Increased *Listeria monocytogenes* Dissemination and Altered Population Dynamics in Muc2-Deficient Mice

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ABSTRACT The mucin Muc2 is a major constituent of the mucus layer that covers the intestinal epithelium and creates a barrier between epithelial cells and luminal commensal or pathogenic microorganisms. The Gram-positive foodborne pathogen *Listeria monocytogenes* can cause enteritis and also disseminate from the intestine to give rise to systemic disease. *L. monocytogenes* can bind to intestinal Muc2, but the influence of the Muc2 mucin barrier on *L. monocytogenes* intestinal colonization and systemic dissemination has not been explored. Here, we used an orogastric *L. monocytogenes* infection model to investigate the role of Muc2 in host defense against *L. monocytogenes*. Compared to wild-type mice, we found that Muc2^{-/-} mice exhibited heightened susceptibility to orogastric challenge with *L. monocytogenes*, with higher mortality, elevated colonic pathology, and increased pathogen burdens in both the intestinal tract and distal organs. In contrast, *L. monocytogenes* burdens were equivalent in wild-type and Muc2^{-/-} animals when the pathogen was administered intraperitoneally, suggesting that systemic immune defects related to Muc2 deficiency do not explain the heightened pathogen dissemination observed in oral infections. Using a barcoded *L. monocytogenes* library to measure intrahost pathogen population dynamics, we found that Muc2^{-/-} animals had larger pathogen founding population sizes in the intestine and distal sites than observed in wild-type animals. Comparisons of barcode frequencies suggested that the colon becomes the major source for seeding the internal organs in Muc2^{-/-} animals. Together, our findings reveal that Muc2 mucin plays a key role in controlling *L. monocytogenes* colonization, dissemination, and population dynamics.

KEYWORDS *Listeria monocytogenes*, Muc2, dissemination, population dynamics

Muc2 is a highly abundant O-glycosylated mucin glycoprotein that is primarily found on the mucosal surface of the intestinal tract and forms a gel-like structure that is the principal component of the mucus layer found at the interface between the intestinal epithelium and lumen (1, 2). Muc2 is synthesized by goblet cells, where it is oligomerized during intracellular trafficking and stored in secretory granules prior to its secretion (3). Although distributed throughout the intestinal tract, the density and structural organization of Muc2 within the mucus layer varies between sites; for example, two thick layers of Muc2 (a "loose" outer layer and a "firm" inner layer) are found in the colon, while only a porous mucus layer is found in the small intestine (1, 4).

A major physiological role of Muc2 is the creation of a physical barrier that segregates the gut microbiota from the intestinal epithelium (5). This barrier function is augmented by the wire mesh-like structure of Muc2 that serves as a scaffold for binding and displaying host-derived antimicrobial peptides and microbial binding proteins (e.g., human β -defensins, Relm- β , and Zg16) (6–8). The extensive O-glycosylation of

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Muc2 exerts both microbe- and host-directed effects that support intestinal homeostasis by supplying nutrients (e.g., carbohydrate moieties) to promote the expansion of gut commensal species and by delivering tolerogenic signals to lamina propria-resident dendritic cells (9, 10). The regulation of Muc2 production and secretion by goblet cells is also integrated into intestinal defense systems against enteric pathogens. For instance, Birchenough et al. found that a subpopulation of goblet cells in the colon releases Muc2-containing granules in response to direct sensing of pathogen-associated molecular patterns (PAMPs) via the intrinsic Nlrp6 (NOD-like receptor family pyrin domain-containing 6)-dependent inflammasome (11). In addition, goblet cells undergo hyperplasia and increase their mucus granule sizes in response to signals generated by other intestinal sentinel cells (e.g., Tuft cells) during infection (12).

The impacts of Muc2 on gut homeostasis and host defense against enteric pathogens have been revealed by studies of mice harboring a targeted knockout of the *Muc2* gene (*Muc2*^{-/-}) (13). These *Muc2*^{-/-} animals exhibit a reduced gap between luminal commensal bacteria and the intestinal epithelium (5, 14), epithelial hyperplasia (13, 14), increased colonic immune cell infiltration (15), altered microbiota (16), and elevated frequencies of colon cancer (13). Similar phenotypes have also been observed in a mouse strain (the Winnie mouse) with a missense mutation in the *Muc2* gene (17, 18). Besides intestinal tract anomalies, *Muc2*^{-/-} mice have systemic inflammation, higher titers of antibodies against bacterial lipopolysaccharide (LPS) and flagellin, and elevated levels of iron in circulation (19). In addition, *Muc2*^{-/-} mice have been found to be more susceptible to challenges with enteric pathogens, including *Citrobacter rodentium*, *Salmonella enterica* serovar Typhimurium, the nematode parasite *Trichuris muris*, and the protozoal parasite *Entamoeba histolytica* (14, 20–22).

L. monocytogenes is a Gram-positive foodborne bacterial pathogen that can cause enteritis as well as additional disease manifestations, such as meningitis, that result from its systemic dissemination from the intestinal tract (23). Observations from a rat ligated ileal loop model revealed that *L. monocytogenes* forms aggregates on intestinal mucus and induces goblet cell degranulation (24). Several *L. monocytogenes* surface proteins have been reported to bind to Muc2 (25), potentially mediating the pathogen's attachment to mucus. Using the mucin-expressing cell line HT29X, Coconnier et al. found that the secreted toxin listeriolysin O (LLO) is the major *L. monocytogenes* component that triggers mucin exocytosis (26, 27). Although goblet cell degranulation is generally recognized as a host defense strategy, *L. monocytogenes* is also thought to target these mucin-secreting cells to gain access to its host receptor in the intestine, E-cadherin (28, 29).

Here, we used *Muc2*^{-/-} mice and an orogastric *L. monocytogenes* infection model to investigate the role of the intestinal mucus layer in host defense against *L. monocytogenes*. Compared to wild-type (WT) mice, *Muc2*-deficient animals had heightened susceptibility to orogastric challenge with *L. monocytogenes*, exhibiting elevated mortality, more severe colonic pathology, and increased bacterial burden in the intestine and distal organs. In contrast, pathogen burdens were similar in *Muc2*^{+/+} and *Muc2*^{-/-} animals after intraperitoneal (i.p.) inoculation of *L. monocytogenes*. By using barcoded *L. monocytogenes* (30), we investigated the impact of Muc2 mucin on *L. monocytogenes* population dynamics during infection. *Muc2*^{-/-} animals had larger pathogen founding population sizes at intestinal and distal sites, and the genetic relatedness between these bacterial populations exceeded that in WT mice. Together, these findings suggest that Muc2 guards against *L. monocytogenes* dissemination from the intestine and demonstrate that the presence of this mucin modulates pathogen population dynamics during infection.

RESULTS

***Muc2*^{-/-} mice have heightened susceptibility to orogastric challenge with *L. monocytogenes*.** To assess the role of Muc2 in host defense against *L. monocytogenes*, littermate offspring of *Muc2*^{+/+} breeders were orogastrically challenged with *L. monocytogenes* 10403S InIA^m, a murinized variant of a human clinical isolate that contains

FIG 1 Muc2^{-/-} mice have heightened susceptibility to orogastric challenge with *L. monocytogenes*. (a) Survival curves of Muc2^{-/-} mice ($n=12$) and WT littermates (Muc2^{+/+}, $n=12$) following orogastric challenge with $\sim 3 \times 10^9$ CFU *L. monocytogenes* 10403S InIA^m. Mice were monitored every 12 h for 8 days. The data are from two independent experiments; the Gehan-Breslow-Wilcoxon test was used to compare the survival curves (****, $P < 0.0001$). (b) Weight of Muc2^{-/-} ($n=14$) and WT ($n=15$) mice during the first 3 days postorogastric inoculation of *L. monocytogenes*. Data are from three independent experiments. Two-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison test were used to assess significance (**, $P < 0.01$; ****, $P < 0.0001$). (c) Muc2^{-/-} mice (right) but not WT mice (left) developed signs of diarrhea at 3 dpi.

an InIA allele with enhanced binding to murine E-cadherin (31). During the 8-day observation period, 92% (11 out of 12) of Muc2^{-/-} mice died postpathogen challenge (Fig. 1a), whereas only 17% (2 out of 12) of WT (Muc2^{+/+}) mice succumbed (Fig. 1a). In addition, Muc2^{-/-} mice began to die earlier than WT mice (Fig. 1a) and lost more weight (Fig. 1b). Several of the Muc2^{-/-} mice developed diarrhea ~ 3 days postinoculation (dpi) (Fig. 1c); in contrast, WT mice do not develop diarrhea in this model. These observations indicate that Muc2 provides protection from the morbidity and mortality of oral *L. monocytogenes* infection in mice.

***L. monocytogenes* infection exacerbates colonic inflammation in Muc2^{-/-} mice.**

The absence of Muc2 is thought to largely eliminate the physical barrier between luminal commensal bacteria and the colonic epithelium, triggering microbially induced intestinal inflammation (15). Colons from *L. monocytogenes*-infected Muc2^{-/-} mice were visibly more swollen than colons from infected WT mice, suggestive of exacerbated intestinal inflammation in the Muc2^{-/-} group (Fig. 2a and b). Furthermore, the masses of the distal colons from both uninfected and infected Muc2^{-/-} mice were greater than those from corresponding WT mice (Fig. 2c); such differences were not observed in the proximal colons from either uninfected or infected animals (Fig. 2d). The elevated mass of the distal colon in Muc2^{-/-} mice is likely attributable to the influx of immune cells and proliferation of local colonic epithelial cells (14), but the factors that restrict these processes to the distal colon are not clear.

Inflammation of the colon is often associated with reduced colon length (32), and the colons of Muc2^{-/-} mice were significantly shorter than those from WT mice at 3 dpi (Fig. 2a and e). In contrast, colon lengths were similar in uninfected Muc2^{-/-} and WT animals (Fig. 2a and e), suggesting that lack of Muc2 mucin by itself is not sufficient to provoke longitudinal colon shrinkage. As *L. monocytogenes* challenge did not alter colon length in WT mice (Fig. 2e), these observations suggest that infection-induced colon shortening in Muc2^{-/-} mice is due to the combined effects of the Muc2 mucin deficit and the presence of the pathogen.

Histological analysis of distal colons from WT and Muc2^{-/-} mice using hematoxylin and eosin (H&E) staining corroborated our visual observations of gross pathology. Notably, in Muc2^{-/-} mice, infection induced massive immune cell infiltration into the colonic lamina propria, heightened erosion of colonic epithelial cells, and increased granulocytes in the colon lumen (Fig. 2f). In marked contrast, there was no detectable immune cell infiltration within the colonic lamina propria of WT mice (Fig. 2f). Overall, with the exception of granulocytes in the lumen of the distal colon in a subset of the WT mice, there was limited pathology observed in colons from WT mice 3 dpi (Fig. 2f). Thus, the absence of Muc2 elevates the intestinal inflammatory response to *L. monocytogenes*.

FIG 2 *L. monocytogenes* infection exacerbates colonic inflammation in *Muc2*^{-/-} mice. (a) Representative images of colons from uninfected (–) and infected (+) *Muc2*^{-/-} and WT mice at 3 days postinoculation (dpi); scale bar, 1 cm. (b) Magnified images of distal colons shown in panel a; scale bar, 1 cm. (c and d) Weight of the distal colon (c) and proximal colon (d) and length of entire colon (e) in uninfected and infected *Muc2*^{-/-} and WT mice at 3 dpi; 9 to 13 mice per group in panels c and d and 8 to 9 mice per group in panel e. (f) Hematoxylin and eosin (H&E)-stained distal colonic tissue from uninfected and infected *Muc2*^{-/-} and WT mice at 3 dpi; the yellow arrows show immune cell infiltration, and the green arrow shows sites of epithelial cell erosion; the blue arrows show granulocytes in the lumen. ANOVA and Fisher multiple-comparison test were used to assess significance in panels c to e (n.s., not significant; **, $P < 0.01$; ****, $P < 0.0001$).

Muc2 mucin modulates *L. monocytogenes* intestinal colonization and dissemination. To address the impact of Muc2 on bacterial colonization patterns, bacterial burdens in the intestines of infected animals were assessed at 3 dpi. Prior to plating, the intestinal samples were treated with gentamicin, an antibiotic that kills extracellular bacteria in the intestinal lumen, but not organisms found within the tissue of the intestine (33). In the small intestine, proximal colon, and distal colon, the *Muc2*^{-/-} mice carried significantly more *L. monocytogenes* than WT mice (Fig. 3a to c). The most dramatic difference was observed in the distal colon, where *Muc2*^{-/-} mice harbored ~1,000 times more *L. monocytogenes* than WT mice (Fig. 3c). Thus, the absence of Muc2-containing mucin in the distal colon markedly augments the accessibility of within-tissue niches to the pathogen at this site.

The burden of *L. monocytogenes* in the mesenteric lymph nodes (MLN), spleen, and liver were also determined to assess the impact of Muc2 deficiency on systemic pathogen dissemination. *Muc2*^{-/-} mice had ~100 times higher *L. monocytogenes* burdens in MLN, spleens, and livers than WT mice at 3 dpi (Fig. 3d to f), suggesting that Muc2 contributes to the barrier that ordinarily limits *L. monocytogenes* dissemination from the gut to distal sites. Furthermore, a higher percentage of *Muc2*^{-/-} mice carried *L. monocytogenes* in bile recovered from the gallbladder (GB) than WT mice 3 dpi (100% versus 45%) (Fig. 3g). Our unpublished observations suggest that there is a positive correlation between hepatic *L. monocytogenes* burden and GB colonization, raising the possibility that the elevated frequency of GB colonization in the Muc2-deficient animals is a consequence of the higher pathogen burden in their livers. Even though the Muc2-deficient animals were more likely to have GB colonization than WT mice, the *L. monocytogenes* burdens in GBs from WT and *Muc2*^{-/-} mice were similar (~10⁷ CFU; Fig. 3g), suggesting that Muc2 does not alter the bacterial carrying capacity of the GB.

Kumar et al. recently reported that *Muc2*^{-/-} mice have an elevated basal level of systemic inflammation (19), suggesting that the absence of a Muc2 barrier alters physiology in distal organs. To test whether *Muc2*^{-/-} mice have deficiencies in controlling systemic *L. monocytogenes* infection, WT and *Muc2*^{-/-} mice were challenged with 10⁵

FIG 3 Elevated *L. monocytogenes* intestinal colonization and dissemination in *Muc2*^{-/-} mice. Burden of *L. monocytogenes* in the ileum (a), proximal colon (b), distal colon (c), MLN (d), spleen (e), liver (f), and gallbladder (g) at 3 dpi in orogastrically infected *Muc2*^{-/-} and WT mice. The intestinal samples (a to c) were treated with gentamicin to kill extracellular bacteria. Data are from three independent experiments with 10 to 15 mice per group. The Mann-Whitney test was used to assess significance (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

CFU of *L. monocytogenes* via the intraperitoneal (i.p.) route. In contrast to our observations with oral inoculation (Fig. 3), similar numbers of *L. monocytogenes* were recovered from the MLN, spleen, and liver in WT and *Muc2*^{-/-} mice 3 days following i.p. inoculation (Fig. 4a to c), suggesting that the absence of Muc2 mucin does not compromise the capacity of these organs to control the infection. In addition, *Muc2*^{-/-} and WT mice exhibited similar weight loss and had comparable colon lengths after i.p. challenge (Fig. 4d and e), suggesting that the susceptibility of *Muc2*^{-/-} mice to oral pathogen challenge is not explained by a systemic immune defect.

Altered *L. monocytogenes* population dynamics in *Muc2*^{-/-} mice. Since we found that the Muc2-containing intestinal mucus barrier modulates *L. monocytogenes* intestinal colonization and dissemination, we leveraged sequence tag-based analysis of microbial populations (STAMP) (30, 34) to investigate the impact of Muc2 on *L. monocytogenes* population dynamics during infection. In this method, DNA-barcoded, but otherwise WT, *L. monocytogenes* organisms are used to calculate the number of bacteria from the inoculum that seed various infection sites (the founding population, or N_b) (30). Notably, in orally infected mice, N_b values from gentamicin-treated proximal and distal colon samples were significantly higher in *Muc2*^{-/-} than WT animals (Fig. 5a and b). The difference was particularly pronounced in the distal colon, where N_b was ~2,000 in *Muc2*^{-/-} versus ~50 in WT mice (Fig. 5b), suggesting that Muc2 provides an especially stringent barrier for the pathogen at this site. Presumably, the absence of the Muc2 mucin facilitates pathogen access to permissive niches within colonic tissue. Additional factors, such as the increased influx of immune cells and their accumulation in the colonic lamina propria of *Muc2*^{-/-} mice (Fig. 2f), may provide additional niches for *L. monocytogenes* growth, since the pathogen is known to replicate in these cells (35).

N_b sizes in internal organs also differed between *Muc2*^{-/-} and WT animals. N_b values in MLNs, spleens, and livers of *Muc2*^{-/-} mice were higher than those in WT mice but only reached statistical significance in MLNs (Fig. 5c to e). These observations are consistent with the idea that the Muc2 mucin barrier imposes a bottleneck on *L. monocytogenes* dissemination from the intestine. In contrast, even though 100% of *Muc2*^{-/-} mice harbored *L. monocytogenes* in their GBs, N_b values in GBs from *Muc2*^{-/-} mice were extremely low (mean N_b , 3; Fig. 5f), as observed before in BALB/c WT mice (30),

FIG 4 *Muc2*^{-/-} and WT littermate mice have similar pathogen burdens and disease manifestations after intraperitoneal challenge with *L. monocytogenes*. *Muc2*^{-/-} ($n=8$) and WT ($n=8$) mice were challenged with 1×10^5 CFU of *L. monocytogenes* 10403S InIA^m via the intraperitoneal route. Pathogen burdens recovered from MLN (a), spleen (b), and liver (c) at 3 dpi. (d) Weight of *Muc2*^{-/-} and WT mice for 3 days following i.p. inoculation. (e) Colon lengths were measured at 3 dpi.

suggesting that *Muc2* does not contribute to the host barrier that restricts *L. monocytogenes* access to the GB.

By calculating changes in the relative frequency of barcodes, STAMP also enables comparison of the genetic relatedness between bacterial populations recovered from different sites (34). In WT mice, bacterial populations from the proximal (Fig. 5g to i) or distal (Fig. 5j to l) colon were relatively distinct from those resident in the MLN, spleen,

FIG 5 *L. monocytogenes* population dynamics differ in *Muc2*^{-/-} and WT mice. Founding population size (N_b) of *L. monocytogenes* recovered from proximal colon (a) and distal colon (b) of *Muc2*^{-/-} ($n=11$) and WT ($n=13$) mice at 3 dpi; data are from three independent experiments. N_b of *L. monocytogenes* recovered from MLN (c), spleen (d), and liver (e) of *Muc2*^{-/-} ($n=6$) and WT ($n=4$) mice at 3 dpi. (f) N_b of *L. monocytogenes* recovered from bile of *Muc2*^{-/-} mice at 3 dpi; data ($n=10$) are from three independent experiments. (g to l) Genetic relatedness of *L. monocytogenes* recovered from different sites of *Muc2*^{-/-} ($n=6$) and WT ($n=4$) mice 3 dpi. Mann-Whitney tests were used to assess significance in panels a to k (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

and liver (i.e., low genetic relatedness values). These low values are similar to those we found in BALB/c mice (30), which are more permissive to *L. monocytogenes* infection than C57BL/6 mice, and indicate that *L. monocytogenes* disseminates from the intestinal tract to distal organs using multiple independent routes (30, 36). In contrast, in Muc2^{-/-} mice, these comparisons revealed a statistically significant higher degree of relatedness between bacterial populations recovered from either the proximal (Fig. 5g to i) or distal (Fig. 5j to l) colon and those from the MLN, spleen, and liver than found in WT animals. These observations suggest that the increases in absolute and founding *L. monocytogenes* population sizes in the proximal and distal colon (Fig. 3b and c and 5a and b) that are associated with the absence of Muc2 mucin also enable the colon to become the major source for seeding the internal organs. Collectively, these findings reveal that the absence of Muc2 mucin alters *L. monocytogenes* population dynamics during infection.

DISCUSSION

Here, we found that Muc2^{-/-} mice have heightened susceptibility to orogastric challenge with *L. monocytogenes*. Compared to WT mice, animals lacking Muc2 exhibited elevated mortality, more severe colonic pathology, and increased pathogen burdens in the intestine as well as in distal organs following oral inoculation of *L. monocytogenes*. The heightened sensitivity of Muc2^{-/-} mice appears to be dependent on the route of infection, since we found that *L. monocytogenes* burdens were equivalent in WT and Muc2-deficient animals when the pathogen was administered intraperitoneally. Furthermore, our experiments with barcoded *L. monocytogenes* demonstrated that Muc2 restricts *L. monocytogenes* founding population sizes, particularly in the colon. In the absence of Muc2, the colon becomes the dominant site from which the pathogen disseminates to distal organs. Together, these observations reveal that Muc2 mucin plays a critical role in controlling *L. monocytogenes* colonization, dissemination, and population dynamics.

The elevated susceptibility of Muc2^{-/-} mice to *L. monocytogenes* challenge is likely attributable to the physical absence of the mucus barrier in the intestine as well as to the indirect effects of the absence of Muc2, including changes in the composition of the microbiota in these mice (16). Muc2 is the dominant mucin component of the mucus barrier that physically limits the access of commensal as well as pathogenic microorganisms to the epithelial surface of the intestine (5, 37); a thinned/absent mucus layer that lacks Muc2 likely facilitates the pathogen's capacity to approach and ultimately invade intestinal epithelial cells. This host defense mechanism has been postulated for other enteric pathogens, including *Citrobacter rodentium* and *Salmonella* Typhimurium, where increased direct contact of these pathogens with the colonic epithelium of Muc2^{-/-} mice was observed (14, 21). Additionally, increased mucus flow during enteric infection expels pathogens from the epithelial surface and is thought to be a host defense mechanism (38). Consistent with this idea, impairment of mucin exocytosis from goblet cells, e.g., by deletion of vesicle-associated membrane protein 8 (VAMP8), leads to heightened host susceptibility to enteric pathogens *Citrobacter rodentium* and *Entamoeba histolytica* (39, 40). Although the function of goblet cell secretory granules in Muc2^{-/-} mice has not been precisely described, a "flush out" strategy may be ineffective in Muc2^{-/-} mice given the absence of mucus to support pathogen excretion. Altered mucus homeostasis due to lack of Muc2 production and secretory granule formation could also lead to goblet cell dysfunction, a phenotype that may increase *L. monocytogenes*' access to its host receptor, E-cadherin (41).

Besides Muc2's critical physical role in intestinal defense, its absence has several indirect consequences that likely also contribute to the susceptibility of Muc2^{-/-} mice to oral infection with *L. monocytogenes*. Using streptomycin-treated mice, Becattini et al. discovered that gut commensal microbes (e.g., *Clostridiales* such as *C. saccharogumia*, *C. ramosum*, and *C. hathewayi*) confer colonization resistance and protection against oral *L. monocytogenes* challenge (42). It is plausible that changes in the

composition of the microbiota in *Muc2*^{-/-} mice (16) increase their susceptibility to oral *L. monocytogenes* challenge through direct antagonism of the pathogen or by other effects on the host. For example, alterations in the intestinal microbiota that accompany *Muc2* deficiency could also drive or synergize with the elevated basal colonic inflammation in these animals (15) in a way that contributes to their heightened susceptibility to oral infection with *L. monocytogenes*.

Mucin glycans can also serve as sources of nutrition for pathogens and are important regulators of bacterial pathogenicity (43–45). For example, O-glycans on MUC5AC can suppress the virulence of *Pseudomonas aeruginosa*, facilitating its clearance in a porcine burn wound model (44). The absence of such interkingdom regulatory signals in *Muc2*^{-/-} mice might modulate the *L. monocytogenes* virulence gene program and, thus, impact *L. monocytogenes* systemic dissemination.

Kumar et al. recently reported that *Muc2*^{-/-} mice have elevated basal levels of systemic inflammation and circulatory iron, conditions that may promote growth of disseminated bacteria and were associated with increased susceptibility to i.p. challenge with LPS (19). This suggested that animals with whole-body knockouts of *Muc2* have systemic defects in their response to PAMPs. However, such changes did not appear to alter the capacity of *Muc2*^{-/-} mice to control systemic infection caused by i.p. administration of *L. monocytogenes*, as pathogen burdens were similar after using this route of infection in WT and *Muc2*^{-/-} mice. Further work will be required to decipher the potential extraintestinal contributions of *Muc2* to host defense against pathogens, which may be challenge and tissue specific.

The structure of the mucus layer varies in different regions of the intestinal tract; the small intestine is covered by a thin, porous mucus layer, while the colon is heavily coated with two layers of *Muc2*-containing mucus (1). We found that the effects of the absence of *Muc2* were particularly marked in the distal colon, where there was a drastic increase in the burden of within-tissue *L. monocytogenes*; there was only a moderate elevation in the pathogen burden in the ileum and proximal colon. Consistent with our observations, in a *Muc2*^{-/-} mouse model of spontaneous colitis, the distal colon was found to have more neutrophilic infiltrates than the proximal colon (15). The distal colon is distinguished by a thick inner mucus layer that is not easily penetrated by microbes (37, 46, 47) and a population of goblet cells that supports the rapid renewal of mucus within this inner layer (48). The severe phenotype we observed in the distal colon supports the idea that this region of the intestine is particularly reliant on *Muc2*-producing goblet cells for defense.

Muc2 deficiency not only affects the pathogen burden in host tissues but also alters the pathogen's population dynamics. Our previous (30) and present observations, in two different strains of mice, are consistent with the hypothesis that *L. monocytogenes* disseminates from the intestinal tract to distal organs using multiple independent routes, a pattern that has been referred to as episodic spread (30). Notably, the pathogen's dissemination pattern was altered in the *Muc2*^{-/-} mice. The genetic relatedness of the *L. monocytogenes* populations recovered from the colon and distal organs (MLN, spleen, and liver) was significantly higher than that observed in WT mice, suggesting that the colon is the source for a larger fraction of organisms that disseminate in *Muc2*^{-/-} versus WT mice. We also observed a marked increase in both the *L. monocytogenes* burden and founding population size in the colon of *Muc2*^{-/-} mice. Together, these observations suggest that *Muc2* in the colon is a critical host restriction factor that ordinarily prevents *L. monocytogenes* colonization and invasion of colonic tissue. The absence of this physical host barrier along with changes in colonic microenvironment facilitate *L. monocytogenes* proliferation and, thus, enable the colon to become the chief reservoir for dissemination. A deeper understanding of the consequences of and mechanisms by which perturbations of intestinal structure and function modify host defense could provide insight to guide new treatment strategies for disorders where intestinal insults promote systemic disease presentations, such as inflammatory bowel disease.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Listeria monocytogenes* strain used in this study (Lm-STAMP-200 library; see Table S1 in the supplemental material) is a barcoded derivative (30) of *L. monocytogenes* 10403S InIA^m, a strain where internalin A contains two amino acid substitutions that increase its capacity to bind murine E-cadherin (31). For animal studies, aliquots of the Lm-STAMP-200 library were cultured in brain heart infusion (BHI; BD Biosciences) broth with chloramphenicol (Cm; 7.5 μ g/ml) and streptomycin (Sm; 200 μ g/ml) at 37°C for 3 h. Bacteria were pelleted by centrifugation (3,000 $\times$ *g* for 10 min), washed twice with 20 ml phosphate-buffered saline (PBS), and diluted to the indicated concentration in PBS for inoculation.

Animal studies. C57BL/6 Muc2^{-/-} mice used in this study were a gift from Anna Velcich (13) and were bred at the Harvard Institutes of Medicine animal facility. Mice were genotyped at 3 weeks of age, and Muc2^{+/+} (WT) and Muc2^{-/-} mice were cohoused until 10 to 12 weeks of age. Gender- and age-matched littermates that were the offspring of heterozygous Muc2^{+/-} breeders were used throughout the study. As observed by other groups (49), a small fraction of Muc2^{-/-} animals (~10%) in our colony developed spontaneous colon prolapse by 10 to 12 weeks of age; these mice were euthanized and excluded from the study. For orogastric infection studies, WT and Muc2^{-/-} littermates were fasted for 8 h prior to infection by gavage (18-gauge feeding needle) with $\sim 3 \times 10^9$ CFU of barcoded *L. monocytogenes* (200 μ l) suspended in a 300- μ l mixture of 200 mM CaCO₃. For intraperitoneal infection studies, WT and Muc2^{-/-} littermates were administered $\sim 1 \times 10^5$ CFU of barcoded *L. monocytogenes* (200 μ l) in PBS via i.p. injection. Mouse body weights were measured prior to inoculation and daily postinoculation for 3 days. To assess gross intestinal pathology, animals were euthanized 3 dpi, and the length and weight of colons were measured. For survival curve analysis after orogastric inoculation, animals were monitored twice per day for 8 days.

To assess bacterial burden, animals were euthanized 3 dpi, and organs (ileum [distal one-third of the small intestines], colon, MLN, spleen, liver, and GB) were collected. Colonic tissues were equally divided into two parts of approximately equal length, which were designated proximal colon (adjacent to cecum) and distal colon (adjacent to anus). Proximal colons, distal colons, and ilea were cut open longitudinally, incubated in Dulbecco's modified Eagle medium (DMEM) with 100 μ g/ml gentamicin for 2 h to kill the extracellular bacteria, transferred to 50-ml conical centrifuge tubes (Corning), and washed with 25 ml of PBS five times on a rotator. The intestinal segments, MLN, spleen, and liver were homogenized in sterile PBS using a beat-beater (BioSpec Products). To collect bile, GBs were transferred to Eppendorf tubes containing 1 ml of PBS and ruptured with 23-gauge needles (Becton, Dickinson). For CFU enumeration, all of the homogenates/samples were serially diluted, plated on BHI-Sm plates, and kept at 37°C for 48 h. For STAMP analysis, homogenates/samples were directly plated on 245-by-245-mm square BHI-Sm plates (Corning).

Histological analysis. Distal colons from WT and Muc2^{-/-} animals 3 dpi were fixed in 4% paraformaldehyde (PFA) for 2 h, transferred to 70% ethanol, and kept at 4°C overnight. Samples were embedded in Tissue-Tek OCT solution (Sakura Finetek) and sliced into 10- μ m sections using a Leica CM1860 UV cryostat. Samples were stained with hematoxylin and eosin, mounted with Organo/Limonene mounting medium (Sigma), and scanned ($\times 200$ magnification) using a Nikon confocal microscope.

STAMP protocol. Calculation of *Nb* and genetic relatedness was performed as previously described (30, 34). Briefly, *L. monocytogenes* colonies from the indicated organs were washed off the BHI-Sm plates with PBS. Cells were pelleted and genomic DNA was extracted (Wizard Genomic DNA purification kit; Promega) from $\sim 1 \times 10^{10}$ bacteria. The region that harbors the 30-bp barcodes was amplified from genomic DNA using primer PLM30 and primer PLM6-P29 (Table S1). The PCR products were purified (MinElute; Qiagen) and quantified (Qubit dsDNA HS assay kit; Life Technologies). Purified PCR products were combined in equimolar concentrations and sequenced on an Illumina MiSeq (MiSeq reagent kit V2, 50-cycle; Illumina) using primer PLM49. Reaper-12-340 was used to discard sequence reads with low quality ($\leq Q30$) and to trim the sequence following the barcode (50). The trimmed sequences were clustered with QIIME (version 1.6.0) using pick_otus.py with a sequence similarity threshold of 0.9 (51). *Nb* was then calculated using a custom R script. Genetic distance was estimated using the Cavalli-Sforza chord distance method (52) as described by Abel et al. (34). Genetic relatedness is one minus genetic distance.

Ethics statement. Animal experiments in this study were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (53) and were approved by the Brigham and Women's Hospital IACUC (2016N000416). Mice were euthanized by isoflurane inhalation followed by cervical dislocation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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