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Host-Microbial Interactions | Full-Length Text

FlgM is required to evade NLRC4-mediated host protection against flagellated *Salmonella*

Américo Harry López-Yglesias,[1] Chun-Chi Lu,[1] Marvin A. Lai,[1] Ellen K. Quarles,[1] Xiaodan Zhao,[1] Adeline M. Hajjar,[2] Kelly D. Smith[1]

AUTHOR AFFILIATIONS See affiliation list on p. 15.

ABSTRACT *Salmonella enterica* serovar Typhimurium is a leading cause of gastroenteritis worldwide and a deadly pathogen in children, immunocompromised patients, and the elderly. *Salmonella* induces innate immune responses through the NLRC4 inflammasome, which has been demonstrated to have distinct roles during systemic and mucosal detections of flagellin and non-flagellin molecules. We hypothesized that NLRC4 recognition of *Salmonella* flagellin is the dominant protective pathway during infection. To test this hypothesis, we used wild-type, flagellin-deficient, and flagellin-overproducing *Salmonella* to establish the role of flagellin in mediating NLRC4-dependent host resistance during systemic and mucosal infections in mice. We observed that during the systemic phase of infection, *Salmonella* efficiently evades NLRC4-mediated innate immunity. During mucosal *Salmonella* infection, flagellin recognition by the NLRC4 inflammasome pathway is the dominant mediator of protective innate immunity. Deletion of *flgM* results in constitutive expression of flagellin and severely limits systemic and mucosal *Salmonella* infections in an NLRC4 inflammasome-dependent manner. These data establish that recognition of *Salmonella's* flagellin by the NLRC4 inflammasome during mucosal infection is the dominant innate protective pathway for host resistance against the enteric pathogen and that FlgM-mediated evasion of the NLRC4 inflammasome enhances virulence and intestinal tissue destruction.

KEYWORDS *Salmonella*, inflammasome, flagellin, NLRC4, innate immunity, mucosal immunity, inflammation

Salmonella is the causative agent in salmonellosis and is one of the main causes of gastrointestinal bacterial infections worldwide. Consumption of contaminated food is responsible for the majority of *Salmonella* infections and is the leading cause of foodborne-related deaths in the United States (1). During the initial phase of the infection, the bacteria travels to the intestine where it encounters a protective layer of mucus lining the intestines (2). After breaking through the mucus layer, *Salmonella* infects the intestinal epithelium and lamina propria myeloid cells, which are capable of detecting the bacteria through innate pattern recognition receptors (PRRs) (3–6). PRRs recognize highly conserved structures of bacteria, such as rod and needle proteins from the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (TTSS) and flagellin monomers that compose the flagellar filament (7–14).

Salmonella's flagellin, encoded by *fljB* and *fliC*, is a potent ligand that elicits a robust innate immune response by activating the caspase-1 (CASP1)-dependent inflammasome (14–16). Inflammasome recognition of flagellin is dependent on the detection of the conserved site, located on the carboxyl terminus of the protein, by NAIP5 and NAIP6 (NAIP5/6) (7, 8, 12). Flagellin recognition by NAIP5/6 leads to the formation of a multiprotein complex, resulting in the activation of the NLRC4-CASP1-dependent inflammasome (17). It has also been shown that the adaptor molecule ASC (apoptotic speck protein containing a caspase recruitment domain, encoded by *Pycard*) can

Editor Manuela Raffatellu, University of California San Diego School of Medicine, La Jolla, California, USA

Address correspondence to Kelly D. Smith, kelsmith@uw.edu.

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associate with the NLRC4 inflammasome, resulting in efficient production of interleukin (IL)-1β; however, ASC-independent NLRC4 -CASP1 inflammasome activation still results in cell death via pyroptosis (18). Flagellin-mediated NLRC4-dependent activation results in biologically active IL-1β and IL-18, eicosanoids, and gasdermin-D-mediated pyroptotic cell death (19, 20).

The SPI-1 TTSS is a critical virulence factor that enables *Salmonella* to colonize and successfully replicate in the host's intestinal epithelial cells (IECs) (21). Described as a needle-like structure, the TTSS delivers critical effector proteins, which allows *Salmonella* to create a favorable environment for colonization within IECs (22, 23). To counteract these virulence factors, the host uses PRRs, NAIP1, and NAIP2 (NAIP1/2), which recognize the needle and rod proteins, encoded by *prgI* and *prgJ*, both of which are required for the TTSS needle complex assembly (8, 9, 12). Similar to flagellin, needle and rod recognition leads to the formation of the NLRC4-CASP1 inflammasome, resulting in NLRC4-dependent IL-1β production and pyroptotic cell death (8, 9, 12). During infection of intestinal epithelial cells, *Salmonella* expresses SPI-1 TTSS and rapidly downregulates SPI-1 TTSS and flagellar synthesis upon traversing the epithelium and entering the lamina propria (24).

NLRC4 inflammasome recognition of *Salmonella* by IECs and phagocytes has been extensively studied and shown to be critical for innate host protection. It has been demonstrated that *Salmonella*-triggered activation of the NLRC4 inflammasome in IECs not only results in the production of cytokine, eicosanoids, and pyroptosis but also leads to rapid expulsion of IECs from the intestinal epithelium (3, 6). More recently, it has been illustrated that NAIP recognition of *Salmonella's* pathogen-associated molecular patterns is critical for restricting the dissemination of the enteric pathogen (24). Deficiency in the NAIP/NLRC4 inflammasome results in increased *Salmonella* growth in intestinal tissues and luminal contents and tumor necrosis factor (TNF)-dependent destruction of the intestinal epithelium (25, 26). Unlike in mice, which utilize NAIP5/6 and NAIP1/2 to detect cytosolic *Salmonella* flagellin and the SPI-1 TTSS needle and rod proteins, humans only have one functional NAIP. It has been shown that similar to mouse NAIPs, human NAIP in macrophages can recognize cytosolic flagellin, and the needle and rod proteins, PrgI and PrgJ, triggering the NLRC4 inflammasome response (27, 28). In human intestinal epithelial cells, the CASP4-dependent non-classical inflammasome is the primary mediator of epithelial cell intrinsic inflammasome responses to *Salmonella* infection (29).

To evade NLRC4 recognition, *Salmonella's* expression of flagellin is tightly regulated by PhoPQ and ClpXP sensors and FlgM, which downregulate flagellin production *in vivo* in macrophages but not in epithelial cells (24, 30–34). Experimental evidence suggest that SPI-1 TTSS is required for the intracellular translocation of flagellin, similarly regulated by two-component sensors, and is silenced during intracellular and systemic infection (35). We hypothesized that flagellin recognition by the NLRC4 inflammasome is the dominant pathway for host protection during mucosal *Salmonella* infection, when both flagellin and SPI-1 are upregulated to promote intestinal colonization and infection. To test our hypothesis, we used mutant strains of *Salmonella*, including an attenuated strain of *Salmonella* that is unable to repress flagellin production (Δ*flgM*), in combination with mice deficient in inflammasome components. Our results reveal that *Salmonella* FlgM is required to successfully evade recognition by the NLRC4 inflammasome and that the NLRC4 inflammasome protects against mucosal *Salmonella* infection primarily through the recognition of flagellin.

MATERIALS AND METHODS

Bacterial strains

The experiments were performed using wild-type (WT) *Salmonella enterica* serovar Typhimurium strain SL1344 (from Brad Cookson, University of Washington) (36), flagellin deficient (Δ*fliC/fljB*, from Brad Cookson University of Washington) (15), flagellin

overexpressing (Δ*flgM*, a gift from Kelly Hughes) (15), SPI-1 deficient (ΔSPI-1, from Kelly Hughes), flagellin overexpressing and SPI-1 deficient (Δ*flgM*/SPI-1 and ΔSPI-1 from Kelly Hughes), flagellin-deficient and lacking control of flagellin repression (Δ*flgM/fliC/fljB*), and flagellin overexpressing and lacking the flagellar basal body (Δ*flgM/flgB* and Δ*flgB* from Kelly Hughes). Δ*flgM* mutant from Kelly Hughes was then transferred into Δ*fliC/fljB*, ΔSPI-1, ΔSPI-2, and Δ*flgB* SL1344 strains using P22 phage (37). Δ*fliC/fljB* mutant from Brad Cookson was then transferred into ΔSPI-1 and SL1344 strains using P22 phage. The deletion of *flgM* was confirmed by PCR. Bacteria were grown in Luria broth (LB) at 37°C with aeration.

Mouse infection

C57BL/6 mice were purchased from Jackson Labs and housed in our facilities at the University of Washington. *Casp1*$^{-/-}$ *Casp11*$^{129mt/129mt}$ (referred to as *Casp1/11*$^{-/-}$), *Nlrc4*$^{-/-}$, and *Nlrp3*$^{-/-}$ (generated by Genetech) were bred in our specific pathogen-free animal facilities (38). Animals were housed under standard barrier conditions in individually ventilated cages. Sex- and age-matched mice 8–14 weeks old were used for infections throughout this study. All oral infections were performed as previously described (15). In brief, 1 day before infection, food was withdrawn 4 h prior to oral administration of 20 mg of streptomycin (39). Food was replaced, and 20 h after streptomycin treatment, food was withdrawn again for 4 h prior to orally infecting mice with 1,000 colony-forming units (CFUs) of *S*. Typhimurium. Food was replaced immediately after infection. The *Salmonella* inoculum was prepared by back-diluting an overnight culture 1:50 in LB + 50 µg/mL of streptomycin. After 4 h, the concentration of bacteria was measured and diluted in cold phosphate buffered saline (PBS) to a concentration of 1×10^4 CFU/mL, and CFU of the inoculum was verified by plating on LB agar plates with 50 µg/mL streptomycin. Five days post-infection, mice were sacrificed by CO_2 asphyxiation, and tissues [intestine, mesenteric lymph node (mLN), spleen, and liver] were promptly removed. Bacterial burden was assessed by weighing and homogenizing the tissues in PBS with 0.025% Triton X-100 and plating dilutions of the samples on MacConkey agar plates with streptomycin (50 µg/mL). Prior to homogenization, the ceca were scraped and blotted to remove fecal content. *Salmonella* inoculum for systemic intraperitoneal (i.p.) infections were prepared as previously described, and 1,000 CFUs of *S*. Typhimurium were administered intraperitoneally. Five days post-infection, mice were sacrificed by CO_2 asphyxiation, tissues (spleen and liver) were promptly removed, and bacterial burden was assessed as previously described.

Quantitative histological assessment

Formalin-fixed tissue was embedded in paraffin using standard protocols. Thick sections (4 µm) were stained with hematoxylin and eosin using standard procedures. A blinded pathologist examined the slides and scored them according to the following criteria. Scores were assigned for changes to the cecum as follows: submucosal expansion, 0 = no significant change, 1 = <25% of the wall, 2 = 25–50% of the wall, 3 = >50% of the wall; mucosal neutrophilic infiltrate, 0 = no significant infiltrate, 1 = mild neutrophilic inflammation, 2 = moderate neutrophilic inflammation, 3 = severe neutrophilic inflammation; lymphoplasmacytosis, 0 = no significant infiltrate, 1 = focal infiltrates (mild), 2 = multifocal infiltrates (moderate), 3 = extensive infiltrates involving mucosa and submucosa (severe); goblet cells, 0 = >28/high power field (HPF), 1 = 11–28/HPF, 2 = 1–10/HPF, 3 = <1/HPF; and epithelial integrity, 0 = no significant change, 1 = desquamation (notable shedding of epithelial cells into the lumen), 2 = erosion (loss of epithelium with retention of architecture), 3 = ulceration (destruction of lamina propria). Crypt loss was estimated by a blinded pathologist as a fraction of cecal epithelium devoid of crypts.

Macrophage cytotoxicity assay

Thioglycollate-elicited peritoneal macrophages were plated in a 96-well plate at a concentration of 5×10^5 macrophages/well in Roswell Park Memorial Institute (RPMI)

1640 medium with L-glutamine and 10% fetal bovine serum. *S.* Typhimurium was grown overnight in LB medium and back-diluted the next day 1:50 in LB medium and grown for 3–4 h. The bacteria were centrifuged and the pellet resuspended to the final desired concentration. Macrophages were infected with the desired multiplicity of infection and centrifuged at 250 × *g* for 5 min, and the infection was allowed to progress for an hour. Gentamicin (50 µg/mL) was added after an hour to kill extracellular bacteria. After an additional hour, the supernatants were removed and cytotoxicity was measured using Cytotox 96 kit (Promega).

Statistics

Significance was obtained by using the software GraphPad Prism (San Diego, CA). One-way analysis of variance (ANOVA) was used when comparing three groups or more, using post-Tukey's multiple comparison test. Two-way ANOVA was used when comparing three groups or more at multiple time points, using Tukey's multiple comparison test. Statistical analyses of survival curve were done using log-rank (Mantel-Cox) test. In all graphs, significance was established and represented using the following system: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

RESULTS

S. Typhimurium requires FlgM to evade inflammasome detection during i.p. infection

The bacterial protein flagellin can be detected by NAIP5/6, which activates the NLRC4-dependent inflammasome. To evade recognition, *Salmonella* downregulates flagellin expression during host invasion. We tested whether the NLRC4 inflammasome contributes to the control of systemic i.p. infection. To identify the role of NLRC4 in host resistance against *S.* Typhimurium *in vivo*, we used mice lacking CASP1/11 (*Casp1/11*$^{-/-}$), NLRC4 (*Nlrc4*$^{-/-}$), and NLRP3 (*Nlrp3*$^{-/-}$). We observed that upon i.p. infection with *S.* Typhimurium SL1344 (WT), there was no difference in the bacterial burden in the spleens or livers of mice deficient in the examined inflammasome components compared to C57BL/6 (B6) controls (Fig. 1A). Similarly, there was no difference in bacterial burden in the spleens and livers between B6 and the inflammasome-deficient strains of mice infected with flagellin-deficient (Δ*fljB/fliC*) *S.* Typhimurium (Fig. 1B).

Salmonella FlgM is an anti-sigma factor that binds FliA and prevents the expression of class III flagellar genes (33, 40). Upon completion of the flagellar basal body, FlgM is secreted and FliA is released to activate class III promoters, resulting in completion of the flagellar assembly (33, 40). Deletion of *flgM* results in constitutive expression of flagellar class III genes and disruption of autogenous regulation of flagellar assembly (33). *flgM*-deficient (Δ*flgM*) *S.* Typhimurium expresses more flagellin protein, has more flagella than WT *Salmonella*, and is attenuated in mice (15, 33). We predicted that the attenuated phenotype observed in mice infected with Δ*flgM S.* Typhimurium is dependent on the response to flagellin by the NLRC4 inflammasome. In striking contrast to both WT and Δ*fljB/fliC Salmonella* infections, Δ*flgM* intraperitoneally infected *Nlrc4*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice had significantly elevated bacterial burden in both the spleen and liver compared to B6 and *Nlrp3*$^{-/-}$ animals (Fig. 1C). Although NLRP3 has been implicated in *Salmonella* detection during mucosal infection, we observed no phenotype for NLRP3 in host protection against i.p. infection by WT, Δ*fljB/fliC*, or Δ*flgM Salmonella* (41–43). Overall, these data are in agreement with Miao and colleagues, demonstrating that the NLRC4-CASP1/11 inflammasome is a critical mediator of flagellin detection (44) and that FlgM repression of flagellar synthesis efficiently evades NLRC4-CASP1/11-mediated defense during i.p. infection of WT *Salmonella*.

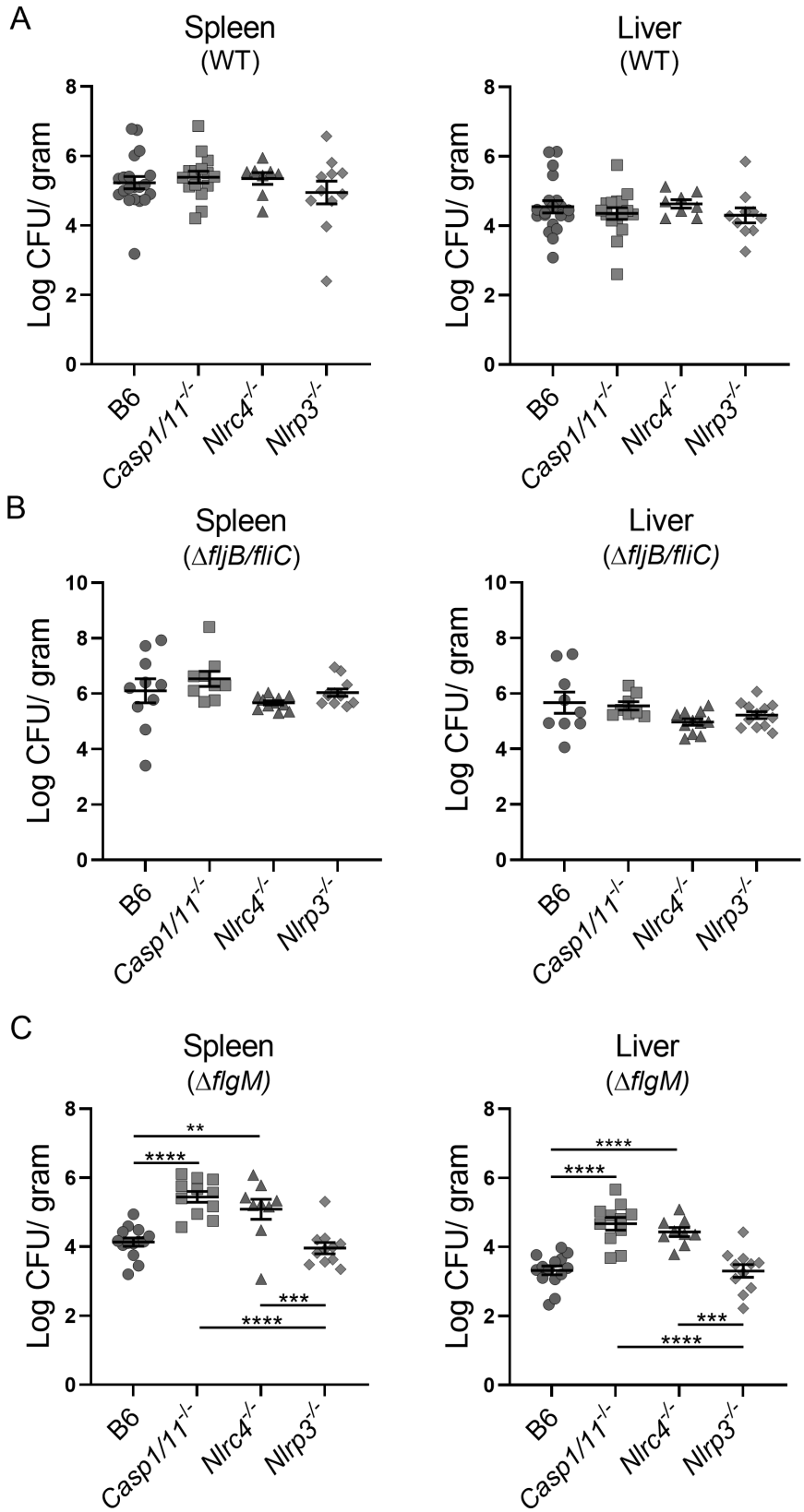

FIG 1 *S.* Typhimurium requires flgM to evade inflammasome detection during intraperitoneal infection. Bacterial burden of B6 (n = 10–20), *Casp1/11*$^{-/-}$ (n = 9–15), *Nlrc4*$^{-/-}$ (n = 8–11), and *Nlrp3*$^{-/-}$ (n = 11–12) mice intraperitoneally infected with 1,000 CFUs of WT SL1344 (A), Δ *fljB/fliC* (B), or Δ *flgM S.* Typhimurium

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FIG 1 (Continued)
(C) in the spleen and liver on day 5 post-infection. Statistical analyses were done using one-way analysis of variance with post-Tukey's multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$, **** P<0.0001. Error bars indicate standard error of the mean.

The NLRC4-CASP1/11 inflammasome is critical for limiting mucosal inflammation and systemic *Salmonella* infection

Flagellin is a key protein for bacterial motility; yet, *Salmonella* is capable of restricting flagellin expression based on its anatomical location in the host (24, 31). Therefore, we investigated the role of the NLRC4 inflammasome during oral infection in streptomycin-pretreated mice. We observed rapid mortality in *Casp1/11*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice compared to B6 controls orally infected with WT *S*. Typhimurium (Fig. 2A). Next, we measured the bacterial burden of *Casp1/11*$^{-/-}$, *Nlrc4*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice orally infected with WT *S*. Typhimurium. Our results showed *Casp1/11*$^{-/-}$ mice had significantly elevated bacterial burden in the cecum compared to B6 animals (Fig. 2B). Yet, we observed no statistically significant difference in the cecal bacterial burden between B6 and *Nlrc4*$^{-/-}$ mice (Fig. 2B). We also observed that B6, *Casp1/11*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice had significantly elevated cecal bacterial burden compared to *Nlrp3*$^{-/-}$ animals (Fig. 2B). CASP1/11- and NLRC4-deficient animals had significantly elevated bacterial burden in the mLN, spleen, and liver compared to B6 and *Nlrp3*$^{-/-}$ mice (Fig. 2B). Compared to B6 animals, infected *Nlrp3*$^{-/-}$ mice had similar amounts of CFUs in the mLN and spleen but significantly elevated CFUs in the liver (Fig. 2B). Histological examination revealed marked inflammation in all mice (Fig. 2C and D) and significantly augmented tissue injury in mice lacking either CASP1/11 or NLRC4 (Fig. 2E). In agreement with Sellin and colleagues, our results reveal that the NLRC4-CASP1/11 inflammasome plays a critical role in limiting intestinal tissue injury, as well as bacterial colonization of systemic sites during oral infections (3).

The NLRC4 inflammasome protects against oral infection with *flgM*-deficient *Salmonella*

We have previously shown that CASP1/11 is critical for limiting the bacterial burden of mice orally infected with *flgM*-deficient *Salmonella* (15). Therefore, we hypothesized that the NLRC4 inflammasome is also required for limiting growth of *Salmonella* lacking FlgM. To test our hypothesis, we orally infected inflammasome-deficient mice with Δ*flgM S*. Typhimurium. Our results show that in the absence of CASP1/11 or NLRC4, there is a significant increase of *S*. Typhimurium in the cecum, mLN, spleen, and liver, compared to B6 and *Nlrp3*$^{-/-}$ mice (Fig. 3A). Furthermore, Δ*flgM* orally infected *Casp1/11*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice displayed significant tissue destruction in the cecum (Fig. 3B through D). These data establish the NLRC4-dependent inflammasome as essential for controlling bacterial burden and limiting intestinal pathology during infection.

The flagellar basal body is required for FlgM-mediated attenuation during mucosal infection

We next defined the requirement for *flgM*-deficient *Salmonella*-mediated inflammasome activation. To generate functional flagella, flagellin proteins are secreted through the flagellar basal body and polymerize into filaments (40). It has also been shown that flagellin activation of the inflammasome requires the SPI-1 TTSS, suggesting that flagellin is also secreted through SPI-1 (35). To characterize the flagellar basal body's, SPI-1's, and SPI-2's roles in mediating flagellin-dependent *Salmonella* pathogenesis, we deleted SPI-1, *flgM* and SPI-1, *flgM* and *flgB*, *flgM* and flagellin (*fliC* and *fljB*), SPI-2, or *flgM* and SPI-2. B6 mice were orally infected with WT, Δ*flgM*, ΔSPI-1, Δ*flgM*/SPI-1, Δ*flgM*/*flgB*, Δ*flgM*/*fljB*/*fliC*, ΔSPI-2, or Δ*flgM*/SPI-2 *Salmonella*, and their bacterial burdens were assessed. In the ceca and mLN, we observed that the absence of both FlgM and SPI-1 resulted in a significant decrease of pathogen burden compared to B6 mice infected with WT, Δ*flgM*, and ΔSPI-1 (Fig. 3E; Tables 1 and 2). Additionally, in the spleen and liver, we observed no significant difference in the bacterial burden of B6 mice infected with Δ*flgM* and Δ*flgM*/SPI-1 (Fig.

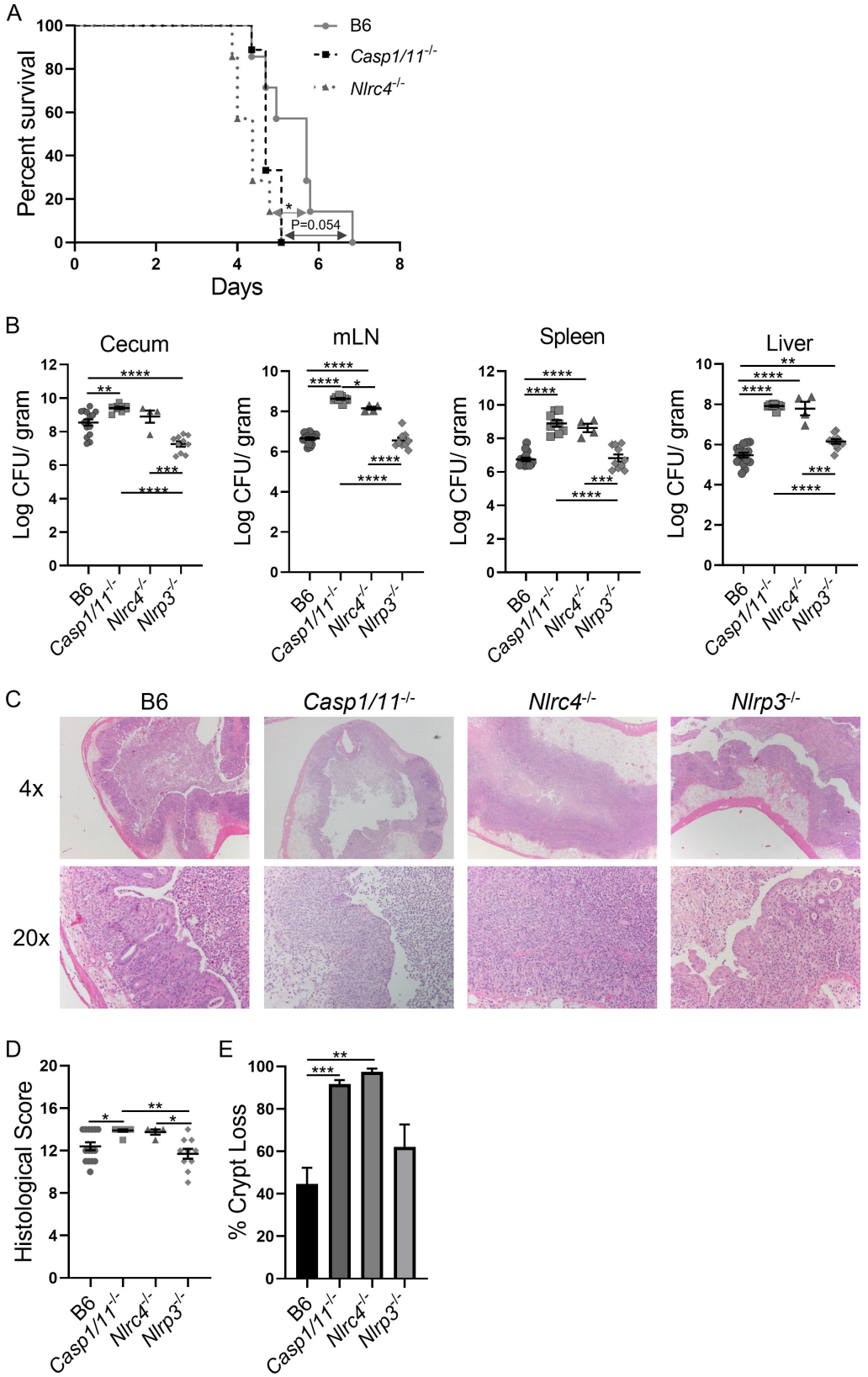

FIG 2 The NLRC4-CASP1/11 inflammasome is critical for limiting systemic *Salmonella* infection. Survival of B6 (n = 7), *Casp1/11*$^{-/-}$ (n = 9), and *Nlrc4*$^{-/-}$ (n = 7) mice that were orally infected with 1,000 CFUs of WT SL1344 *S.* Typhimurium (A). Bacterial burden of B6 (n = 15), *Casp1/11*$^{-/-}$ (n = 9), *Nlrc4*$^{-/-}$ (n = 4), and *Nlrp3*$^{-/-}$ (n = 10) mice orally infected with 1,000 CFUs
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FIG 2 (Continued) of WT SL1344 *S.* Typhimurium in the cecum, mLN, spleen, and liver on day 5 post-infection (B). Representative histology of the cecum infected with WT SL1344 *S.* Typhimurium (C). Histological scores for changes in the cecum (D). Frequency of crypt loss in the cecum (E). Survival curve is a combination of two independent experiments involving at least three mice per group. Statistical analyses of survival curve were done using log-rank (Mantel-Cox) test. Statistical analyses were done using one-way analysis of variance with post-Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Error bars indicate standard error of the mean.

3E; Tables 3 and 4). Moreover, B6 mice infected with Δ*flgM*/SPI-1 had significantly lower pathogen burden in the spleen and liver compared to ΔSPI-1 infected B6 animals (Fig. 3E; Tables 3 and 4). Conversely, in the absence of FlgM and the flagellar basal body, our results showed a significant increase of the bacterial burden in the mLN, spleen, and liver compared with mice infected with Δ*flgM* and Δ*flgM*/SPI-1 (Fig. 3E; Tables 2 to 4). We also observed that the attenuated phenotype of Δ*flgM Salmonella* was eliminated in the absence of flagellin expression (Fig. 3E). Next, we observed that deletion of SPI-2 or *flgM* and SPI-2 resulted in lower bacterial burden in the ceca from infected B6 mice but did not reach statistical significance (Fig. 3E; Table 1). Finally, B6 mice infected with ΔSPI-2 and Δ*flgM*/SPI-2 had significantly lower pathogen burden in the mLN, and both ΔSPI-2 and Δ*flgM*/SPI-2 were undetectable in the spleen and liver (Fig. 3E; Tables 2 to 4).

NLRC4 provides modest protection against aflagellate *Salmonella*

To determine the role of NLRC4 inflammasome-mediated detection of non-flagellin molecules in host resistance, we orally infected *Casp1/11*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice with flagellin-deficient *Salmonella*. Compared with B6 mice, *Nlrc4*$^{-/-}$ animals had significantly greater bacterial burden in the mLN, spleen, and liver (Fig. 4A). *Casp1/11*$^{-/-}$ mice also significantly displayed elevated bacterial burdens compared with B6 animals, in the mLN and the liver, but in the spleen, it did not reach statistical significance (Fig. 4A). Histological analysis revealed no significant difference in inflammation between any strain of mice (Fig. 4B and C); however, *Nlrc4*$^{-/-}$ and B6 mice did have slightly reduced tissue injury compared with CASP1/11-deficient animals (Fig. 4D). Overall, these data suggest that non-flagellin molecules are recognized by the NLRC4 inflammasome pathway but have a more limited role in restricting bacterial dissemination compared with flagellin.

To assess the overall changes in bacterial burden across all performed experiments, we compiled and compared CFU from B6, *Casp1/11*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice orally infected with either WT, Δ*flgM*, or Δ*fljB*/*fliC Salmonella*. Our analyses demonstrate that the NLRC4-CASP1/11 inflammasome is critical for the recognition of flagellin and significantly limits the bacterial burden in peripheral tissues such as the mLN, spleen, and liver (Fig. 4E). To a lesser extent, non-flagellin molecules recognized by the NLRC4 inflammasome pathway also restrict bacterial growth in peripheral tissues. In addition, flagellin-dependent motility enhances the virulence of *S.* Typhimurium, which is also seen when comparing infection of *motA*-deficient *Salmonella* to WT and aflagellate *Salmonella* (Fig. S1A).

NLRC4-CASP1/11-mediated intestinal inflammation requires flagellin and SPI-1

To define the requirements for *Salmonella* efficiently activating the inflammasome, we tested *Salmonella* genes that are critical for oral infection in streptomycin-pretreated mice. *In vitro* CASP1/11-dependent killing of macrophages requires both flagellin and SPI-1 expressions (Fig. 5A). Similarly, during oral infection in streptomycin-treated mice, enhanced virulence of *Salmonella* in *Casp1/11*$^{-/-}$ mice is dependent on both flagellin and SPI-1 (Fig. S2A). Augmented tissue inflammation of injury in *Casp1/11*$^{-/-}$ mice relative to B6 mice was also dependent on SPI-1 and flagellin (Fig. S1B), suggesting flagellin and SPI-1-dependent non-flagellin molecules are both required for enhanced virulence and to trigger intestinal pathology.

To assess the role of flagellin and SPI-1-dependent non-flagellin molecules in activating the NLRC4 inflammasome, we infected mice with *Salmonella* lacking both flagellin and the entire SPI-1 needle complex (ΔSPI-1/*fljB*/*fliC*). We observed no

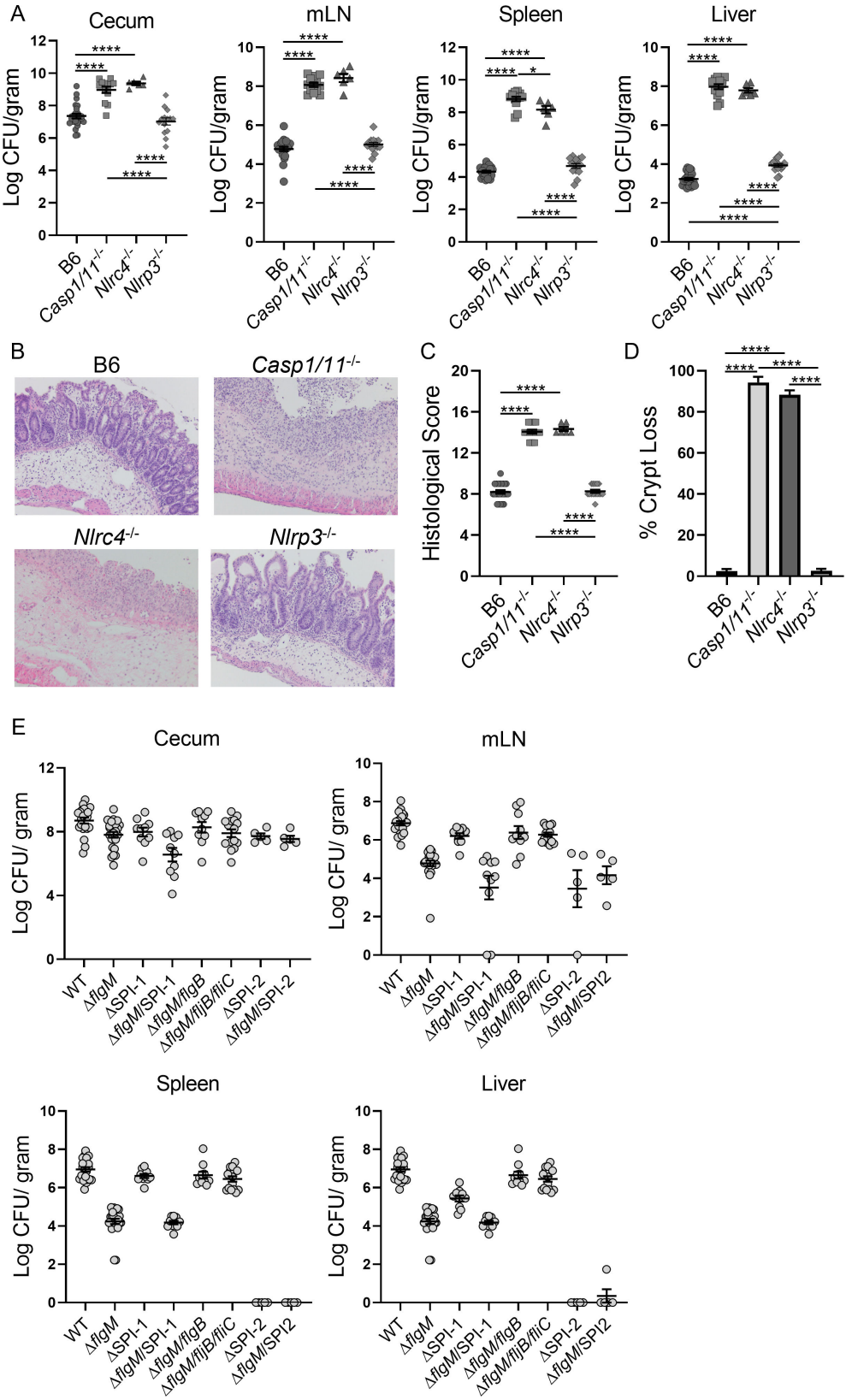

FIG 3 The NLRC4-CASP1/11 inflammasome is required to mediate the attenuation of flgM-deficient *S.* Typhimurium. Bacterial burden of B6 ($n = 24$), *Casp1/11*$^{-/-}$ ($n = 13$), *Nlrc4*$^{-/-}$ ($n = 6$), and *Nlrp3*$^{-/-}$ ($n = 14$) mice orally infected with 1,000 CFUs of *Δ flgM S.* Typhimurium in the cecum, mLN, spleen, and liver on day 5 post-infection (A). Representative histology (20×) of the cecum

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FIG 3 (Continued) Infected with *ΔflgM S.* Typhimurium (B). Histological scores for changes in the cecum (C). Frequency of crypt loss in the cecum (D). Bacterial burden of B6 mice orally infected with 1,000 CFUs of WT (n = 22) *ΔflgM* (n = 24), ΔSPI-1 (n = 10), *ΔflgM*/SPI-1 (n = 10), *ΔflgM/flgB* (n = 10), *ΔflgM/fljB/fliC* (n = 15), ΔSPI-2 (n = 5), or *ΔflgM*/SPI-2 (n = 5) *S.* Typhimurium in the cecum, mLN, spleen, and liver on day 5 post-infection (E). Statistical analyses were done on using one-way analysis of variance with post-Tukey's multiple comparison test. *P < 0.05, ****P < 0.0001. Error bars indicate standard error of the mean.

differences between the bacterial burden of intraperitoneally infected B6 and *Nlrc4*$^{-/-}$ mice in both the spleen and liver (Fig. 5B). In the ceca, spleen, and liver, we observed no differences between the pathogen burden of orally infected B6 and *Nlrc4*$^{-/-}$ mice (Fig. 5C). However, in the mLN, we did observe a significant difference in the pathogen burden between *Nlrc4*$^{-/-}$ mice and both *Casp1/11*$^{-/-}$ and B6 animals (Fig. 5C). Yet, these results showed a subtle increase of CFUs in orally infected *Casp1/11*$^{-/-}$ compared to both B6 and *Nlrc4*$^{-/-}$ animals in all tissues (Fig. 5C). Notably, examination of intestinal histology revealed that the absence of both flagellin and SPI-1 alleviated intestinal inflammation and tissue injury in all strains of mice (Fig. 5D through F). These data illustrate that flagellin and SPI-1 are required for efficient cecal colonization and *Salmonella*-induced intestinal inflammation and mucosal tissue injury.

DISCUSSION

Previously, we demonstrated that flagellin recognition by CASP1/11 controls infection of *flgM*-deficient *S.* Typhimurium and limits intestinal inflammation and injury (15). It has been shown that *Salmonella*-mediated activation of the NLRC4 inflammasome has distinct roles during systemic and mucosal infections through the detection of flagellin and non-flagellin molecules (7–10, 12, 14). In this article, we provide a more comprehensive understanding of how innate recognition of *S.* Typhimurium flagellin by the NLRC4 inflammasome is essential for mucosal protection against the enteric pathogen. Notably, the inflammasome was not required for host defense against *Salmonella* infection when mice were infected via the i.p. route (Fig. 1A). This is likely due to the efficient downregulation of inflammasome ligands (flagella and SPI-1 TTSS) during the systemic phase of infection. In contrast, the NLRC4 inflammasome is critical for prevention of *Salmonella*-induced intestinal tissue injury and systemic dissemination during mucosal infection (Fig. 2B). These data confirm that innate recognition of flagellin and SPI-1 TTSS structural proteins by the NLRC4-CASP1/11 inflammasome is critical to limit bacterial burden and intestinal pathology during mucosal infections in mice.

Previous studies have shown contradicting results as to the role of the NLRP3 inflammasome in innate immunity against *S.* Typhimurium. The data reported by De Jong et al., Hausmann et al., and Tenthorey et al. are consistent with our own, indicating a limited role for NLRP3 in *Salmonella* resistance (24, 43, 45). However, Broz and colleagues' data indicate that innate recognition of *Salmonella* by the NLRP3 inflammasome plays a significant albeit redundant role with NLRC4 to limit *Salmonella* infection (41).

TABLE 1 Statistical analysis of the cecum from Fig. 3E[a]

Cecum	WT	*ΔflgM*	ΔSPI-1	*ΔflgM*/SPI-1	*ΔflgM/flgB*	*ΔflgM/fljB/fliC*	ΔSPI-2	*ΔflgM*/SPI-2
WT								
ΔflgM	*							
ΔSPI-1	NS[b]	NS						
ΔflgM/SPI-1	****	*	*					
ΔflgM/flgB	NS	NS	NS	**				
ΔflgM/fljB/fliC	NS	NS	NS	*	NS			
ΔSPI-2	NS	NS	NS	NS	NS	NS		
ΔflgM/SPI-2	NS	NS	NS	NS	NS	NS	NS	

[a]One-way analysis of variance with post-Tukey's multiple comparison test of B6 mice orally infected with 1,000 CFUs of WT (n = 22), *ΔflgM* (n = 24), ΔSPI-1 (n = 10), *ΔflgM*/SPI-1 (n = 10), *ΔflgM/flgB* (n = 10), *ΔflgM/fljB/fliC* (n = 15), ΔSPI-2 (n = 5), or *ΔflgM*/SPI-2 (n = 5) *S.* Typhimurium in the ceca on day 5 post-infection from Fig. 3E. *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars indicate standard error of the mean.
[b]NS, not significant.

TABLE 2 Statistical analysis of the mLN from Fig. 3E[a]

mLN	WT	Δ*flgM*	ΔSPI-1	Δ*flgM*/SPI-1	Δ*flgM*/*flgB*	Δ*flgM*/*fljB*/*fliC*	ΔSPI-2	Δ*flgM*/SPI-2
WT								
Δ*flgM*	****							
ΔSPI-1	NS[b]	**						
Δ*flgM*/SPI-1	****	*	****					
Δ*flgM*/*flgB*	NS	***	NS	****				
Δ*flgM*/*fljB*/*fliC*	NS	***	NS	****	NS			
ΔSPI-2	****	NS	****	NS	****	****		
Δ*flgM*/SPI-2	****	NS	**	NS	**	**	NS	

[a]One-way ANOVA with post-Tukey's multiple comparison test of B6 mice orally infected with 1,000 CFUs of WT ($n = 22$), Δ*flgM* ($n = 24$), ΔSPI-1 ($n = 10$), Δ*flgM*/SPI-1 ($n = 10$), Δ*flgM*/*flgB* ($n = 10$), Δ*flgM*/*fljB*/*fliC* ($n = 15$), ΔSPI-2 ($n = 5$), or Δ*flgM*/SPI-2 ($n = 5$) *S.* Typhimurium in the mLN on day 5 post-infection from Fig. 3E. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Error bars indicate standard error of the mean.
[b]NS, not significant.

Discrepancies between studies may be due to the limited number of mice tested and the potential differences in gut microbiota that can influence oral infections. The preponderance of the data does not support a significant role for the NLRP3 inflammasome during oral or systemic *Salmonella* infection in mice.

Using Δ*flgM S.* Typhimurium, our data demonstrate that potent activation of the NLRC4 inflammasome pathway substantially limits bacterial burden and intestinal tissue damage (Fig. 3A through D). Our results demonstrate that FlgM-deficient *Salmonella* are markedly attenuated in their ability to infect systemic sites (mLN, liver, and spleen), where SPI-1 expression is suppressed and the SPI-2 inflammasome is induced. It has been shown *in vitro* that secretion of flagellin through the SPI-1 TTSS activates the inflammasome, and *in vivo* studies reported that forced expression of flagellin attenuates *Salmonella* and requires the SPI-2 TTSS and the NLRC4 inflammasome (44). Our results indicate that secretion of flagellin through the flagellar basal body is required for NLRC4-dependent protection against FlgM-deficient *Salmonella*. During mucosal infection, when SPI-1 and flagella are expressed, the NLRC4 inflammasome may detect flagellin that is secreted into the cytosol through the SPI-1 TTSS or liberated from flagella (Fig. 3E). As *Salmonella* transitions into the systemic phase of infection, where it is resides predominantly in myeloid cells with SPI-1 repressed and SPI-2 expressed, aberrant production of flagella due to FlgM deficiency is sufficient to activate the NLRC4 inflammasome (Fig. 3E). Leakage of flagellin out of damaged *Salmonella*-containing vacuoles or escape of *Salmonella* into the cytosol is a possible mechanism for FlgB-dependent cytosolic delivery of flagellin and NLRC4 inflammasome activation.

The critical role for NLRC4 during mucosal *Salmonella* infection most likely reflects the need for flagella and the SPI-1 TTSS to efficiently invade host IECs and access other mucosal host cells, such as dendritic cells and macrophages. It has been previously shown by both Sellin et al. and Hausmann et al. that NLRC4 is critical to restrict

TABLE 3 Statistical analysis of the spleen from Fig. 3E[a]

Spleen	WT	Δ*flgM*	ΔSPI-1	Δ*flgM*/SPI-1	Δ*flgM*/*flgB*	Δ*flgM*/*fljB*/*fliC*	ΔSPI-2	Δ*flgM*/SPI-2
WT								
Δ*flgM*	****							
ΔSPI-1	NS[b]	****						
Δ*flgM*/SPI-1	****	NS	****					
Δ*flgM*/*flgB*	NS	****	NS	****				
Δ*flgM*/*fljB*/*fliC*	NS	****	NS	****	****			
ΔSPI-2	****	****	****	****	****	****		
Δ*flgM*/SPI-2	****	****	****	****	****	****	NS	

[a]One-way ANOVA with post-Tukey's multiple comparison test of B6 mice orally infected with 1,000 CFUs of WT ($n = 22$), Δ*flgM* ($n = 24$), ΔSPI-1 ($n = 10$), Δ*flgM*/SPI-1 ($n = 10$), Δ*flgM*/*flgB* ($n = 10$), Δ*flgM*/*fljB*/*fliC* ($n = 15$), ΔSPI-2 ($n = 5$), or Δ*flgM*/SPI-2 ($n = 5$) *S.* Typhimurium in the spleen on day 5 post-infection from Fig. 3E. ****$P < 0.0001$. Error bars indicate standard error of the mean.
[b]NS, not significant.

TABLE 4 Statistical analysis of the liver from Fig. 3E[a]

Liver	WT	Δ*flgM*	ΔSPI-1	Δ*flgM*/SPI-1	Δ*flgM*/*flgB*	Δ*flgM*/*fljB*/*fliC*	ΔSPI-2	Δ*flgM*/SPI-2
WT								
Δ*flgM*	****							
ΔSPI-1	****	****						
Δ*flgM*/SPI-1	****	NS[b]	****					
Δ*flgM*/*flgB*	NS	****	****	****				
Δ*flgM*/*fljB*/*fliC*	NS	****	****	****	NS			
ΔSPI-2	****	****	****	****	****	****		
Δ*flgM*/SPI-2	****	****	****	****	****	****	NS	

[a]One-way ANOVA with post-Tukey's multiple comparison test of B6 mice orally infected with 1,000 CFUs of WT ($n = 22$), Δ*flgM* ($n = 24$), ΔSPI-1 ($n = 10$), Δ*flgM*/SPI-1 ($n = 10$), Δ*flgM*/*flgB* ($n = 10$), Δ*flgM*/*fljB*/*fliC* ($n = 15$), ΔSPI-2 ($n = 5$), or Δ*flgM*/SPI-2 ($n = 5$) *S.* Typhimurium in the liver on day 5 post-infection from Fig. 3E. ****$P < 0.0001$. Error bars indicate standard error of the mean.
[b]NS, not significant.

systemic dissemination of *Salmonella* within the first 36 h of oral infection of IECs (3, 24). During oral WT *Salmonella* infection, SPI-1 and flagellin are both targeted by the NLRC4 inflammasome. Using Δ*flgM S.* Typhimurium, our data indicate that recognition of flagellin by the NLRC4-CASP1/11 inflammasome significantly reduces intestinal pathology and tissue injury (Fig. 3); likewise, intestinal tissue damage is augmented by deleting flagellin in *Salmonella* to levels seen in *Nlrc4*$^{-/-}$ or *Casp1/11*$^{-/-}$ mice (Fig. 4). SPI-1 is required to induce maximal intestinal inflammation and injury, and this is independent of the CASP1/11-inflammasome (Fig. S2B). These results indicate that flagellin and SPI-1 are critical triggers of intestinal inflammation and injury through inflammasome-dependent and -independent pathways. Deleting both flagellin and SPI-1 alleviated *Salmonella*-mediated intestinal pathology and tissue damage but did not prevent systemic dissemination of the bacteria (Fig. 5). Thus, our data are in agreement with the previous reports by both Sellin et al. and Hausmann et al., which demonstrate that NLRC4 recognition of *Salmonella* flagellin is essential to restrict bacterial dissemination and growth (3, 24).

The limited phenotype for *S.* Typhimurium mutants lacking flagellin expression in B6 mice can be attributed to a concomitant loss of motility. This is most readily observed when looking at infection of B6 mice by Δ*motA S.* Typhimurium (Fig. S1A). When amotile flagellin-sufficient bacteria are compared to amotile flagellin-deficient bacteria, loss of flagellin expression results in enhanced virulence. Thus, motility enhances *Salmonella*'s virulence, which is offset by increased host resistance through the detection of flagellin by the NLRC4 inflammasome. NLRC4 inflammasome-induced inflammation also benefits *Salmonella* colonization of the gut and increases transmissibility (46, 47), providing additional benefits for maintenance of flagellin-dependent motility in the face of host innate immune surveillance.

Since *Casp1/11*$^{-/-}$ mice lack both canonical and non-canonical inflammasome pathways, the mucosal injury observed in these mice is independent of both the caspase-1 and caspase-11 inflammasomes. Epithelial cell intrinsic NAIP-NLRC4-CASP1 activation has been shown to induce cellular expulsion of infected enterocytes into the intestinal lumen, preventing *S.* Typhimurium infection of lamina propria mononuclear phagocytes, thereby restricting *Salmonella* dissemination to systemic tissues (3). We have previously shown that enhanced IL-12 and interferon gamma (IFN-γ) production by lamina propria leukocytes correlates with tissue injury (15). In addition, *Salmonella* accumulates more readily in lamina propria macrophages in the absence of CASP1/11 (15). Thus, deficiency in the NLRC4-CASP1 inflammasome may promote the accumulation of *Salmonella* within lamina propria macrophages and IFN-γ-dependent immunopathology.

Our study demonstrates that SPI-1 and flagellin are critical for efficient mucosal infection and *Salmonella*-induced intestinal inflammation and injury. The NLRC4 inflammasome targets these virulence pathways to limit mucosal infection, inflammation, and tissue injury in mice. Constitutive production of flagellin in Δ*flgM S.*

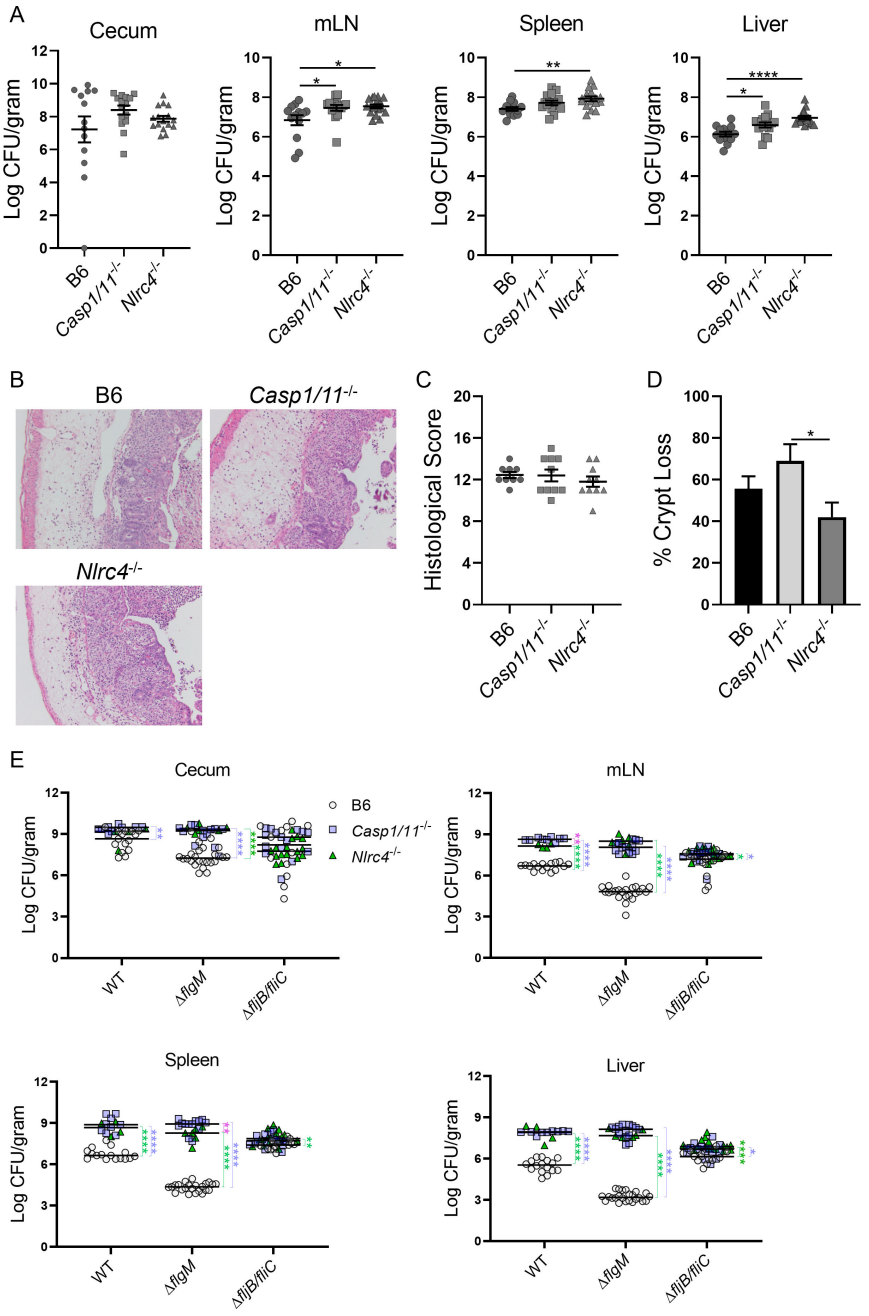

FIG 4 NLRC4-CASP1/11-mediated host protection is dependent on flagellin expression. Bacterial burden of B6 (n = 13), *Casp1/11*$^{-/-}$ (n = 15), and *Nlrc4*$^{-/-}$ (n = 16) mice orally infected with 1,000 CFUs of *ΔfljB/fliC S.* Typhimurium in the cecum, mLN, spleen, and liver on day 5 post-infection (A). Representative histology (20×) of the cecum infected with *ΔfljB/fliC S.* Typhimurium (B). Histological scores for changes in the cecum (C). Frequency of crypt loss in the cecum (D). Composite analysis of WT SL1344, *ΔflgM*, and *ΔfljB/fliC S.* Typhimurium infections in B6, *Casp1/11*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice (E; i.e., blue = WT vs *Nlrc4*$^{-/-}$; green = WT vs *Casp1/11*$^{-/-}$; pink = *Nlrc4*$^{-/-}$ vs *Casp1/11*$^{-/-}$). Statistical analyses were done on using one-way analysis of variance with post-Tukey's multiple comparison test. *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars indicate standard error of the mean.

Typhimurium prevents excessive tissue injury, inflammation, and systemic spread in a NLRC4 inflammasome-dependent manner and is a strategy to attenuate *Salmonella* while preserving the expression of this important virulence factor and target of innate

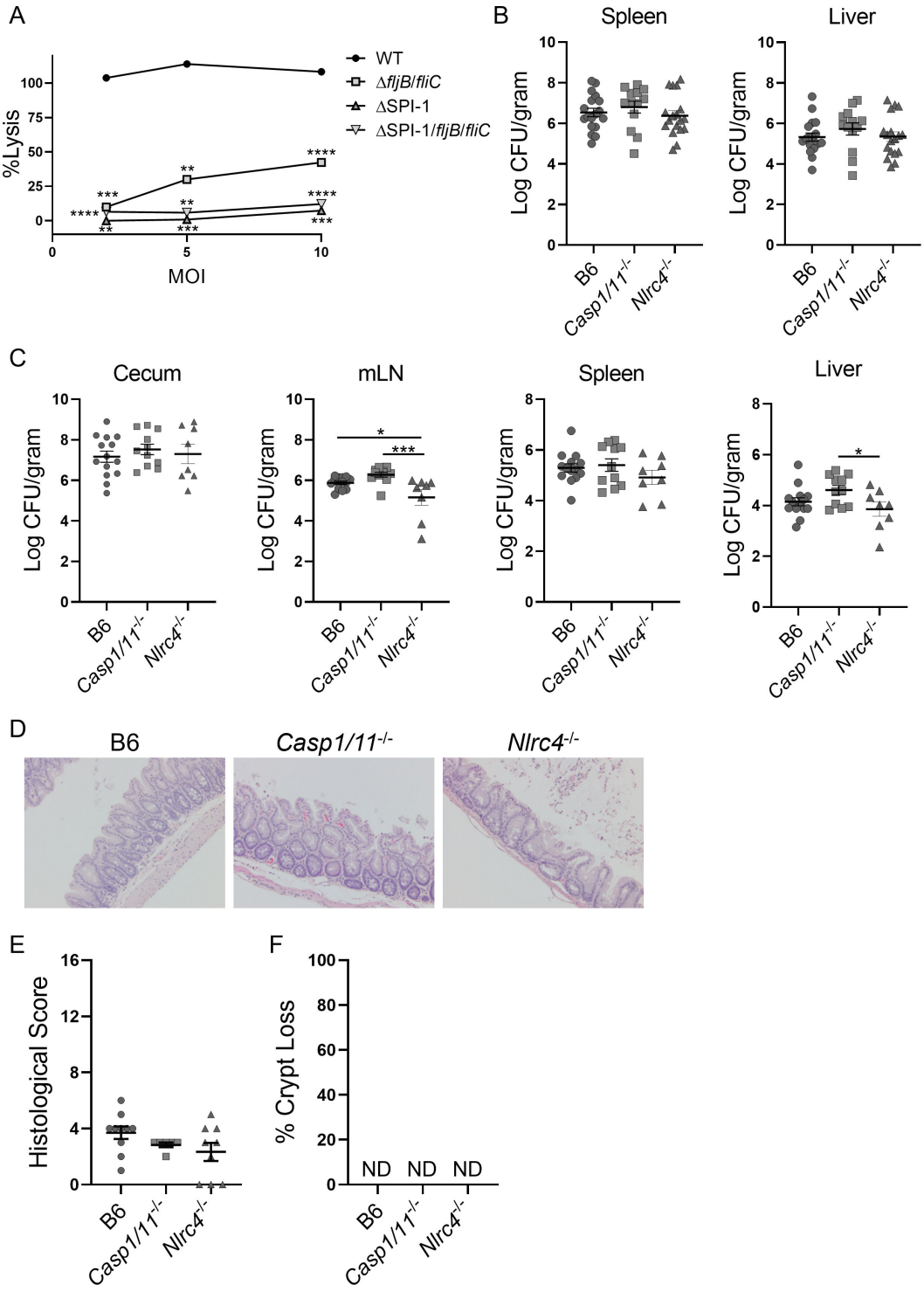

FIG 5 Flagellin-independent NLRC4-CASP1/11-mediated intestinal inflammation is SPI-1-dependent. *Salmonella*-induced cell death in thioglycollate elicited peritoneal macrophages measured by lactate dehydrogenase (LDH) release assay (A). Bacterial burden of B6 (n = 18), *Casp1/11*$^{-/-}$ (n = 13), and *Nlrc4*$^{-/-}$ (n = 18) mice intraperitoneally infected with 1,000 CFUs of *ΔflgM*/SPI-1 *S.* Typhimurium in the spleen and liver on day 5 post-infection (B). Bacterial burden of B6 (n = 14), *Casp1/11*$^{-/-}$ (n = 11), and *Nlrc4*$^{-/-}$ (n = 8) mice orally infected with 1,000 CFUs of *ΔflgM*/SPI-1 *S.* Typhimurium in the cecum, mLN, spleen, and liver on day 5 post-infection (C). Representative histology (20×) of the cecum infected with *ΔflgM*/SPI-1 *S.* Typhimurium (D). Histological scores for changes in the cecum (E). Frequency of crypt loss in the cecum (F). Statistical analyses were done using two-way ANOVA with Tukey's multiple comparison test (A) or one-way ANOVA with post-Tukey's multiple comparison test. *P < 0.05, ***P < 0.001 (B, C, E, F). Error bars indicate standard error of the mean. ND, not detected.

and adaptive immunity. Since the human NAIP-NLRC4 inflammasome has also been demonstrated to be an important mechanism of flagellin recognition, our results suggest that attenuation of *Salmonella* by deletion of *flgM* may produce a novel live attenuated vaccine that both augments innate immunity and increases bioavailability of protective antigens. Similar strategies to create *Salmonella* with constitutive SPI-1 expression may behave similarly to provoke protective innate immunity while augmenting expression of important targets for adaptive immune responses.

AUTHOR AFFILIATIONS

[1]Department of Laboratory Medicine and Pathology, University of Washington, Seattle, Washington, USA
[2]Department of Comparative Medicine, University of Washington, Seattle, Washington, USA

PRESENT ADDRESS

Américo Harry López-Yglesias, Department of Microbiology and Immunology, Indiana University School of Medicine-Terre Haute, Terre Haute, Indiana, USA

AUTHOR ORCIDs

Américo Harry López-Yglesias http://orcid.org/0000-0001-6797-2179
Kelly D. Smith http://orcid.org/0000-0002-2368-4957

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Américo Harry López-Yglesias, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing | Chun-Chi Lu, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft | Marvin A. Lai, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft | Ellen K. Quarles, Methodology | Xiaodan Zhao, Methodology | Adeline M. Hajjar, Investigation, Resources | Kelly D. Smith, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing

ETHICS APPROVAL

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 Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington (protocol: 4031–01, Mucosal Immunity).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1 and S2 and legends (IAI00255-23-s0001.docx). (S1) WT SL1344, fljB/fliC KO, or motA KO *S.* Typhimurium mouse infection and (S2) Bacterial burden of B6 and Casp1/11$^{-/}$

⁻ mice orally infected with WT SL1344, fljB/fliC KO, SPI-1 KO, or SPI-1/fljB/fliC KO *S.* Typhimurium.

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