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Salmonella enterica Serovar Typhimurium Pathogenicity Island 2 Is Necessary for Complete Virulence in a Mouse Model of Infectious Enterocolitis

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***Salmonella* species cause a wide range of disease in multiple hosts. *Salmonella enterica* serovar Typhimurium causes self-limited intestinal disease in humans and systemic typhoid-like illness in susceptible mice. The prevailing dogma in murine *S. enterica* serovar Typhimurium pathogenesis is that distinct virulence mechanisms—*Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2)—perform distinct roles in pathogenesis, the former being important for invasion and intestinal disease and the latter important for intracellular survival and systemic persistence and disease. Although evidence from bovine infections has suggested that SPI2 has a role in ileal disease, there is no evidence that SPI2 is important for inflammation in a disease that more closely recapitulates human colitis. Using *S. enterica* serovar Typhimurium strains that lack functional type III secretion systems, we demonstrate that SPI2 is essential for complete virulence in murine infectious enterocolitis. Using a recently characterized murine model (M. Barthel,S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W. D. Hardt, Infect. Immun. 71:2839–2858, 2003), we demonstrate that SPI1 mutants are unable to cause intestinal disease 48 h after infection and that SPI2-deficient bacteria also cause significantly attenuated typhlitis. We show that at the peak of inflammation in the cecum, SPI2 mutants induce diminished intercellular adhesion molecule 1 expression and neutrophil recruitment but that wild-type and mutant *Salmonella* are similarly distributed in the lumen of the infected organ. Finally, we demonstrate that attenuation of intestinal inflammation is accompanied by resolution of typhlitis in the mutant, but not wild-type, infections. Collectively, these results indicate that SPI2 is needed for enterocolitis, as well as for systemic disease.**

Salmonella species are facultative intracellular gram-negative bacteria that cause a wide array of disease including systemic disease and enterocolitis in a multitude of hosts (reviewed in [2]). Murine infection with *Salmonella enterica* serovar *typhimurium* has been used predominantly to model human typhoid (caused by *Salmonella enterica* serovar Typhi), while bovine infection with *Salmonella enterica* serovar Dublin or *S. enterica* serovar Typhimurium has been a prevailing model of intestinal disease. These models have been exploited to gain critical insight into the pathogenesis of disease cause by salmonellae, including (for example) that invasion-associated genes are required for intestinal secretory and inflammatory disease, that intracellular survival in both the intestinal epithelium and macrophages is essential for systemic pathogenesis, and that M cells of the ileal Peyer's patches are the site of invasion for systemic infection in murine typhoid prior to dissemination to liver and spleen via the reticuloendothelial system (8, 14, 15, 28). A central hypothetical theme that has emerged as a result of these discoveries is the distinct role of different virulence systems—*Salmonella* pathogenicity island 1 (SPI1) and SPI2—in the pathogenesis of intestinal and systemic disease.

Both of these horizontally acquired genomic islands encode a type III secretion system (TTSS), capable of secreting bacterial proteins into the host cell or extracellular milieu. The prevailing view is that SPI1 is necessary for cell invasion and essential for intestinal disease, while SPI2 is required for intracellular survival and persistence in target organs such as the spleen and liver (reviewed in reference 22). Soon after the identification of these virulence systems, it was demonstrated that in mice, SPI1-deficient *S. enterica* serovar Typhimurium is incapable of causing systemic disease following oral infection but is not attenuated when introduced intraperitoneally (10, 28), while SPI2 mutants inoculated by the latter route are attenuated for systemic infection (23) but maintain intestinal virulence in cows and rabbits after oral infection (7, 25).

Whether SPI2 plays a role in intestinal inflammatory disease caused by *S. enterica* serovar Typhimurium is unclear. It has been shown with cattle that diarrhea caused by *S. enterica* serovar Typhimurium is SPI1 but not SPI2 dependent (25) and that *Salmonella* strains lacking SPI2 regulatory genes and wild-type (WT) *Salmonella* are equally pathogenic in a rabbit model of gastroenteritis (7). However, it has also been reported that SPI2 plays at least some role in bovine inflammatory disease (3, 25). Bispham et al. (3) demonstrated that bacteria lacking the SPI2 translocon component *sseD* or the SPI2 TTSS appa-

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ratus protein encoded by the *ssaT* gene induced less fluid secretion into bovine ileal loops inoculated with mutant bacteria than did WT and that there was diminished neutrophilic influx into infected tissue.

There are significant practical and theoretical limitations of these models, however. Due to the technically demanding nature of bovine experiments, the number of experimental animals used in these experiments is limited. Furthermore, cattle used are typically outbred and show significant variation in disease severity, depending on age. In addition, although investigations have demonstrated disease in the ilea of these animals, the role of SPI2 in tissues more commonly affected in human *S. enterica* serovar Typhimurium infection, e.g., the cecum and colon, have not been investigated. Consequently, it is difficult to extrapolate the findings of these studies to human disease.

A recently characterized model of infectious cecal inflammation (typhlitis) in mice provides an alternate model for the study of human *S. enterica* serovar Typhimurium-induced intestinal disease with significant advantages over the bovine model (1). Following oral administration of streptomycin, mice challenged with *S. enterica* serovar Typhimurium display signs of intestinal inflammatory pathology with many histopathological similarities to human disease, including severe inflammation in the large bowel with little or no inflammatory pathology in the ileum (1, 4, 17). This model has been utilized to demonstrate that *Salmonella*-induced intestinal inflammation requires bacterial activity dependent on the SPI1 effector *sipA*, as well as functional flagella and chemotaxis (1, 11, 24). Antibiotic treatment is a risk factor for acquiring *S. enterica* serovar Typhimurium colitis (6), and the dependence of inflammation on streptomycin treatment is inferred to be due to alterations in the host microflora, resulting in environmental changes and decreased colonization resistance (20, 21).

In this study, we exploited the streptomycin pretreatment model to show that SPI2 is required for complete intestinal virulence in the cecum and colon in vivo in a disease model that shares clinical and histopathological features with human disease.

MATERIALS AND METHODS

Bacterial culture. *Salmonella enterica* serovar Typhimurium SL1344 (wild type [WT]), *invA* mutant (*invA*::*kan* SB103; SPI1), and *ssaR* mutant (Δ*ssaR*; SPI2) (5) were grown with overnight shaking (200 rpm) in 3 ml Luria-Bertani (LB) broth with 50 μg/ml streptomycin ± 50 μg/ml kanamycin at 37°C for 18 h.

Mouse experiments. Inbred 10-week-old C57BL/6 mice (Jackson Laboratories) were deprived of food and water for 4 h prior to administration of 20 mg of streptomycin/mouse by oral gavage. Two hours subsequently, food and water were provided ad libitum. Twenty hours after oral streptomycin treatment, food and water were once again withdrawn for 4 h, after which 3×10^6 or 3×10^8 bacteria in 100 μl LB broth were administered by oral gavage. Control mice were given 100 μl sterile LB broth. Water and food were provided ad libitum. Mice were euthanized with CO_2 at designated time points, and tissues were harvested aseptically for bacterial enumeration and histopathology. All animal experiments were conducted in a manner consistent with the ethical requirements of the Animal Care Committee at the University of British Columbia.

Bacterial enumeration. Tissues were collected at various time points into 1.5 ml sterile PBS and homogenized with a tissue homogenizer (Polytron MR 21; Kinematica). Serial dilutions of the resulting mixture were plated on LB agar plates containing 100 μg/ml streptomycin. The threshold of detection was 50 CFU per organ.

Histopathology. Colons, ceca, and ilea of experimental animals were fixed in 3% formalin for 18 h, followed by 18 h in 70% ethanol prior to being embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), or they were fixed for 3 h in 3% paraformaldehyde, prior to being embedded and cryosectioned.

Pathological scores were determined by averaging six fields/sample as follows, based on a revision of previously published methods (quantitave and qualitative criteria were separated) and descriptions of human disease.

(i) Lumen. Pathological scores were determined as follows (scores are given in parentheses after each category): empty (0), necrotic epithelial cells (scant, 1; moderate, 2; dense, 3), and polymorphonuclear leukocytes (PMNs) (scant, 2; moderate, 3; dense, 4).

(ii) Surface epithelium. Pathological scores were determined as follows (scores are given in parentheses after each category): no pathological changes (0); mild, moderate, or severe regenerative changes (1, 2, or 3, respectively); patchy or diffuse desquamation (1 or 2); PMNs in epithelium (1); and ulceration (1).

(iii) Mucosa. Pathological scores were determined as follows (scores are given in parentheses after each category): no pathological changes (0); rare (<15%), moderate (15 to 50%), or abundant (>50%) crypt abscesses (1, 2, or 3, respectively); presence of mucinous plugs (1); presence of granulation tissue (1).

(iv) Submucosa. Pathological scores were determined as follows (scores are given in parentheses after each category): no pathological changes (0); mononuclear cell infiltrate (1 small aggregate, >1 aggregate, or large aggregates plus increased single cells) (0, 1, or 2, respectively); PMN infiltrate (no extravascular PMNs, single extravascular PMNs, or PMN aggregates) (0, 1, or 2, respectively); mild, moderate, or severe edema (0, 1, or 2, respectively).

Immunohistochemistry. Paraffin-embedded tissues were deparaffinized in xylene (twice for 5 min each time); rehydrated in 100%, 95%, and 70% ethanol (each, 5 min); then washed in phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-BSA; BSA from Sigma). Sections were outline with a wax pen and blocked for 30 min in 10% goat serum in PBS-BSA at room temperature. Sections were then washed three times in PBS-BSA, prior to incubation overnight at 4°C or 1 h room temperature with a primary antibody (anti-*Salmonella* lipopolysaccharide [LPS], 6.8 μg/ml) and anti-intercellular adhesion molecule 1 (anti-ICAM-1), 5μg/ml; BD Biosciences). Sections were then washed three times in PBS-BSA, prior to incubation with the appropriate fluorochrome- or streptavidin-conjugated secondary antibodies (0.1 mg/ml, 30 min at room temperature). Imaging was performed with a Zeiss Axioskop epifluorescence microscope and MetaMorph software for fluorescent images and with a Zeiss Axiostar microscope with a Nikon Powershot G5 camera. Light and fluorescent images were cropped and scaled in Adobe Photoshop, version 7.0.1.

Mast cell staining. Paraffin-embedded sections were deparaffinized as above prior to treatment with the Naphthol AS-D Chloroacetate Esterase staining kit (Sigma). Sections were treated for 15 min to visualize mast cells or for 1 h to visualize neutrophils as previously described (29).

Quantitative measures of inflammation. Mast cells from 100 sequential crypts were counted at a magnification of ×400. Goblet cells were enumerated from 10 random high-powered fields spanning muscularis mucosa to surface epithelium with H&E-stained sections. Mucosal and submucosal thicknesses were measured at six evenly spaced points per section for each experimental animal. Mucosal thickness was defined as the distance from the surface epithelium to the inner edge of the muscularis mucosa. Submucosa thickness was defined as the distance from muscularis mucosa to the muscularis externa. Averages for each mouse were compared.

Statistical analysis. Total pathological scores were compared using Mann-Whitney U and Kruskall-Wallis nonparametric tests. Bacterial load was compared using analysis of variance and Tukey's multiple comparison posttests. All analyses were performed using Graphpad Prism version 3.0.

RESULTS

***S. enterica* serovar Typhimurium elicits intestinal inflammation that is most pronounced in the cecum of streptomycin-pretreated mice.** To assess induction of intestinal inflammation in response to *S. enterica* serovar Typhimurium, we infected Nramp1^S C57BL/6 mice orally with 3×10^8 WT *S. enterica* serovar Typhimurium SL1344 24 h after oral gavage with 20 mg streptomycin. We observed extensive inflammatory changes in the large bowels of WT *Salmonella*-infected mice similar to those reported previously (1), as well as bacterial translocation to liver and spleen and ultimately fatal systemic disease (Fig. 1; data not shown). Common features in the intestine following *Salmonella* infection included neutrophilic

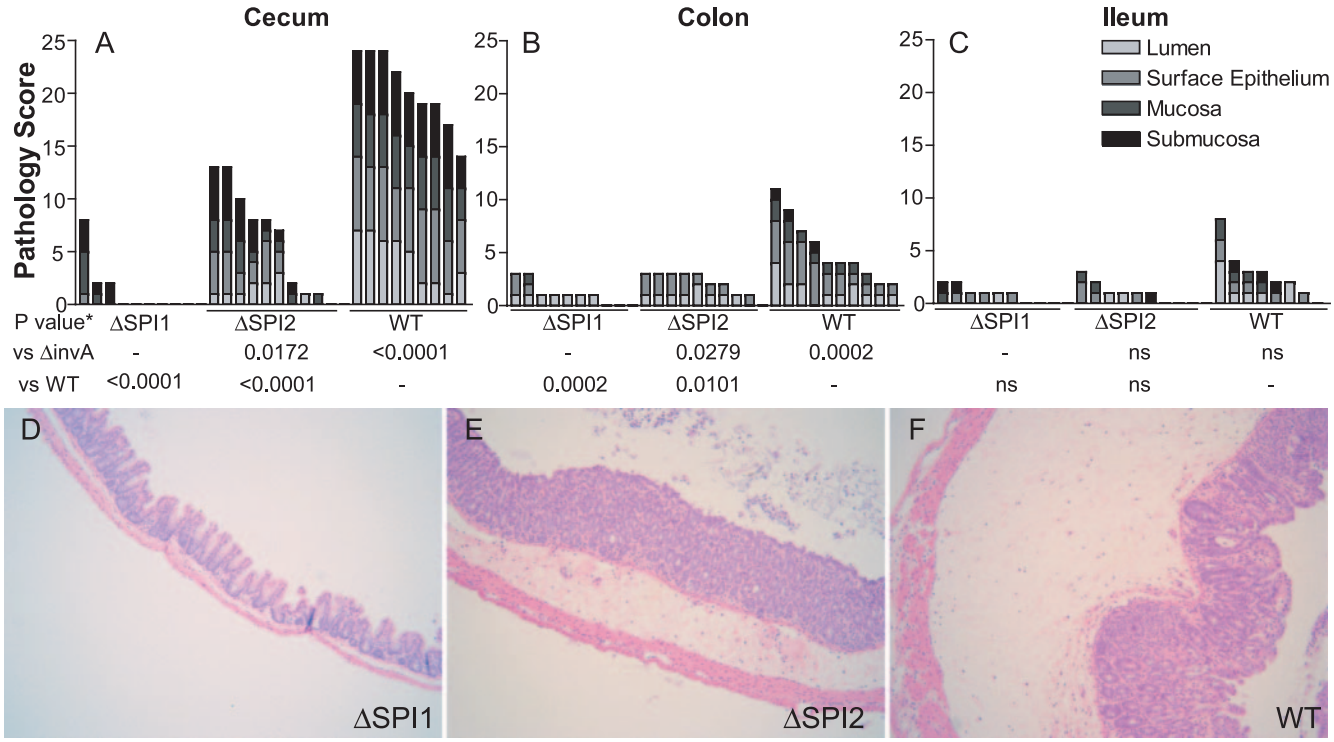

FIG. 1. *Salmonella enterica* serovar Typhimurium-elicited enterocolitis is most severe in the ceca of wild-type infected mice and is partially attenuated in the absence of a SPI2 TTSS. Streptomycin-treated C57Bl/6 mice were infected with 3×10^8 wild-type, SPI1, or SPI2 mutant *Salmonella enterica* serovar Typhimurium organisms. Histopathology scores from WT-, SPI1 mutant-, and SPI2 mutant-infected ceca (A), colons (B), and ilea (C) plus representative H&E-stained ceca (D to F) are shown. Histopathology was most severe in the ceca of all infected mice and was completely attenuated in ΔinvA-infected mice. Inflammation in the ceca and colons of mice infected with SPI2 mutants was statistically intermediate between WT- and SPI1-infected mice by the Kruskall-Wallis test ($P < 0.0001$). *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. (A to C) Each bar represents a single mouse. Images are shown at a magnification of ×200.

infiltratation into the intestinal lumen, surface epithelial erosion and/or desquamation, inflammatory infiltratation into the lamina propria and submucosa, crypt abscesses, and submucosal edema. These features were absent from control mice given LB broth alone. Epithelial proliferation in the mucosa as assessed by immunohistochemistry with antiproliferating cell nuclear antigen (data not shown) was increased, resulting in increased mucosal thickness (Fig. 1F; Table 1). Submucosal edema was also evident (Fig. 1F; Table 1). Inflammatory features including mast cell recruitment and loss of goblet cells were common at 48 h in infected mice (Table 1) but absent from controls. Pathological changes were also uncommon in mice infected with WT *S. enterica* serovar Typhimurium but not pretreated with streptomycin (data not shown). Bacterial translocation to the liver and spleen occurred as early as 6 h postinfection, and bacterial colonization of these sites occurred consistently by 24 h.

At 48 h postinfection, intestinal inflammation was maximal (Fig. 1; data not shown). Consistent with previous studies (1), we observed that WT *S. enterica* serovar Typhimurium-elicited intestinal inflammation at 48 h was most severe in the cecum, with less severe inflammation in the colon and little or no inflammatory change in the ileum (Fig. 1A to C). We focused subsequent comparisons of intestinal pathology on

TABLE 1. Inflammatory pathology in the ceca of WT, SPI1, and SPI2 mutant *S. typhimurium* serovar Typhimurium-infected mice

Pathological change	Strain[a]			*P* value (ANOVA)[b]
	ΔSPI1	ΔSPI2	WT	
Log_{10} CFU/g colon	7.4 ± 0.4	7.7 ± 0.6	7.8 ± 0.7	NS
Cecal wt (g)	$0.47 \pm 0.10^{2**3***}$	$0.34 \pm 0.09^{1**3***}$	$0.16 \pm 0.05^{1***2***}$	<0.0001
Submucosal edema (% wall thickness)	$22 \pm 6^{3*}$	26 ± 11	$37 \pm 15^{1*}$	0.0298
Mucosa (μm)	$90 \pm 13^{3***}$	$115 \pm 15^{3***}$	$192 \pm 52^{1***2***}$	<0.0001
Goblet cells/HPF[c]	$38 \pm 19^{2*3***}$	$12 \pm 1^{1*3***}$	$6 \pm 7^{1***2***}$	<0.0001
Mast cells/100 crypts	$1.3 \pm 1.4^{3**}$	$1.2 \pm 1.2^{3**}$	$15 \pm 12^{1**2**}$	0.0006

[a] Values shown represent means ± standard deviation for each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Tukey's multiple comparison test; [1], versus ΔSPI1; [2], versus ΔSPI2; [3], versus WT.
[b] ANOVA, analysis of variance.
[c] HPF, high-powered field.

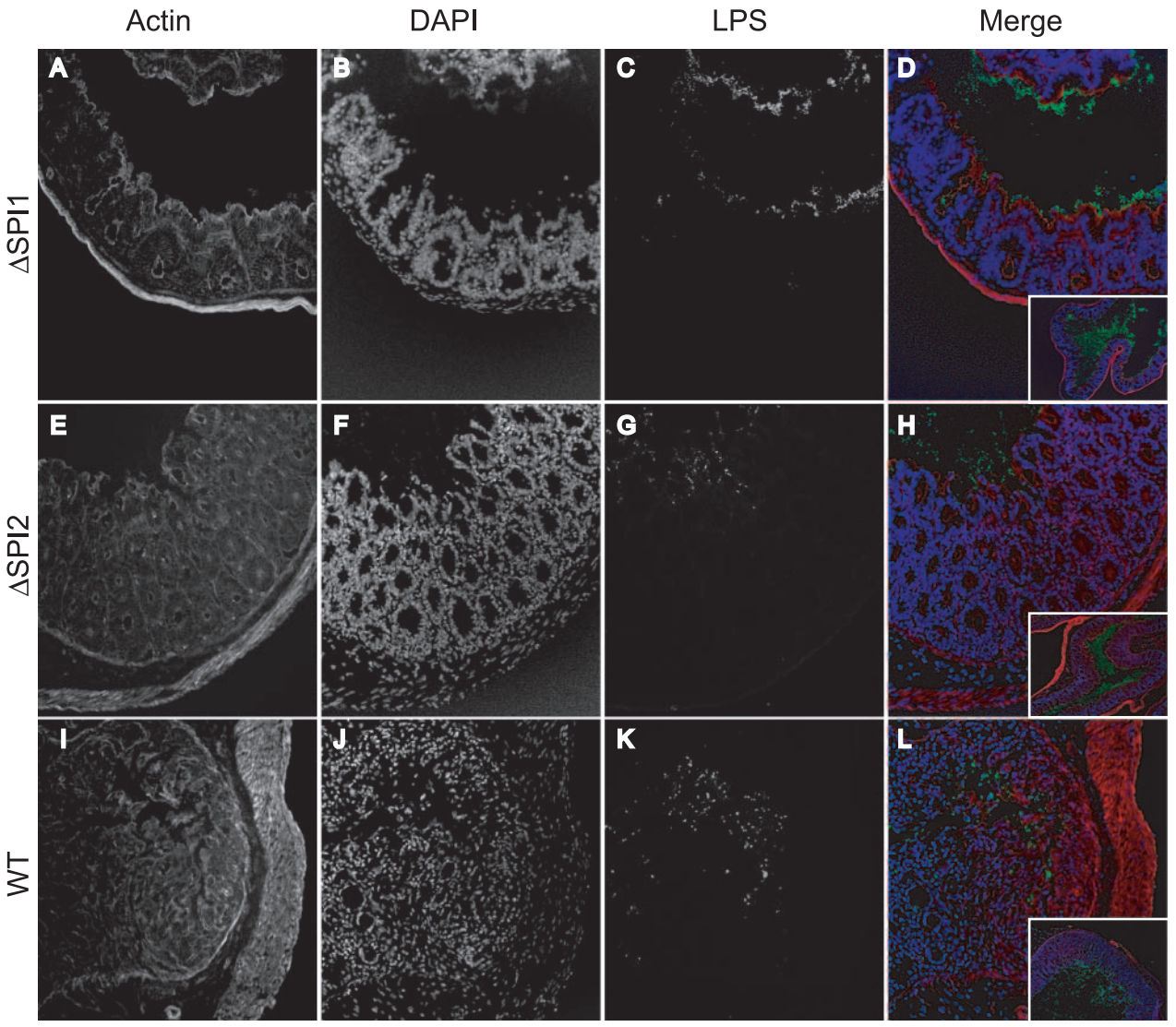

FIG. 2. *S. enterica* serovar Typhimurium in the ceca of infected mice is primarily extracellular and infiltrates the mucosa as inflammation progresses. Bacterial localization of SPI1 (A to D), SPI2 (E to H), and WT (I to L) *S. enterica* serovar Typhimurium 48 h after infection in the ceca of streptomycin-treated C57BL/6 mice. Paraformaldehyde-fixed optimal cutting temperature compound-embedded cryosections were stained for actin (red in merge) (A, E, and I), nuclei (with DAPI [4′,6′-diamidino-2-phenylindole; blue in merge) (B, F, and J), or against *Salmonella* LPS (green in merge) (C, G, and K). Mucosal infiltration was more common as inflammation worsened (merges shown in panels D, H, and K). Bacteria were not seen in the mucosa of SPI1-infected mice but were occasionally present in mucosa of SPI2-infected mice and common in the mucosa of WT-infected mice. Images are a pseudocolor and are shown at ×200 magnification. Insets are shown at ×100 magnification.

the ceca at this time point, due to the consistent severity of histopathology.

SPI2 contributes to cecal inflammation in *S. enterica* serovar Typhimurium infection. To assess the role of SPI2 in intestinal disease, we compared intestinal pathology at 48 h in mice infected with WT *S. enterica* serovar Typhimurium or bacterial strains lacking a functional SPI1 or SPI2 TTSS. In agreement with previous studies, we observed that a functional SPI1 TTSS is essential to elicit intestinal inflammation (1, 11). Significant histopathological changes were absent from all SPI1-infected mice in all three tissues examined (Fig. 1 A to C), and histopathology scores were statistically indistinguishable from uninfected mice (data not shown). Mucosal hypertrophy and submucosal edema were absent from SPI1-infected mice, and mast cell and goblet cell numbers were not different than uninfected controls (Table 1; data not shown).

Intestinal inflammation in the absence of SPI2 type III secretion was also significantly attenuated compared to WT ($P < 0.0001$; Fig. 1 and Table 1). In all three tissues assessed, intestinal pathology was intermediate between SPI1- and WT-infected mice (Fig. 1; Table 1). The intermediate phenotype associated with SPI2 mutant infection was not due to the absence of one specific pathological feature but rather reflected the diffuse attenuation of the inflammatory phenotype in all measured parameters (Fig. 1A; Table 1). This intermediate pathology was not due to differences in bacterial content of the tissues examined, as bacterial loads of all strains were comparable at the time point assessed (Table 1), suggesting

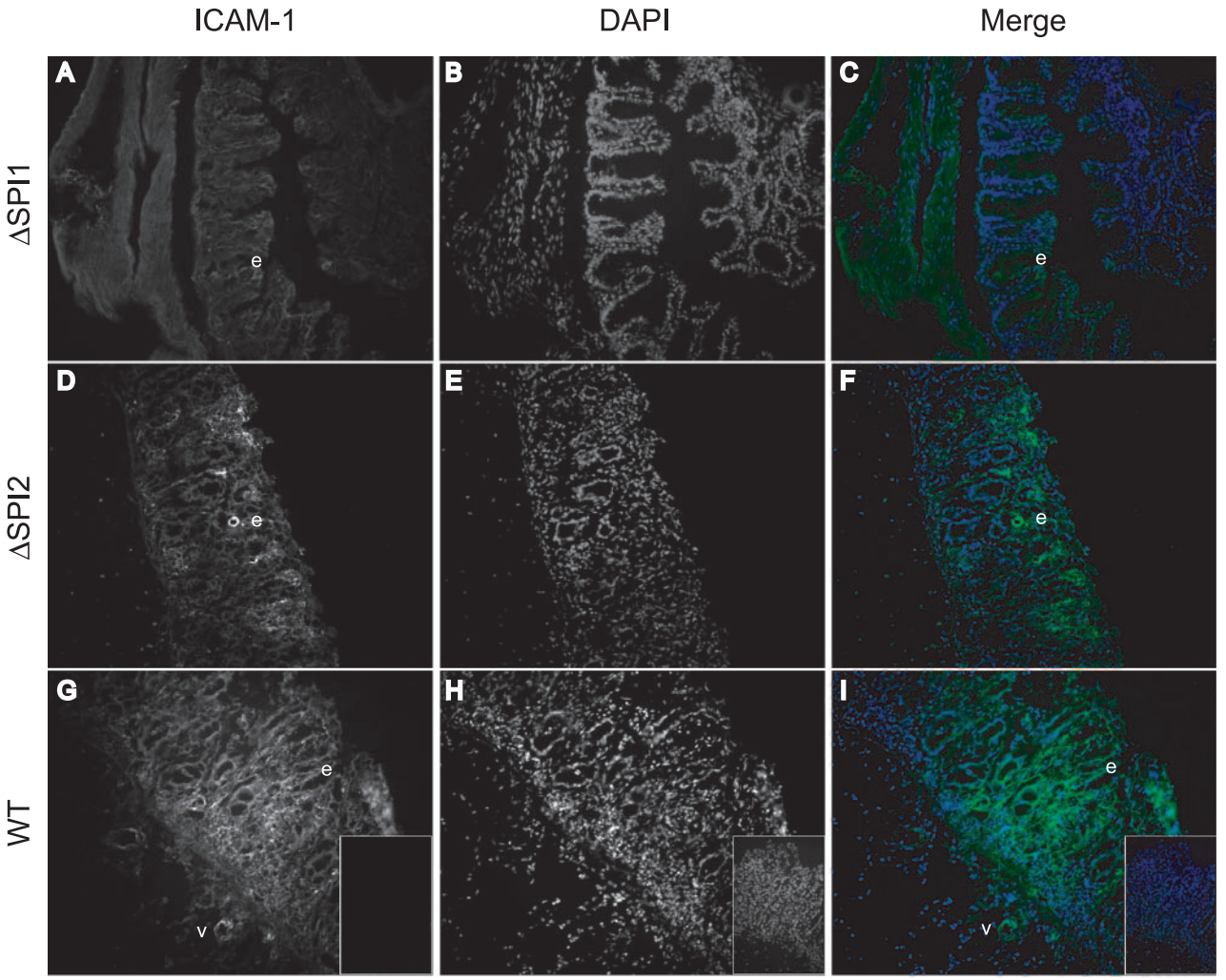

FIG. 3. SPI2 but not SPI1 mutant *S. enterica* serovar Typhimurium induces ICAM-1 and neutrophil recruitment in the intestines of streptomycin-treated mice 48 h after infection. Streptomycin-treated mice were infected with SPI1 (A to C), SPI2 (D to F), or WT (G to I) *S. enterica* serovar Typhimurium for 48 h. Ceca were retrieved, paraformaldehyde fixed, OCT embedded, and cryosectioned prior to staining for ICAM-1 (green in merge) (A, D, and G) and nuclei (DAPI; blue in merge) (B, E, and H) by immunohistochemistry. Isotype control antibody staining of WT-infected mice was not significant (insets). ICAM-1 expression was evident in the epithelium and submucosal vessels of both WT- and SPI2-infected mice but decreased in the latter. Ceca of SPI1-infected animals showed minimal ICAM-1 expression. e, epithelium; v, submucosal vessel. Pictures are representative of eight mice/group and four tissue sections per mouse.

that SPI2 is actively involved in the induction of colitis and typhlitis.

***S. enterica* serovar Typhimurium in the ceca of infected mice is predominantly luminal, and bacterial presence in the mucosa coincides with severe mucosal inflammation.** To assess whether intestinal inflammation was attenuated in SPI2 mutants due to changes in the distribution of the bacteria within the gut, we assessed bacterial localization by immunohistochemistry. Twenty-four hours after streptomycin administration, mice were infected with WT, SPI1, or SPI2 *S. enterica* serovar Typhimurium as described above. Tissues were harvested 48 h after infection and stained for *Salmonella* LPS, actin, and nuclei. As observed previously, mice infected with SPI1, SPI2, and WT *Salmonella* displayed no, moderate, or severe inflammatory pathology, respectively (Fig. 2D, H, and L). Bacterial staining was predominantly confined to the lumen of infected tissues in mice infected with all bacterial strains (Fig. 2, insets). Bacteria that were in close association with the epithelium and mucosa were present in all infected mice. However, infiltration of bacteria into the deep mucosa was confined to areas in which there was moderate or severe inflammation (Fig. 2H and L). Severe inflammation was common in the absence of infiltrating bacteria, suggesting that bacterial penetration deep into the mucosa was a consequence rather than a cause of inflammation. The number of infiltrating bacteria of each strain tested correlated directly with the overall severity of intestinal pathology. Intracellular bacteria were not observed in any infected tissues at this time point.

SPI2 mutants induce ICAM-1 expression and neutrophil recruitment less strongly than WT *S. enterica* serovar Typhimurium. Since SPI2 bacteria were able to associate with the intestinal epithelium, we sought to determine whether the attenuation of typhlitis was due a decreased ability to induce leukocyte recruitment. To do this, mice were treated with

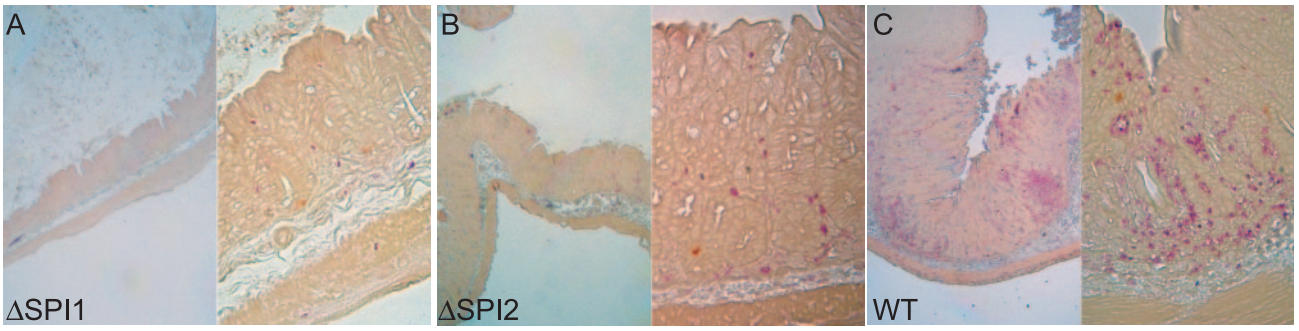

FIG. 4. Neutrophil infiltration is markedly reduced in *S. enterica* serovar Typhimurium-induced typhlitis in the absence of SPI2. Forty-eight hours after infection with SPI1 mutant (A), SPI2 mutant (B), or WT (C) *S. enterica* serovar Typhimurium, ceca were retrieved, fixed, and stained for the neutrophil-specific chloroacetate esterase (red staining; arrows). Neutrophils were rare in ceca infected with SPI1- or SPI2-deficient *S. enterica* serovar Typhimurium (A and B) but abundant in WT-infected tissues. Pictures are representative of eight mice/group and four tissue sections per mouse.

streptomycin and infected as before; after 48 h, ceca were cryosectioned and stained for the CD18-β_2 integrin receptor ICAM-1. In response to infection with WT *S. enterica* serovar Typhimurium, we noted significant ICAM-1 expression in the mucosa and submucosal vasculature, as was previously reported (1). Expression of ICAM-1 was absent from SPI1-infected tissues, confirming that SPI1 activity is essential to induce an inflammatory response in infected intestines. Epithelial cells maintained some ICAM-1 expression in response to SPI2-deficient *Salmonella*, although it was less intense and less extensive than that induced by WT bacteria (Fig. 3D to I). To determine if differences in ICAM-1 expression correlated with differences in neutrophil recruitment, we stained sections of infected ceca with a neutrophil detection kit that detects activity of the neutrophil-specific esterase.

Rare neutrophils were present in all sections but were abundant only in WT-infected ceca (Fig. 4). Occasional small aggregates of neutrophils were present in SPI2-infected ceca, but large aggregates were not observed (Fig. 4B). Infection with WT *Salmonella*, however, resulted in numerous large aggregates and greatly increased single cells in the mucosa, lamina propria, and submucosa (Fig. 4C).

SPI2 mutant *S. enterica* serovar Typhimurium causes transient moderate infectious colitis in streptomycin-treated mice. To test whether the absence of SPI2 type III secretion influences the course of *S. enterica* serovar Typhimurium-induced disease, we orally infected susceptible mice and examined bacterial loads and histopathological changes at 2 and 5 days after infection. A smaller dose (3×10^6) of bacteria was used in these experiments, as WT infection with 10^8 bacteria was fatal before 5 days. Unlike mice infected with WT bacteria, mice infected with SPI2 mutants showed no infection-associated morbidity such as ruffling of fur, wasting, or splenomegaly (Table 2; data not shown). We also observed a dramatic attenuation of intestinal pathology in mice infected with SPI2 mutants compared to WT bacteria. Mice infected with WT *S. enterica* serovar Typhimurium continued to have significant and extensive typhlitis, whereas mice infected with SPI2 mutant bacteria had almost completely recovered from the infection and showed no gross abnormalities and little or no histopathological evidence of active disease (Fig. 5; Table 2).

TABLE 2. Systemic and intestinal pathology at 5 days in WT and SPI2 mutant in serovar Typhimurium-infected mice

Pathological change	Strain[a]		*P* value (*t* test)
	ΔSPI2	WT	
Cecal wt	0.42 ± 0.12	0.22 ± 0.04	0.0036
Submucosal edema (% wall thickness)	16 ± 4	48 ± 9	0.0013
Mucosa (μm)	151 ± 18	271 ± 29	0.0010
Spleen wt	0.12 ± 0.03	0.18 ± 0.02	0.0086

[a] Values shown represent means ± standard deviation for each group.

In addition to significant differences in bacterial load by day 5, WT and SPI2 mutant bacteria were distributed differently by day 5 of infection. While WT bacteria were seen invading tissues extensively by day 5 (Fig. 5D), SPI2 mutant bacteria were exclusively luminal and no mucosal invasion was observed (Fig. 5E).

At 2 days postinfection, bacterial burden in the colon after WT or SPI2 mutant infection was similar. By 5 days, the number of SPI2 mutant S*almonella* organisms was significantly decreased while WT *Salmonella* persisted (Fig. 6A). Bacterial burden in the liver and spleen had also decreased significantly by this time as expected (Fig. 6B and C). These data demonstrate that SPI2 is essential for persistent intestinal disease in *S. enterica* serovar Typhimurium-infected mice.

DISCUSSION

The data presented here are the first demonstration that SPI2 is necessary for complete virulence in a model that recapitulates *S. enterica* serovar Typhimurium-elicited human colitis. Furthermore, our data suggest that SPI2 is necessary for complete intestinal virulence during the bacterium-epithelium interaction and demonstrates that intestinal inflammation is resolved in SPI2 mutant but not WT *S. enterica* serovar Typhimurium-infected mice.

Although there are differences in the overall presentation of murine and human *S. enterica* serovar Typhimurium-elicited intestinal disease, including the absence of diarrhea and the development of systemic typhoid-like infection in mice, this model has numerous advantages over existing models of *Salmonella* enterocolitis, including low cost, availability of mice,

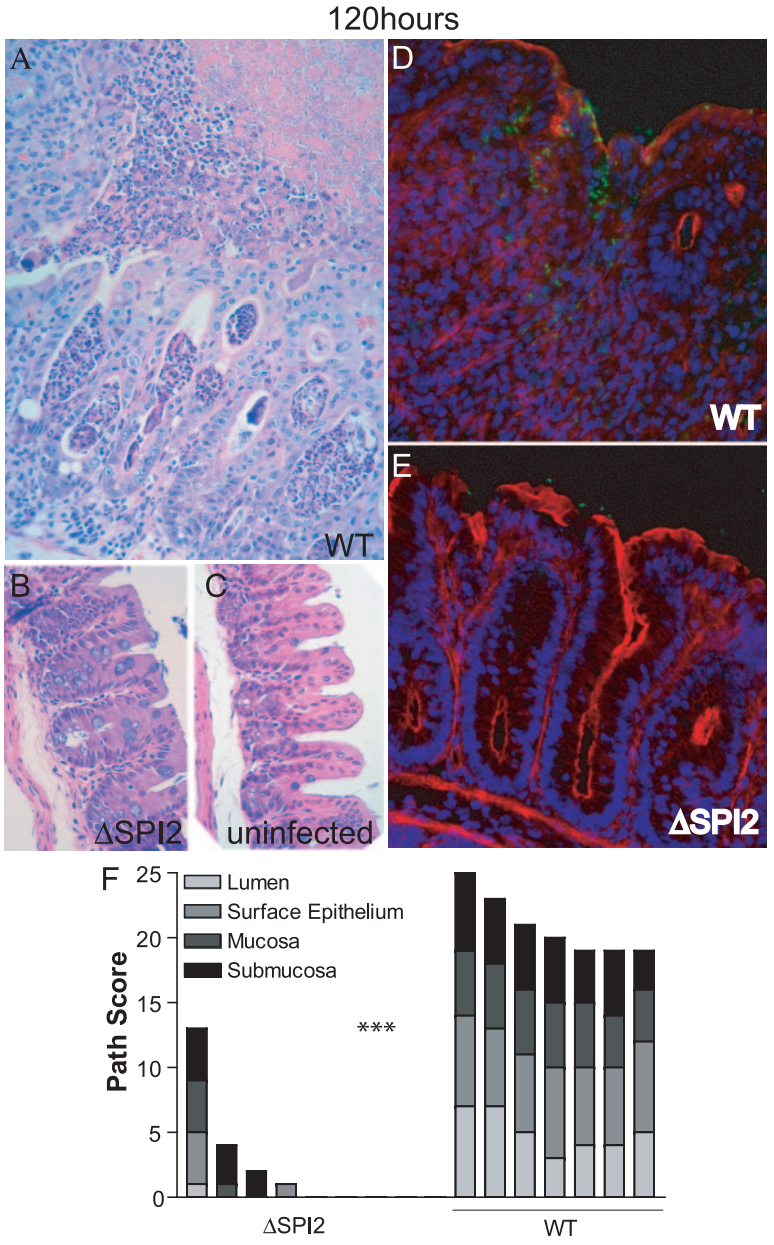

FIG. 5. Cecal inflammation caused by *S. enterica* serovar Typhimurium is controlled by day 5 in the absence of a functional SPI2 TTSS in streptomycin-treated mice. (A to C) Histopathology formalin-fixed, paraffin-embedded sections of ceca were stained with hemotoxylin and eosin 5 days after oral infection with 2×10^6 WT (A) or SPI2 (B) *S. enterica* serovar Typhimurium organisms or after mock infection (LB broth only) (C). Severe inflammatory pathology is evident in WT-infected mice. Pathology is attenuated in SPI2 infected ceca at day 5. (D and E) Evidence of bacterial invasion of the mucosa is present in WT but not SPI2 *S. enterica* serovar Typhimurium-infected animals. Cryopreserved ceca were stained against actin (red), nuclei (blue), and *Salmonella* LPS (green). (F) Histopathology scores show persistent severe pathological changes in WT-infected mice at 5 days but significant recovery in SPI2-infected mice. ***, $P < 0.001$; Mann-Whitney U test. Each bar represents a single mouse. Hematoxylin and eosin images are shown at a magnification of ×400; fluorescent images are shown at a magnification of ×630.

and reproducibility. Like human intestinal disease induced by *S. enterica* serovar Typhimurium, murine inflammation is predominantly colitic with little or no ileal inflammation (4, 17). In contrast, although colitis is observed in cows upon *S. enterica* serovar Typhimurium infection, previously published models of *S. enterica* serovar Typhimurium pathogenesis in cows have focused primarily on inflammatory disease of the ileum. This focus may have arisen from substantial evidence that bacterial invasion in *S. enterica* serovar Typhimurium infection occurs in the M cells of ileal Peyer's patches, a behavior required for systemic virulence (14, 19). Although this may represent an important pathogenic mechanism in murine typhoid, it has not been demonstrated that intestinal invasion per se is essential for intestinal disease. Rather, it has been demonstrated that ileal inflammation requires invasion-associated virulence genes (27, 28) and that intestinal invasion in inoculated ilea occurs in

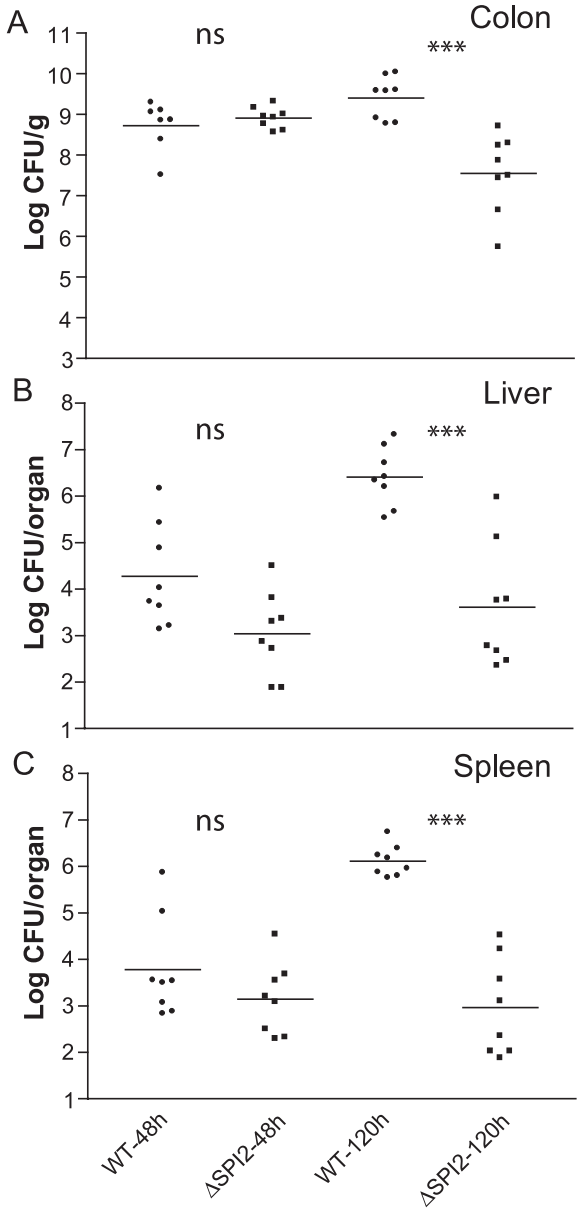

FIG. 6. Bacterial persistence in the intestines, liver, and spleen is significantly diminished between 2 and 5 days after oral SPI2 mutant, but not WT *S. enterica* serovar Typhimurium infection. Mice were infected with 2×10^6 bacteria orally 24 h after oral administration of streptomycin. Intestinal (A) and systemic (B and C) bacterial loads significantly decreased between day 2 and day 5 postinfection with SPI2 *S. enterica* serovar Typhimurium but increased in the liver, spleen, and colon during WT infection. *P* values were determined by Student's *t* test for bacterial load. Bars indicate geometric means. ***, $P < 0.0001$; ns, not significant.

an SPI1-dependent manner (3, 9). It is in this paradigm that conflicting evidence for the involvement of SPI2 in intestinal disease has arisen (3, 7). By exploiting the murine *S. enterica* serovar Typhimurium colitis model, we have demonstrated for the first time that SPI2 is necessary for complete virulence in a model representative of human disease and that SPI2 is necessary for the persistence of infection in murine large intestines.

The SPI2 virulence system is important in a range of bacterial adaptations to the host, including vacuolar remodeling, intracellular survival, and resistance to the host immune response. Although survival of many host defenses is SPI2 dependent, it is not clear whether this role of SPI2 is important in inflammation of the large bowel. The SPI2 dependence of intracellular survival in epithelial cells has been demonstrated in vitro (18), and this may be important in intestinal disease; however, we observed inflammation in this model at a time point at which the vast majority of bacteria were luminal, suggesting that extracellular bacteria may be responsible for intestinal inflammation. Others have shown that the attenuation of intestinal virulence in SPI2 mutant-infected bovine ilea was not accompanied by a decrease in the number of intracellular bacteria (3), and only a small proportion of total intestinal bacterial burden in infected human intestines were intracellular (12).

While it is not clear what role SPI2 plays in intestinal inflammation, our data imply that SPI2 is important for the induction of inflammation at the epithelial interface, rather than in deeper tissues, as ICAM-1 expression, a marker of epithelial inflammation and an inducer of neutrophil recruitment, was diminished in SPI2 mutant infections. The uniform intermediacy of the inflammatory phenotype associated with SPI2 mutant infection suggests a decrease in the dose of inflammatory stimulus in the gut rather than the complete loss of a single virulence strategy. Although bacterial numbers are similar in the entire gut, proinflammatory stimuli such as flagellin may be compartmentalized differently within mice infected with various mutants. Flagellin is an important *S. enterica* serovar Typhimurium inflammatory stimulus (30). *Salmonella* isolates lacking intact flagella are attenuated in this murine colitis model (24), and it has recently been demonstrated that SPI2-mediated vesicular transport is necessary for the transcytosis of flagellin across polarized epithelia (16). The attenuation of cecal inflammation in the absence of SPI2 may be a result of the decreased SPI2-dependent delivery of proinflammatory flagellin to the basolateral epithelium.

In addition to the attenuation of acute cecal inflammation, we have demonstrated that SPI2 mutant *S. enterica* serovar Typhimurium infection is resolved in mice. This is dramatically different from the course of WT infection, in which bacterial colonization of intestinal and systemic sites is maintained over time and intestinal disease continues unresolved, ultimately resulting in fatal infection. Resistance to antimicrobial defenses such as complement and evasion of the phagocyte NADPH oxidase complex within phagocytes is SPI2 dependent (13, 26). While loss of the resistance to soluble antimicrobial defenses may decrease the ability of SPI2-deficient bacteria within the gut to persist while extracellular, failure to survive phagocytic killing may diminish the survival of bacteria within phagocytic cells that have migrated into the intestinal lumen. It is possible, therefore, that SPI2 is necessary for persistence within the intestinal environment itself, without the need for extensive cellular invasion.

We have clearly demonstrated that SPI2 is essential for complete virulence in the large intestines of infected mice. These results parallel the recently published findings of Hapfelmeier et al. (11a). It is important now to carefully consider the dichotomous roles of SPI1 and SPI2 in the intestinal

and/or systemic paradigm of *S. enterica* serovar Typhimurium infection.

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