IFN γ has historically been linked to the development of colitis in *H. hepaticus*-infected *Il10*^{-/-} mice as intestinal inflammation is reduced following administration of neutralizing monoclonal antibodies.²⁹ Recent studies³⁰ have shown that mice deficient in both IFN γ and IL10 still developed colitis following *H. hepaticus* infection suggesting that colitis is likely induced by other pathways. Although IL-17 was linked to colitis in this model, there are more recent reports of mice deficient in both IL-17 and IL-10 developing more severe colitis than mice deficient only in IL-10.²⁷ IL-22 is also overexpressed in *Il10*^{-/-} mice and is associated with the colitis phenotype.²⁸ *Helicobacter* spp.-infected mice deficient in both IL-22 and IL-10 remained colitis free suggesting that IL-22 is a major driver of colitis in this model.²⁸ Given that IFN γ , IL-17 and IL-22 are upregulated following *C. muridarum* infection, it is not surprising that *C. muridarum* is sufficient to induce colitis in *Il10*^{-/-} mice in the absence of *H. hepaticus*.⁶

Interestingly, 2 of 16 *Il10*^{-/-} mice were negative for *C. muridarum* by qPCR at euthanasia despite being qPCR positive following cohousing 70 days prior. One of these 2 mice was coinfecting with *H. hepaticus* while the other was *H. hepaticus* free. Both were singly housed after cohousing, which could have contributed to a reduction in *C. muridarum* burden to undetectable levels by qPCR as they would not have been continuously exposed to *C. muridarum* by a *C. muridarum*-shedding cage mate. Despite negative qPCR results, intestinal pathology was similar to other *C. muridarum*-positive animals. Interestingly, all *C. muridarum*-infected *Il10*^{-/-} were also MOMP IHC negative, which was not observed in any of the other models we evaluated, although *C. muridarum* was detectable in most of the *Il10*^{-/-} mice evaluated via ISH. Of the 2 mice negative for *C. muridarum* by qPCR, one was also negative by ISH. Collectively, these findings indicate that the *C. muridarum* burden in the large intestine decreases temporally in this model with only trace amounts of *C. muridarum* nucleic acid detectable in the colonic mucosa by the end of the experimental period. Given that IL-10 is a potent anti-inflammatory cytokine that modulates immune responses, it is plausible that the exaggerated inflammatory response led to the clearance of most chlamydial inclusions from the intestines. This notion is supported by prior studies³¹ using *Il10*^{-/-} mouse models, which demonstrated a progressive reduction in *C. trachomatis* burden in the lungs beginning 7 days postinfection. Although proinflammatory cytokine levels were not assessed in the colonic mucosa of *C. muridarum*-infected *Il10*^{-/-} mice, it is well established that upregulation of IFN γ production by epithelial cells plays a critical role in host defense by depleting intracellular tryptophan, an essential nutrient for *Chlamydia* replication within host tissues.³² Future studies should elucidate the immunoregulatory pathways influenced by *C. muridarum* in the colon of *Il10*^{-/-} mice.

Finally, we assessed the impact of *C. muridarum* infection on the DSS-treated *Apc*^{Min/+} mouse of colonic neoplasia. Importantly, *C. muridarum* infection significantly decreased tumor burden in the model. We elected to use the DSS-treated model as DSS exposure stimulates colonic neoplasia rather than the small intestinal neoplasia that predominates in untreated *Apc*^{Min/+} mice.^{33,34} As *C. muridarum* infection preferentially colonizes the large intestine, we speculated that it would be more likely to influence the DSS-treated model.⁶ Although the specific mechanism by which DSS impacts colonic tumorigenesis is unknown, the induction of colonic neoplasia is likely influenced by the resulting colitis.³³ Our findings are not surprising considering *C. muridarum* alleviated DSS-induced colitis by stimulating IL-22 production, and the cytokine has been shown to be protective in chemically-induced models of tumorigenesis by decreasing inflammation.^{7,21,35} Interestingly, IL-22 can also be tumorigenic as *Il22*^{-/-}*Apc*^{Min/+} mice develop fewer tumors as compared with *Il22*^{+/+}*Apc*^{Min/+} mice.^{35,36} The mechanism is believed to be a result of the epithelial turnover induced by IL-22 suggesting that multiple pathways could impact tumorigenesis in *C. muridarum*-infected, DSS-treated *Apc*^{Min/+} mice.³⁶ It remains unknown whether *C. muridarum* infection could influence neoplasia in untreated *Apc*^{Min/+} mice. *C. rodentium* infection of *Apc*^{Min/+} mice resulted in increased colonic neoplasia; the mechanism was thought to result from increased epithelial turnover.³⁷ Although *C. muridarum* infection has not been reported to induce epithelial hyperplasia, we observed epithelial hypertrophy in *C. muridarum*-infected *Il10*^{-/-} mice.⁴ Future research could examine the impact of *C. muridarum* in the untreated *Apc*^{Min/+} mouse model.

C. muridarum infection significantly impacted 3 of the 4 gastrointestinal models evaluated in this study providing further and compelling evidence that *C. muridarum* status should be considered when using these models. One reason to consider excluding *C. muridarum* is the sensitivity of available diagnostics for its detection and, more importantly, the relative ease by which *C. muridarum* can be successfully eradicated from most mouse strains using antibiotic therapy.³⁸ This contrasts with other prevalent murine infectious agents, such as MNV, that, while ideally should be excluded, the process of establishing MNV-free colonies would be extremely challenging in that numerous unique strains of mice present in many colonies would need to be rederived. Careful consideration should also be given to using previously *C. muridarum*-infected, antibiotic-treated mice as research subjects as the impact of prior *C. muridarum* infection is unknown. Ideally, offspring of treated mice should be used, although epigenetic multigenerational effects remain possible.

Acknowledgments

We thank Jacqueline “Jackie” Candelier, Sockie Jiao, and the staff of the Laboratory of Comparative Pathology for their assistance with necropsies, histology, immunohistochemistry and in situ hybridization assays, Mohd Anees Ahmed for his assistance with *C. rodentium* culture and infection and Mengze Lyu for his assistance with *Helicobacter hepaticus* culture.

Conflict of Interest

Anthony Mourino and Mert Aydin are employees of Jackson Laboratories, a company that produces and distributes research models and provides research services. David Artis has contributed to scientific advisory boards at Pfizer, Nemagene and the Kenneth Rainin Foundation. The other authors have no competing interest to declare.

Funding

This work was funded in part by the ACLAM Foundation. The Laboratory of Comparative Pathology is supported in part by NIH Grant P30 CA008748. Hiroshi Yano and David Artis are supported by The Allen Discovery Center program, a Paul G. Allen Frontiers Group advised program of the Paul G. Allen Family Foundation, the AGA Research Foundation, the WCM-RAPP Initiative, Cure for IBD, the Weill Cornell Medicine Jill Roberts Institute for Research in IBD, the Kenneth Rainin Foundation, the Sanders Family Foundation, the Rosanne H. Silvermann Foundation, the Glenn Greenberg and Linda Vester Foundation, the Crohn’s & Colitis Foundation (937437), and the National Institutes of Health (K99AI180354, DK126871, AI151599, AI095466, AI095608, AI142213, AR070116, AI172027, DK132244).

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