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Journal

Infection and Immunity, 88(12)

ISSN

0019-9567

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Publication Date

2020-11-16

DOI

10.1128/iai.00407-20

Peer reviewed

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HOST RESPONSE AND INFLAMMATION

Secretion of c-di-AMP by *Listeria monocytogenes* Leads to a STING-Dependent Antibacterial Response during Enterocolitis

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ABSTRACT Stimulator of interferon genes (STING) acts as a cytoplasmic signaling hub of innate immunity that is activated by host-derived or bacterially derived cyclic dinucleotides. *Listeria monocytogenes* is a foodborne, facultative intracellular pathogen that secretes c-di-AMP and activates STING, yet the *in vivo* role of the STING pathway during bacterial pathogenesis remains unclear. In this study, we found that STING-deficient mice had increased weight loss and roughly 10-fold-increased systemic bacterial burden during *L. monocytogenes*-induced enterocolitis. Infection with a *L. monocytogenes* mutant impaired in c-di-AMP secretion failed to elicit a protective response, whereas a mutant with increased c-di-AMP secretion triggered enhanced protection. Type I interferon (IFN) is a major output of STING signaling; however, disrupting IFN signaling during *L. monocytogenes*-induced enterocolitis did not recapitulate STING deficiency. In the absence of STING, the intestinal immune response was associated with a reduced influx of inflammatory monocytes. These studies suggest that in barrier sites such as the intestinal tract, where pathogen-associated molecular patterns are abundant, cytosolic surveillance systems such as STING are well positioned to detect pathogenic bacteria.

KEYWORDS *Listeria monocytogenes*, gastrointestinal infection, innate immunity, monocytes, pathogenesis

Listeria monocytogenes is a Gram-positive bacterium that lives on decaying organic matter in the environment, and upon ingestion by a mammalian host, it transforms into a facultative intracellular pathogen (1). In humans, *L. monocytogenes* causes a foodborne disease that often leads to a mild, self-limiting gastroenteritis, but in rare instances, *L. monocytogenes* infection can become a life-threatening condition that is characterized by bacteremia, meningoencephalitis, and pregnancy loss (2, 3). In mice, infection through the intravenous (i.v.) route leads to highly reproducible bacterial burdens in the spleen and liver. Although the i.v. model recapitulates the severe systemic form of *L. monocytogenes* infection, it completely bypasses the initial intestinal phase of pathogenesis. Like humans, mice are also generally resistant to *L. monocytogenes* infection though the oral route. In mice, administration of doses as high as 10^9 CFU p.o. leads to a mild infection with minor clinical symptoms (4, 5). Recently, it was shown that the induction of intestinal dysbiosis by streptomycin treatment before administering *L. monocytogenes per os* (p.o.) dramatically improved *L. monocytogenes* colonization of mouse intestines (6). In this model, mice develop enterocolitis, diarrhea, and systemically disseminated *L. monocytogenes*. Importantly, bacterial mutants that are unable to escape from a phagosome (LLO-minus) or mediate cell-to-cell spread (ActA-minus) still colonize the intestinal lumen but fail to induce host pathology, demonstrating the requirement for virulence factors in developing intestinal disease and thus establishing the basis for the use of the enterocolitis model to study *L. monocytogenes* pathogenesis (7).

Citation Louie A, Bhandula V, Portnoy DA. 2020. Secretion of c-di-AMP by *Listeria monocytogenes* leads to a STING-dependent antibacterial response during enterocolitis. Infect Immun 88:e00407-20. https://doi.org/10.1128/IAI.00407-20.

Editor Nancy E. Freitag, University of Illinois at Chicago

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Received 3 July 2020
Returned for modification 10 August 2020
Accepted 27 September 2020

Accepted manuscript posted online 5 October 2020
Published 16 November 2020

In this study, we explored the role of innate immune signaling during *L. monocytogenes* enterocolitis. Although much is known about activation of innate immunity during systemic spread, less is known about innate immunity during the natural route of infection. The intestinal tract is populated with a dense and diverse microbial community, and the host immune system can distinguish pathogens from normal members of the microbiota by monitoring for microbial activities that are associated with pathogenesis (8). For example, the presence of c-di-AMP in the host cytosol alerts the innate immune system to the presence of intracellular *L. monocytogenes* (9, 10). C-di-AMP belongs to a broad family of cyclic dinucleotides (CDNs), and c-di-AMP and c-di-GMP are highly conserved among diverse bacteria (11, 12). In *L. monocytogenes*, c-di-AMP functions as an essential allosteric activator of metabolic functions (13). *L. monocytogenes* mutants that lack c-di-AMP are extremely debilitated, making it difficult to define the *in vivo* role of c-di-AMP in pathogenesis and the host immune response (14). Of note, the induction of multidrug resistance (MDR) transporters, which mediate c-di-AMP secretion in *L. monocytogenes*, increases between 4- and 200-fold during intracellular growth relative to induction levels when grown in broth. *L. monocytogenes* encodes at least 8 MDR transporters of the major facilitator superfamily, and deleting 4 accounts for 80% of c-di-AMP secretion (15). Adjacent transcriptional repressors regulate the induction of MDR transporters. For example, TetR regulates *mdrT* expression, and Δ*tetR* mutants have 100-fold-increased induction of *mdrT*, leading to increased c-di-AMP secretion in host cells (16).

There are 3 major pathways of innate immune recognition during *L. monocytogenes* infection (17): a MyD88-dependent pathway that emanates from a phagosome that is triggered by bacterial lipoproteins and nucleic acid released during bacteriolysis (18), DNA-dependent AIM2-mediated inflammasome activation that is induced primarily by the infrequent lysis of cytosolic bacteria (19), and the stimulator of interferon (IFN) genes (STING)-dependent pathway, which is triggered by the secretion of *L. monocytogenes* c-di-AMP (9). In this study, we focused on the role of the STING pathway. STING is an innate immune sensor and cytosolic signaling hub that acts as a cytosolic CDN receptor (20). Both endogenously and bacterially produced CDNs bind and activate STING. In vertebrates, aberrant double-stranded DNA activates cyclic GMP-AMP synthase (cGAS) to produce c-GMP-AMP (21). *L. monocytogenes* c-di-AMP activates STING directly and does not require cGAS (22). STING signaling contributes to the production of type I IFN during *L. monocytogenes* infection (23). The route of infection influences how type I IFN impacts pathogenesis. In the i.v. model, type I IFNs promote bacterial pathogenesis (24–26), but in various p.o. models, type I IFNs have modest antibacterial effects (27, 28). In addition to type I IFN, STING activation also results in NF-κB activation (29), autophagy (30), and STAT6 activation (31). Active STAT6 can lead to the production of the chemokine CCL2 (31), which binds CCR2 on the surfaces of monocytes. CCR2 signaling initiates the egress of monocytes from the bone marrow and entry to the bloodstream (32, 33). Monocytes represent a heterogenous population of phagocytic white blood cells that rapidly traffic to sites of infection. Mice lacking CCR2 or CCL2 or that have undergone monocyte depletion develop high bacterial burdens following i.v. *L. monocytogenes* infection (32, 34, 35). Furthermore, adoptive transfer of monocytes leads to a reduction of systemic *L. monocytogenes* (36). During intestinal infection, monocytes represent the predominant cell type that associates with *L. monocytogenes* and fail to support intracellular replication (37). Together, these findings demonstrate an essential role for monocytes in the immune response to *L. monocytogenes* infection. Given that the activation of a single innate immune sensor leads to a wide array of functional outputs that provide both pro- and antibacterial responses, a common theme for the role of the STING in bacterial pathogenesis has not yet emerged.

In this study, we found that STING activation led to reduced bacterial burden and correlated with the recruitment of monocytes to the intestines during *L. monocytogenes*-induced enterocolitis. This STING-mediated protective response was triggered by the secretion of *L. monocytogenes* c-di-AMP, while disruption of type I IFN signaling during *L. monocytogenes*-induced enterocolitis did not recapitulate STING

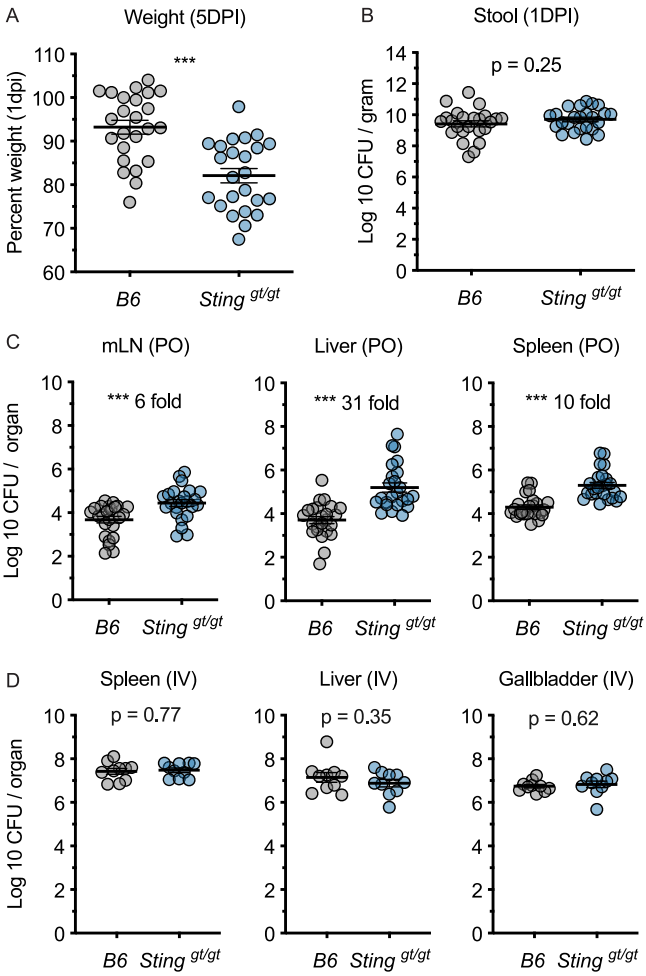

FIG 1 STING activation during the intestinal phase of *L. monocytogenes* infection leads to an antibacterial response. (A to C) *B6* ($n = 24$) and *Sting*$^{gt/gt}$ ($n = 24$) mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU *per os* [p.o.]). (A) Body weights 5 days postinfection expressed as a percentage of initial weight. (B) *L. monocytogenes* CFU recovered from stool samples collected 1 day postinfection. (C) *L. monocytogenes* CFU recovered from the indicated organs 5 days postinfection. (D) *B6* ($n = 10$) and *Sting*$^{gt/gt}$ ($n = 10$) mice were infected with *L. monocytogenes* (10^5 CFU intravenously [i.v.]). *L. monocytogenes* CFU were recovered from the indicated organs at 2 days postinfection. For panels B to D, results are presented as log-transformed values. For all panels, horizontal lines represent means and error bars represent SEM. Asterisks indicate statistically significant differences by two-tailed *t* test (***, $P < 0.001$). Data are pooled from at least two experiments.

deficiency. Collectively, these findings broaden our understanding of the *in vivo* functional outcomes of STING activation and may help us to understand how sensors of the innate immune system coordinate to detect pathogens at barrier sites.

RESULTS

STING activation during the intestinal phase of *L. monocytogenes* infection leads to an antibacterial response. To investigate the role of STING signaling in the intestinal phase of *L. monocytogenes* pathogenesis, we chose to use a recently developed mouse model of *L. monocytogenes*-induced enterocolitis (7). Groups of wild-type C57BL/6 (*B6*) and STING-deficient *Sting*$^{gt/gt}$ mice received a 2-day course of streptomycin (5 mg/ml)-supplemented drinking water and underwent an overnight fast followed by infection p.o. through the voluntarily consumption of a breadcrumb soaked with 10^8 *L. monocytogenes* CFU. At 5 days postinfection, *Sting*$^{gt/gt}$ mice had lost nearly 3 times more weight than *B6* mice (Fig. 1A). The difference in disease severity between *B6* and *Sting*$^{gt/gt}$ mice could not be explained by a change in the ability of *L. monocytogenes* to colonize the intestinal tract in STING-deficient mice, as the number of *L. monocytogenes*

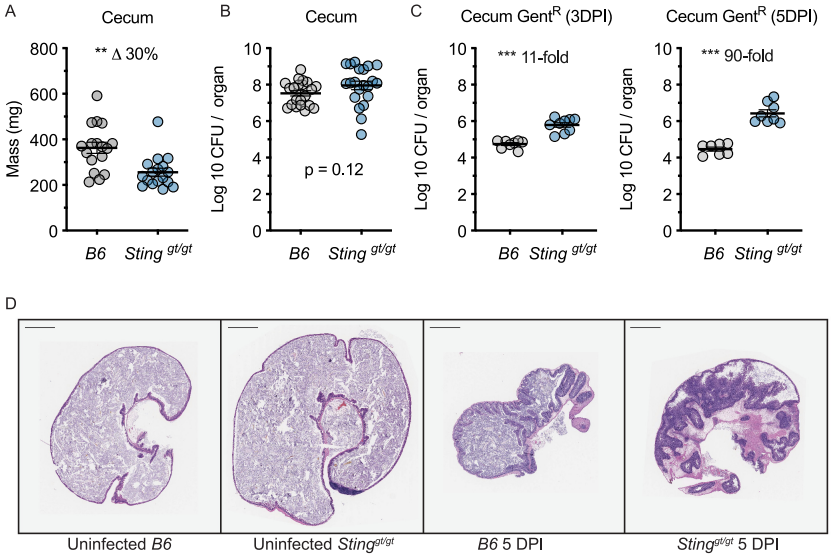

FIG 2 STING activation restricts intracellular *L. monocytogenes* in intestinal tissues. *B6* and *Sting*$^{gt/gt}$ mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU *per os*). (A) Cecal masses of *B6* ($n = 17$) and *Sting*$^{gt/gt}$ ($n = 17$) mice. (B) *L. monocytogenes* CFU recovered from cecal contents at 5 days postinfection for *B6* ($n = 21$) and *Sting*$^{gt/gt}$ ($n = 21$) mice. (C) Gentamicin-resistant *L. monocytogenes* CFU recovered from cecum samples at indicated time points. For 3 days postinfection (3DPI), $n = 8$ and $n = 9$ for *B6* and *Sting*$^{gt/gt}$ mice, respectively; for 5 days postinfection (5DPI), $n = 7$ and $n = 8$ for *B6* and *Sting*$^{gt/gt}$ mice, respectively. For panels B and C, results are presented as log-transformed values. Horizontal lines represent means and error bars represent SEM. Asterisks indicate statistically significant differences by two-tailed *t* test (**, $P < 0.01$;***, $P < 0.005$). Data are pooled from at least two experiments. (D) Hematoxylin and eosin-stained cecal samples collected 5 days postinfection. Scale bars represent 2 mm.

CFU recovered from stool samples collected 24 h postinfection were comparable for both mouse strains (Fig. 1B). From the intestinal tract, *L. monocytogenes* spreads locally to the mesenteric lymph nodes (mLN) and systemically to organs, including the spleen and the liver. At both local and distal sites of dissemination, *Sting*$^{gt/gt}$ mice carried higher bacterial burdens than did *B6* mice: a 6-fold increase in the mLN, a 10-fold increase in the spleen, and a 31-fold increase in the liver (Fig. 1C). Differences in the bacterial burdens of systemic sites were also observed between *Sting*$^{gt/gt}$ and littermate controls, suggesting that the intestinal microbiota could not account for this phenotype (see Fig. S1 in the supplemental material). Interestingly and consistent with previous studies, the bacterial burdens of the spleen, liver, and gallbladder did not significantly differ between *B6* and *Sting*$^{gt/gt}$ mice when *L. monocytogenes* was administered intravenously (Fig. 1D), indicating that STING signaling plays a minor role in controlling bacterial growth during the systemic phase of the infection. Collectively, these results are consistent with a requirement for STING signaling in the intestinal tract to coordinate an immune response that reduces the severity of *L. monocytogenes* infection.

STING activation restricts intracellular *L. monocytogenes* in intestinal tissues. During the collection of tissues to enumerate CFU at 5 days postinfection, we noticed that the ceca of *Sting*$^{gt/gt}$ mice were noticeably smaller and weighed 30% less than those of *B6* mice (Fig. 2A). The difference in weights appeared to be due to a reduction in cecal contents and could have been caused by a reduction in food intake or the development of diarrhea, which was observed in the animal bedding. Given the striking visual difference in the ceca, we considered that this phenotype might relate to the more severe disease exhibited by *Sting*$^{gt/gt}$ mice. Within the intestinal tract, *L. monocytogenes* replicates in the lumen as free-living bacteria, and in the tissues, *L. monocytogenes* replicates intracellularly in host cells. To examine these two populations, the luminal content was collected and plated to obtain the extracellular bacteria fraction,

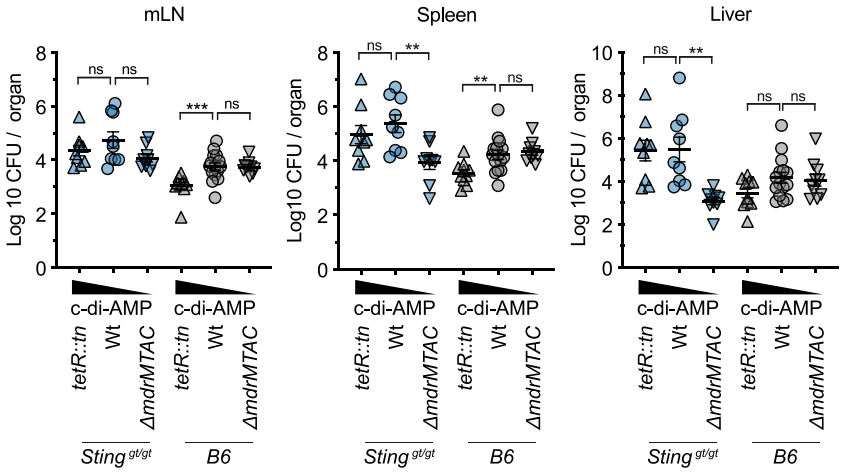

FIG 3 STING activation by *L. monocytogenes*-derived c-di-AMP is antibacterial. *B6* and *Sting*$^{gt/gt}$ mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU of indicated strains *per os*). *L. monocytogenes* CFU were recovered from indicated organs 5 days postinfection. Sample sizes are as follows: for *Sting*$^{gt/gt}$ mice, 10, 15, and 10 for the *tetR*::*tn*, wild-type, and Δ*mdrMTAC* strains, respectively; for *B6* mice, 9 each for the *tetR*::*tn*, wild-type, and Δ*mdrMTAC* strains. Results are presented as log-transformed values. Horizontal lines represent means and error bars represent SEM. Asterisks indicate statistically significant differences by one-way analysis of variance (ANOVA) and Dunnett's posttest (**, $P < 0.01$; ***, $P < 0.005$). Data are pooled from at least two experiments. ns, not significant.

and tissues were washed, treated with gentamicin, and homogenized to obtain the intracellular bacterial fraction. The host cell membrane protects intracellular bacteria from the bactericidal activity of gentamicin; therefore, the gentamicin-resistant bacteria represent the number of intracellular bacteria. At 5 days postinfection, the numbers of extracellular bacteria in the ceca were not significantly different between *B6* and *Sting*$^{gt/gt}$ mice (Fig. 2B). However, the number of intracellular bacteria in the ceca of *Sting*$^{gt/gt}$ mice was increased 11-fold at 3 days postinfection and 90-fold at 5 days postinfection relative to that in *B6* mice (Fig. 2C). These data indicate a requirement for STING signaling in the control of intracellular bacteria in the intestines. Histological samples of the cecum showed no remarkable differences between uninfected *B6* and *Sting*$^{gt/gt}$ mice, but upon *L. monocytogenes* infection, inflammation of the *Sting*$^{gt/gt}$ mouse cecum appeared more diffuse (Fig. 2D). The increase in intracellular *L. monocytogenes* of the *Sting*$^{gt/gt}$ ceca could explain this difference in inflammation.

***L. monocytogenes*-derived c-di-AMP activates STING to provide a protective response.** Many bacterial species, including the commensals that occupy the intestinal tract, produce CDNs (11). We used bacterial and mouse mutants to address the functional significance of c-di-AMP secretion by *L. monocytogenes* during the intestinal phase of infection. *L. monocytogenes* strains that completely lacked c-di-AMP, such as a Δ*dacA* strain, could not be tested because loss of c-di-AMP synthesis leads to severe growth defects (14). As an alternative, we used a strain that is deficient in four MDR transporters (Δ*mdrMTAC*) and secretes 7-fold less c-di-AMP than a wild-type strain (15). We also evaluated the *tetR*::*tn* strain, in which the transcriptional repressor of *mdrT* is disrupted by a transposon insertion, resulting in increased *mdrT* expression and a 20-fold increase in c-di-AMP secretion (16). The contribution of host-derived CDNs was ruled out because bacterial burdens of *Cgas*$^{-/-}$ mice and *B6* mice did not significantly differ when infected with wild-type *L. monocytogenes* (Fig. S2).

To investigate the impact of increased c-di-AMP secretion on virulence, we compared a Δ*tetR* mutant to wild-type *L. monocytogenes* in *B6* mice. The *tetR*::*tn* bacterial burdens were 5-fold lower in the spleen and mLN than in mice infected with wild-type *L. monocytogenes*. However, the bacterial burdens of *Sting*$^{gt/gt}$ mice infected with *tetR*::*tn* or wild-type *L. monocytogenes* did not significantly differ, indicating that *L. monocytogenes*-derived c-di-AMP contributes to a STING-mediated antibacterial response (Fig. 3).

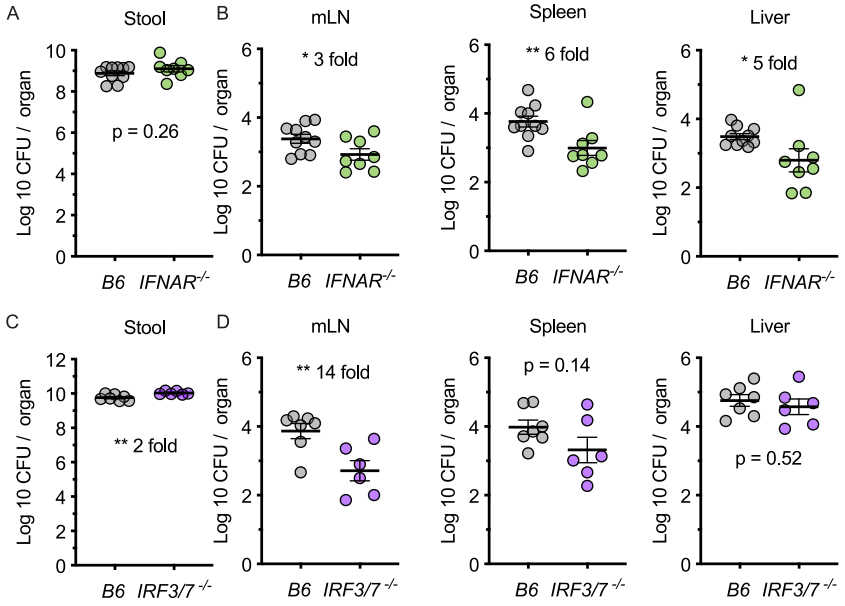

FIG 4 Disrupting IFN signaling during *L. monocytogenes*-induced enterocolitis leads to reduced bacterial loads at systemic sites. (A and B) *B6* ($n = 10$) and *Ifnar*$^{-/-}$ ($n = 8$) mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU *per os*). (A) *L. monocytogenes* CFU recovered from stool samples collected 1 day postinfection. (B) *L. monocytogenes* CFU recovered from the indicated organs at 5 days postinfection. (C and D) *B6* ($n = 7$) and *Irf3/7*$^{-/-}$ ($n = 6$) mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU *per os*). (C) *L. monocytogenes* CFU recovered from stool samples collected 1 day postinfection. (D) *L. monocytogenes* CFU recovered from the indicated organs at 5 days postinfection. Results are presented as log-transformed values. Horizontal lines represent means and error bars represent SEM. Asterisks indicate statistically significant differences by two-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are pooled from at least two experiments.

We next investigated the effects of decreased c-di-AMP secretion on virulence. To deduce STING-independent effects of deleting four MDR transporters on virulence, we evaluated Δ*mdrMTAC* and wild-type *L. monocytogenes* in *Sting*$^{gt/gt}$ mice. Compared to wild-type *L. monocytogenes* in *Sting*$^{gt/gt}$ mice, the bacterial burdens of Δ*mdrMTAC* in *Sting*$^{gt/gt}$ mice were 240-fold lower in the liver and 25-fold lower in the spleen and trended lower in the mLN, indicating a role for MDR transporters in virulence that is independent of STING signaling. To evaluate the STING-dependent antibacterial response in a setting of low *L. monocytogenes*-derived c-di-AMP, we compared Δ*mdrMTAC* and wild-type *L. monocytogenes* in *B6* mice. The bacterial burdens of *B6* mice infected with Δ*mdrMTAC* and wild-type *L. monocytogenes* did not significantly differ (Fig. 3). In the absence of a STING-independent virulence defect, we would have expected the Δ*mdrMTAC* strain to have increased virulence by evading STING activation. However, because we observed a significant STING-independent virulence defect, the bacterial burden in *B6* mice represents a STING-dependent phenotypic rescue. These observations suggest that the extent by which the Δ*mdrMTAC* strain induces a STING-dependent antibacterial response is minimal compared to that of wild-type *L. monocytogenes*, and the potential benefit of reduced STING activation is neutralized by the STING-independent virulence defect of the Δ*mdrMTAC* strain. Taken together, these results provide genetic evidence for the initiation of a STING-mediated antibacterial response by *L. monocytogenes*-derived c-di-AMP.

Disrupting IFN signaling during *L. monocytogenes*-induced enterocolitis leads to reduced bacterial loads at systemic sites. *L. monocytogenes* infection elicits a robust STING-dependent type I IFN response (23). To address the contributions of type I IFN to a protective response during *L. monocytogenes*-induced enterocolitis, groups of *B6* and *Ifnar*$^{-/-}$ mice received streptomycin pretreatment and 10^8 CFU of *L. monocytogenes* p.o. *L. monocytogenes* CFU recovered from stool samples revealed no differences in the abilities to colonize the intestinal tracts of *B6* and *Ifnar*$^{-/-}$ mice (Fig. 4A).

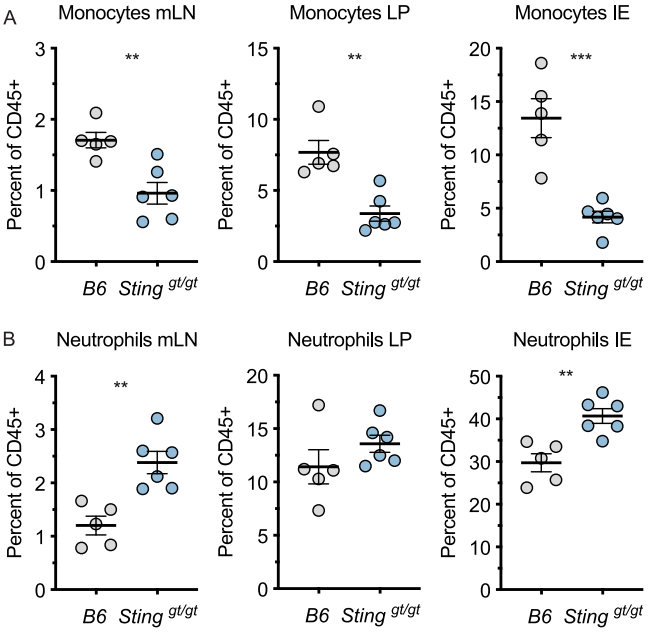

FIG 5 Reduced monocyte recruitment in the absence of STING signaling. (A and B) *B6* ($n = 5$) and *Sting*$^{gt/gt}$ ($n = 6$) mice were pretreated with streptomycin and infected with *L. monocytogenes* (10^8 CFU *per os*). (A) Monocyte counts at 3 days postinfection of indicated tissues by flow cytometry. (B) Neutrophil counts at 3 days postinfection of indicated tissues by flow cytometry. Horizontal lines represent means and error bars represent SEM. Asterisks indicate statistically significant differences by two-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$). Data are pooled from at least two experiments.

In stark contrast to the case with *Sting*$^{gt/gt}$ mice, the mLN, spleens, and livers of *Ifnar*$^{-/-}$ mice contained significantly lower bacterial burdens than those of *B6* mice (Fig. 4B). We also examined *Irf3/7*$^{-/-}$ mice, which lack two transcription factors that act downstream of STING signaling to induce transcription of type I IFN (38). *L. monocytogenes* colonized the intestinal tracts of *Irf3/7*$^{-/-}$ mice slightly better than those of *B6* mice, but at 5 days postinfection, we observed 14-fold fewer CFU in mLN of *Irf3/7*$^{-/-}$ mice than of *B6* mice and no statistically significant differences in the spleen and liver (Fig. 4C and D). Collectively, these data suggest that the type I IFN response does not contribute to protection during *L. monocytogenes*-induced enterocolitis. Consistent with what had been reported with i.v. *L. monocytogenes* infection (24–26), the type I IFN response has a detrimental effect in controlling bacterial growth.

Reduced monocyte recruitment in the absence of STING signaling. STING signaling leads to the production of chemokines which mobilize immune cells to sites of inflammation (31). To investigate the functional consequences of chemokine production by STING signaling during *L. monocytogenes*-induced enterocolitis, we characterized the immune cell composition of the colon and mLN by flow cytometry. Colon samples from mice were separated into fractions containing cells associated with the epithelial layer (intraepithelial IE) and cells of the lamina propria (LP). Under homeostatic conditions, the mLN and colon contained very few neutrophils (CD11b$^+$ LyG$^+$) and monocytes (CD11b$^+$ Ly6C$^+$), but during *L. monocytogenes* infection, the intestines became markedly infiltrated with neutrophils and monocytes (Fig. 5; see Fig. S3 for gating strategy). Compared to those of *B6* mice, both the mLN and colons of *Sting*$^{gt/gt}$ mice at 3 days postinfection contained roughly half the number of monocytes, indicating that the monocyte response was dependent on STING signaling (Fig. 5A). We also observed an increase in the presence of neutrophils associated with the colonic epithelium of *Sting*$^{gt/gt}$ mice (Fig. 5B). Given that efficient clearance of systemic *L. monocytogenes* is highly dependent on monocytes and that neutrophils appear to be dispensable (35), our observations are consistent with a model in which STING signaling contributes to the recruitment of monocytes to the sites of infection, where they

participate in an immune response that effectively eliminates intracellular bacteria in the intestinal tract, leading to reduced systemic disease.

DISCUSSION

The results of our study revealed that STING signaling provides a protective immune response that limits the severity of *L. monocytogenes*-induced enterocolitis as measured by weight loss and bacterial burdens at systemic sites. We observed that disrupting STING signaling during infection impaired monocyte recruitment to intestinal tissues. We also found that the magnitude of protection was dependent on the level of c-di-AMP secretion by *L. monocytogenes*. Although type I IFN is a widely accepted output of STING signaling, disrupting IFN signaling during intestinal infection did not reproduce the increased susceptibility observed in STING-deficient mice; in contrast, loss of IFN signaling led to reduced bacterial loads in systemic sites. Together, our results support a model in which the secretion of c-di-AMP by *L. monocytogenes* in the cytosol of host cells in the intestinal tract activates STING and initiates a monocyte response that inhibits the local replication of intracellular bacteria, leading to a reduced systemic burden. To further advance this model, additional experiments will be needed to delineate the signaling cascades that lead to the functional consequences of STING activation during *L. monocytogenes* infection. In addition, experiments to characterize the cell types that initiate the immune response to *L. monocytogenes* infection and the cell types that represent the *L. monocytogenes* intracellular niche within the distal intestinal tract will advance our understanding of *L. monocytogenes* pathogenesis.

Given the general consensus that monocytes play an essential role in the elimination of *L. monocytogenes in vivo* (35), the impaired monocyte response of *Sting*$^{gt/gt}$ mice provides a likely explanation for increased bacterial burden and increased disease severity. This dependency of the monocyte response on STING signaling was also observed in studies using i.v. *L. monocytogenes* infections, in which STING signaling contributes to monocyte recruitment in the liver (36). Observations from viral infections offer a possible mechanism for how STING regulates the monocyte response. Infections with Sendai virus, vesicular stomatitis virus, and herpes simplex virus 1 demonstrate that the *in vitro* and *in vivo* production of CCL2, a key monocyte chemokine, occurs through STING signaling in a STAT6-dependent manner (31). Conceivably, monocyte recruitment during *L. monocytogenes* infection also relies on STAT6-dependent CCL2 production. At the site of infection, monocytes acquire the capacity to produce inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) (39). The cellular identity of this cell population remains to be fully defined, but these cells constitute the major source of TNF-α and iNOS during *L. monocytogenes* infection (39, 40). iNOS and TNF-α are potent effector molecules that mediate bacterial killing through cell intrinsic and extrinsic mechanisms. Mice that lack either iNOS or TNF-α are highly susceptible to intravenous *L. monocytogenes* infection (41–43). Future experiments will be needed to test the direct links between STING, STAT6, CCL2, monocyte recruitment, and antibacterial activity.

Although type I IFN is a major output of STING signaling during *L. monocytogenes* infection, disruption of type I IFN signaling during *L. monocytogenes*-induced enterocolitis resulted in reduced bacterial burdens, suggesting that in this setting, type I IFN benefits the pathogen. The promotion of *L. monocytogenes* pathogenesis by type I IFN has been previously reported for the i.v. mouse model (24–26). In studies in which infection occurred through the oral route, the propathogen effect of type I IFN was not observed, and in some instances, type I IFN helped to control bacterial growth (27, 28). An important distinction from previously reported infections by the oral route is that the enterocolitis model used in this study leads to robust intestinal colonization by *L. monocytogenes* and notable intestinal inflammation. An explanation that may help to reconcile this discrepancy in the effects of type I IFN on *L. monocytogenes* pathogenesis comes from studies of *Mycobacterium tuberculosis*, another intracellular pathogen. Mice carrying the "sensitive" allele of the "super susceptibility to tuberculosis 1" loci (*Sst1*^{S}) have decreased survival following *M. tuberculosis* infection that is associated with

enhanced production of type I IFN, and susceptibility to *M. tuberculosis* is rescued by disrupting type I IFN signaling (44, 45). *B6* mice carry the resistant SstI allele, and disrupting type I IFN in B6 mice has a modest effect. Interestingly, *Sst1*^{S} mice are also more susceptible to *L. monocytogenes* infection (46); it is tempting to speculate that type I IFN explains this phenotype as well. Together, these observations suggest that type I IFN can drive *L. monocytogenes* pathogenesis when sufficient type I IFN is produced, which can be influenced by multiple factors, such as route of infection, host genetics, and pathogen burden.

In the CDN-STING dialog between host and microbe, our results provide a clear benefit from the host's perspective; however, consideration of this interaction from the microbe's perspective is also warranted. The expression of MDR transporters which secrete c-di-AMP is induced by intracellular growth of *L. monocytogenes*. The transcriptional induction of all MDR transporters is at least 4-fold higher, with some transporters being induced up to 200-fold more than in bacteria grown in broth (15). Given that the *L. monocytogenes* genome also encodes at least two phosphodiesterases that degrade c-di-AMP, what benefit does c-di-AMP secretion afford (47, 48)? According to the "Trojan horse" hypothesis of bacterial dissemination, infected monocytes help disseminate bacteria to the brain and placenta (49–51). In humans and livestock, invasive listeriosis is characterized by neurological involvement and miscarriage (52). *L. monocytogenes* transmission can occur through consumption of bacteria present in high numbers in the placenta during miscarriage. Therefore, we suggest the possibility that STING activation by *L. monocytogenes*-derived c-di-AMP leads to the recruitment of monocytes to sites of infection, and then infected monocytes help disseminate the infection to privileged tissues such as the brain and placenta. Perhaps *L. monocytogenes* has evolved to make a trade-off between immune evasion and dissemination.

In our efforts to define the roles of c-di-AMP secretion by *L. monocytogenes* in the host immune response, we revealed a striking STING-independent virulence phenotype associated with the Δ*mdrMTAC* strain. Infected *Sting*$^{gt/gt}$ mice had 240-fold-decreased bacterial burdens in the liver when infected with the Δ*mdrMTAC* strain compared to the wild type. In *L. monocytogenes*, MDR transporters contribute to bile resistance (53), and the liver is the site of primary bile acid biosynthesis. Interestingly, bile appears to transcriptionally regulate nearly 400 *L. monocytogenes* genes (53). Although more work is needed, a proper response to bile appears to be a critical feature of *L. monocytogenes* pathogenesis.

Regulation of autophagy by STING signaling predates the emergence of the type I IFN response (54), and *L. monocytogenes* can be targeted by cell-autonomous defenses that require autophagy machinery. For these reasons, autophagy and related mechanisms might contribute to STING-mediated antibacterial responses. Entry to the host cytosol results in damage to the *L. monocytogenes*-containing vacuole, which serves as a signal to eliminate the bacteria through a process called LC3-associated phagocytosis. Additionally, during transit through the intestinal tract or blood, *L. monocytogenes* can become coated in complement C3, and when internalized by host cells, complement C3 recruits ATG16L through a direct interaction and directs the bacterium to degradative compartments (55). Cytosolic *L. monocytogenes* is further subjected to the activity of ubiquitin ligases and autophagy adaptors to initiate a process called xenophagy which entraps the bacteria in membranes before delivery to degradative compartments (56). While these processes are distinct, these mechanisms likely act in concert to provide adequate defense because many pathogens possess virulence factors that neutralize these antibacterial responses. The activity of two *L. monocytogenes* phospholipases C decreases the pool of phosphatidylinositol 3-phosphate, which serves as a critical signal that recruits autophagy machinery (57). *L. monocytogenes* can also avoid autophagy by using actin-based motility to evade ubiquitin ligases and to escape from LC3^{+} membranes (58). Studies with macrophages suggest that these countermeasures are largely effective because only 30% of *L. monocytogenes* colocalize with autophagy machinery (56). The mechanisms by which *L. monocytogenes* is targeted by xenophagy appear to be distinct from the mechanisms by which STING signaling induces au-

TABLE 1 Strains used in this study

Strain	Description	Reference
10403S	Wild-type *L. monocytogenes*	63
DP-L5387	*tetR*::*Tn*	16
DP-L7247	Δ*mdrMTAC*	15

tophagy. STING-induced activation of autophagy machinery is TBK1 and Beclin 1 independent, whereas the xenophagy of *L. monocytogenes* requires both factors (30, 56). Further investigation will be needed to determine the extent of cross talk between STING signaling and cell-autonomous defenses that require autophagy machinery. Of particular interest, how does a diffusible molecule like CDN regulate the selective targeting of bacteria in the cytosol?

Pattern recognition receptors (PRRs) recognize conserved pathogen-associated molecular patterns (PAMPs), but many nonpathogens also contain these ligands (59). Therefore, mechanisms must be in place to distinguish pathogens from commensals in order to avoid unnecessary activation of the immune system (8, 64). Here, we showed that STING signaling provides a protective response following *L. monocytogenes* infection through the p.o. route, but with infection through the i.v. route, STING signaling appeared to be dispensable. This discrepancy could reflect differences in the tissue-specific requirements for immune response activation. In the context of disseminated disease, where bacteria are present in the spleen or liver, the detection of PAMPs in these tissues would be teleologically sufficient to discriminate pathogens from commensals because commensals do not typically reach internal organs. In the context of mucosal barriers, where PAMPs are abundant, external surveillance systems alone would not be sufficient, as commensals would trigger immune system activation. Cytosolic surveillance systems such as STING possess an additional requirement for activation: the PAMP needs to be present in the host cytosol. Many pathogens rely on host cytosol access to carry out virulence strategies, and these activities can inadvertently introduce PAMPs into the cytosol. Therefore, cytosolic surveillance provides a mechanism to distinguish the presence of a pathogen from that of a commensal. Further experimentation is needed to fully define the cell types in the intestines that are responsible for initiating the immune response to *L. monocytogenes*. Given the differential requirements of tissues to assess microbe encounters, it is possible that the activity of other immune sensors can compensate for STING function during disseminated disease, but at the initial site of infection in the mucosal barrier, STING signaling could provide more pronounced contributions to immune responses.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council of the National Academy of Sciences (60). All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (UC Berkeley; AUP-2016-05-8811).

Mice. All mice were specific pathogen free, maintained under a 12-h light-dark cycle (7 a.m. to 7 p.m.), and given a standard chow diet (Harlan irradiated laboratory animal diet) *ad libitum*. Within each experiment, mice of all genotypes were age matched at 8 to 12 weeks old and had been cohoused for at least 2 weeks at the start of infections. *Ifnar*$^{-/-}$ (the Jackson Laboratory; 028288) mice were provided by R. Vance. *Irf3/7*$^{-/-}$ (61) mice were provided by G. Barton. *Sting*$^{gt/gt}$ (23) and *Cgas*$^{-/-}$ (62) mice were previously described. All mouse strains were bred at UC Berkeley except for C57BL/6 mice, which were purchased from the Jackson Laboratory. All mice were maintained at the University of California, Berkeley, animal facility according to institutional guidelines for animal care. Mouse work was not subjected to randomization or data blinding.

Bacterial strains and growth conditions. All *L. monocytogenes* strains used in this study were derived from streptomycin-resistant wild-type 10403S (Table 1) and propagated in filter-sterilized brain heart infusion (BHI) medium (BD) at 37°C with shaking and without antibiotics unless otherwise stated in Materials and Methods. Cell density was spectrophotometrically measured by optical density at a wavelength of 600 nm (OD_{600}). Frozen bacterial stocks were stored at −80°C in BHI medium plus 40% glycerol. Culture medium supplements were used at the following concentrations: streptomycin at 200 μg/ml, nalidixic acid at 15 μg/ml, LiCl at 6 mg/ml, and glycine at 6 mg/ml.

***L. monocytogenes*-induced enterocolitis.** Prior to infection, 5 mg/ml of streptomycin sulfate was added to the drinking water. After 32 h, mice were transferred to fresh cages, and chow was removed

to initiate an overnight fast. At 48 h after streptomycin was added to the water, mice were isolated, fed a 3-mm piece of bread soaked with 3 μl of butter and an inoculum of *L. monocytogenes* in phosphate-buffered saline (PBS), and returned to cages containing standard drinking water and chow. Following infection, stool samples were collected and homogenized in PBS by vortexing for 5 min at 4°C, and dilutions were plated. In instances where streptomycin was not sufficient to restrict growth of intestinal bacteria, plates were supplemented with nalidixic acid, LiCl, and glycine. To confirm the identity of colonies recovered from feces, PCR for *actA* was performed. To determine bacterial burden in organs, mice were euthanized and tissues were collected. Livers were homogenized in 10 ml of 0.1% IGEPAL (Sigma CA-630), and ceca, mLN, and spleens were homogenized in 2 ml, of 0.1% IGEPAL. Dilutions of homogenates were plated to enumerate CFU.

Gentamicin treatment. Cecal tissues were cut longitudinally, washed with cold PBS, and incubated in RPMI medium (Gibco) containing 5% fetal calf serum, HEPES, L-glutamine, and 100 μg/ml of gentamicin for 45 min at 37°C. Tissues were washed by placing the sample into 10 ml of PBS on a rotator at 4°C for 20 min, and washing was repeated 6 times. Tissues were homogenized in 2 ml of 0.1% IGEPAL, and dilutions were plated.

Histology. For hematoxylin and eosin staining, tissues were fixed in buffered 4% paraformaldehyde (PFA). Histology was performed by HistoWiz Inc. (Brooklyn, NY) using a standard operating procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 4 μm. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole-slide scanning ($\times$40 magnification) was performed on an Aperio AT2 instrument (Leica Biosystems).

Flow cytometry. Single-cell suspensions were incubated with Fc Block (eBiosciences; clone 93) and fixable viability stain (BD-Horizon), followed by staining with antibodies to surface markers: CD45 (30-F11), Ly6C (HK1.4), Ly6G (IA8), CD11b (M1170), CD64 (x-54-4/7.1), CD11c (N418), and major histocompatibility complex class II (MHC-II; M5/144.15.2). Samples were analyzed on a LSRFortessa (BD Biosciences) with five lasers (351 nm, 405 nm, 488 nm, 561 nm, and 640 nm). Samples were gated by FSC-A and SSC-A to exclude debris, FSC-H and FSC-W for single cells, and to exclude dead cells. Data analysis was performed using FlowJo (Treestar).

Tissue dissociation. Colons were opened, thoroughly cleaned with PBS, and incubated for 20 min in 20 ml of Hanks balanced salt solution (HBSS; Ca^{2+}/Mg^{2+} free) supplemented with 2% fetal calf serum (FCS), 10 mM HEPES, and 5 mM dithiothreitol. Supernatants were discarded, and intestines were incubated for 15 min in 10 ml of HBSS (Ca^{2+}/Mg^{2+} free) supplemented with 2% FCS, 10 mM HEPES, and 5 mM EDTA solution. This step was repeated twice using fresh solution. Supernatants were collected and represent the intraepithelial fraction. Next, intestines were incubated for 10 min in 20 ml of HBSS (with Ca^{2+}/Mg^{2+}) supplemented with 3% FCS and 10 mM HEPES. After incubation, intestines were gently vortexed, cut into small pieces, and incubated for 30 min in 5 ml of HBSS (with Ca^{2+}/Mg^{2+}) supplemented with 3% FCS, 10 mM HEPES, 100 μg/ml of Liberase, and 30 μg/ml of DNase I. All incubations were performed with gentle rocking at 37°C. After digestion, samples were passed through a 100-μm filter and washed. The resulting cell pellet was resuspended in 5 ml of 40% Percoll (Sigma-Aldrich) and centrifuged at 2,000 rpm for 20 min at 20°C. The debris on the surface was aspirated away and the remainder was washed and stained for flow cytometry. mLN were gently dissociated over a 70-μm nylon filter using a syringe plunger and washed with PBS with 3% FCS.

Statistical analysis. Statistical analyses were carried out with GraphPad Prism software (version 7.0a). See the figure legends for details.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

ACKNOWLEDGMENTS

Research reported in this publication was supported by funding from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (1P01 AI063302 and 1R01 AI27655 to D.A.P.) and the National Institutes of Health Immunology and Pathogenesis Program training grant (2T32 AI 100829-06A1 to A.L.).

We thank Russell E. Vance and Gregory M. Barton for helpful discussions and sharing mice. We thank Claire O'Leary for providing technical advice. We thank Brittney N. Nguyen and Samuel H. Light for critically reading the manuscript.

Conceptualization, A.L. and D.A.P.; Methodology, A.L. and D.A.P.; Investigation, A.L., V.B., and D.A.P., Analysis, A.L. and D.A.P.; and Writing, A.L. and D.A.P.

We declare no competing interests.

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