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Differences in the expression of SPI-1 genes pathogenicity and epidemiology between the emerging *Salmonella enterica* serovar Infantis and the model *Salmonella enterica* serovar Typhimurium

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Background: *Salmonella enterica* serovar Infantis (*S.* Infantis) is one of the ubiquitous serovars of the bacterial pathogen *S. enterica* and recently has been emerging in many countries worldwide. Nonetheless, not much is known about its epidemiology, host adaptation, and virulence.

Methods: Epidemiological and molecular approaches were used together with tissue-culture and mouse models to conduct phenotypic comparison with the model *S. enterica* serovar Typhimurium.

Results: We show that *S.* Infantis is more frequently associated with infections in infants <2 years old and prone to cause significantly less invasive infections than serovar Typhimurium. Moreover, although *S.* Infantis adheres better to host cells and highly colonizes mouse intestines soon after infection, it is significantly less invasive and induces much lower inflammation and disease in vivo than *S.* Typhimurium. These differences were associated with lower expression of *Salmonella* pathogenicity island (SPI) 1 genes in *S.* Infantis than in *S.* Typhimurium.

Conclusions: Our results demonstrate previously unknown differences in the epidemiology, virulence pathway expression, and pathogenicity between two highly abundant *Salmonella* serovars and suggest that native variation in the expression of the SPI-1 regulon is likely to contribute to epidemiological and virulence variation between genetically similar nontyphoidal *Salmonella* serovars.

Keywords. *Salmonella enterica*; salmonellosis; virulence; pathogenicity; host-pathogen interactions; gastroenteritis; *Salmonella*-pathogenicity islands; invasion.

Salmonella enterica subsp. enterica is a gram-negative, ubiquitous pathogen that can infect a wide range of animal and human hosts. This versatile bacterial species comprises >2600 serovars that can differ in their adaptation to various hosts and the disease they cause. In general, *Salmonella* can cause 3 types of diseases in humans: gastroenteritis, enteric (typhoid) fever, and nontyphoid extraintestinal (invasive) disease with bacteremia [1]. The annual estimated global burden of gastroenteritis due to *Salmonella* infections is 78.7 million cases, and each year 59 000 persons lose their lives because of nontyphoidal serovar (NTS) infections, mainly in developing countries [2]. In healthy humans, infection with NTS results in most cases in a localized self-limiting inflammation of the terminal ileum and colon, known as gastroenteritis. Nevertheless, more severe complications, such as invasive infection or bacteremia, may result in about 5% of individuals infected with NTS [3].

Active *Salmonella* invasion into host cells involves the function of a conserved type 3 secretion system (T3SS) encoded on *Salmonella* pathogenicity island (SPI) 1 and numerous translocated effector proteins, which are directly injected into the host cell cytoplasm. The interaction of the *Salmonella* effectors with various host pathways results in rearrangement of the host cell cytoskeleton and destruction of epithelial cell junctions [4]. Invasion of NTS via the intestinal barrier elicits a strong T-helper 1 immune response and recruitment of phagocytes in an interleukin 8–dependent manner in immunocompetent individuals. Neutrophil recruitment, production of reactive oxygen and nitrogen species, synthesis of antimicrobial peptides, and the bactericidal activity of the phagocytes

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effectively prevent dissemination of *Salmonella* to systemic sites and restrict the infection to the mucosa of the ileum and colon [5].

The *Salmonella* serovars Typhimurium and Infantis (*S.* Typhimurium and *S.* Infantis) are among the most prevalent serovars worldwide. In the European Union, *S.* Infantis was ranked third in the prevalence order, after serovars Enteritidis and Typhimurium [6]. In the United States, at 2016 *S.* Typhimurium and *S.* Infantis were ordered third and sixth, respectively, in the occurrence hierarchy, and serovar Infantis presented the largest increase in occurrence (165.8%) since 2006 [7]. From 2008 to 2015, *S.* Infantis was the most predominant serovar isolated from both clinical (human) and poultry sources in Israel [8, 9]. In recent years, the emergence of *S.* Infantis has been further reported in many other countries, including Belgium, France [10], Germany [11], Hungary [12], Honduras [13], Japan [14], and Australia [15], indicating that *S.* Infantis is an emerging cause of gastroenteritis in multiple areas of the developed world.

Considering the high prevalence of *S.* Infantis and its global clinical impact, it is rather surprising that not much is known about the interactions of *S.* Infantis with host cells and its virulence potential. In the current article, we show significant distinctions in the epidemiology, virulence-associated phenotypes, and pathogenicity of *S.* Infantis compared with the model *Salmonella* serovar, *S.* Typhimurium, and we demonstrate that these differences are associated with lower expression of the SPI-1 regulon in *S.* Infantis compared with *S.* Typhimurium.

METHODS

Epidemiology

Salmonellosis is a reportable disease in Israel by law. All microbiology laboratories nationwide are required to submit identified *Salmonella* isolates from all sources to the National *Salmonella* Reference Center, where serological identification according to the–White–Kauffmann–Le Minor scheme [16] is conducted. In the current study, 33 924 clinical (human) and 29 342 poultry *Salmonella* isolates, reported to the National *Salmonella* Reference Center in 2006–2017, were anonymously analyzed. *Salmonella* isolates obtained from normally sterile sites, including blood, urine, and cerebrospinal fluid, were considered extraintestinal infections, whereas stool isolates were defined as gastroenteritis cases.

Bacterial Strains and Primers

Bacterial strains and clinical isolates used in this study are listed in Supplementary Table 1. Primers used in the study are listed in Supplementary Table 2.

Biofilm Formation

The ability of *Salmonella* strains to form biofilm was assessed as reported elsewhere [17] and as explained in the Supplementary Data.

Host Cell Adhesion, Invasion, and Replication

Host cell infection with *Salmonella* strains was performed using the gentamicin protection assay, as described elsewhere [18, 19]. For more details, see Supplementary Data.

Transepithelial Electrical Resistance

Caco-2 cells (2×10^5) were seeded on Transwell permeable supports (Corning) with a pore size of 3 μm. Cells were grown for 21 days until transepithelial electrical resistance (TEER), measured with a Millicell ERS-2 Volt-Ohm meter (Millipore), reached a plateau, and they were infected with *Salmonella* strains at a multiplicity of infection of 10.

Differential Expression of *Salmonella* Genes

RNA was extracted from *Salmonella* cultures grown aerobically to the late logarithmic phase by the Qiagen RNAprotect Bacteria Reagent and the RNeasy mini kit (Qiagen). First-strand complementary DNA synthesis was performed using 200 ng of RNA and the iScript complementary DNA synthesis kit (Bio-Rad Laboratories). Real-time polymerase chain reaction (PCR) reactions and data analysis were performed as described elsewhere [20]. More details are provided in the Supplementary Data.

Salmonella Infection in the Mouse Model

Female C57BL/6 mice (Charles River) were infected at the age of 8 weeks. Food and water were provided ad libitum. Streptomycin (20 mg per mouse) was given by oral gavage 24 hours before infection with 3×10^6 *Salmonella* in 100 μL of HEPES buffer. Control mice were orally gavaged with 100 μL of HEPES buffer. Mice were euthanized, and tissues were harvested aseptically for bacterial enumeration and histopathological evaluation at 1 or 4 days after infection. Tissues were homogenized in 1 mL of phosphate-buffered saline and serial dilutions of the homogenates were plated on MacConkey agar plates to calculate bacterial tissue burdens.

Histology

Tissues were fixed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin. Sections (5 μm) were stained with hematoxylin-eosin (HE). Tissue pathology was scored pathologically as described elsewhere [21].

RESULTS

Epidemiology of S. Infantis and S. Typhimurium infections

To gain new insight into the clinical and epidemiological characteristics of *S.* Infantis, we performed pairwise comparison with the well-studied *S.* Typhimurium, considered the NTS model serovar. To this end, we analyzed 33 924 clinical (human) infections caused by these serovars in Israel during 12 years between 2006 and 2017. Throughout this period, 7838 (23%) and 1654 (4.8%) were culture-confirmed cases of *S.* Infantis and *S.* Typhimurium, respectively. The other dominant serovars

contributing to human salmonellosis were Enteritidis (25%), Virchow (5%), and Hadar (4%). Similarly, of 29 342 poultry-originated *Salmonella* isolates examined during the same period, 7557 (26%) and 732 (2.5%) of the *S.* Infantis and *S.* Typhimurium isolates, respectively, were identified (Figure 1A). These data demonstrate a similar occurrence of serovar Infantis in clinical cases and in poultry isolates and highlight the contribution of this serovar to nontyphoidal *Salmonella* infections in humans and poultry.

Next, we analyzed the association of *S.* Typhimurium and *S.* Infantis infections with patient age. Interestingly, this analysis showed that although both serovars are prevalent among children <10 years old, as the serovar designation "Infantis" implies, *S.* Infantis is indeed more frequently associated with infections in infants <2 years old. In contrast, the proportion of *S.* Typhimurium is significantly higher in children aged 2–10 years old (Figure 1B).

Moreover, *S.* Typhimurium was found to cause >2-fold invasive disease than *S.* Infantis, because 4.2% of all *S.* Typhimurium human infections are extraintestinal, whereas only 1.8% of *S.* Infantis salmonellosis cases are invasive (Figure 1C). These epidemiological observations, which were based on a large cohort of cases, indicate different serovar-dependent clinical characteristics associated with *S.* Infantis compared with *S.* Typhimurium.

Comparison of Virulence-Associated Phenotypes

To identify possible bacterial mechanisms that may be involved in the clinical and epidemiological differences found between serovars Infantis and Typhimurium, we subsequently compared main virulence-associated phenotypes, known to contribute to *Salmonella* pathogenicity. These phenotypes were compared between the well-characterized virulent reference strain of *S.* Typhimurium SL1344 [22] and the *S.* Infantis strain 119944, which has been characterized as a prototypic strain of the clonal emergence of *S.* Infantis [8, 9, 23].

Virulence-associated phenotypes, including biofilm formation (Supplementary Figure 1*A*) and intramacrophages replication in both RAW264.7 (Supplementary Figure 1*B*) and J774 (Supplementary Figure 1C) cells, showed similar capability between *S.* Typhimurium and *S.* Infantis, suggesting that these phenotypes may not contribute to the clinical differences observed between these serovars.

Next, we examined the ability of *S.* Infantis and *S.* Typhimurium to adhere to and invade host cells. Aviv et al [8] showed elsewhere that these phenotypes are affected by the presence of the resistance-virulence *S.* Infantis plasmid pESI; hence, a genetically characterized *S.* Infantis strain that lacks pESI (strain 335-3) was also included in these assays. Adherence and invasion of *S.* Infantis 119944 and 335-3 strains in comparison with *S.* Typhimurium SL1344 were studied using the gentamicin protection assay in 4 cell lines, including chicken fibroblasts DF-1, swine intestinal epithelial cells IPI-2I, human intestinal Caco-2 cells, and HeLa human epithelial cells.

As a negative control for the invasion experiments, we included a *S.* Typhimurium *invA* mutant strain, deficient in nonphagocytic host cell invasion [24]. Overall, adhesion experiments showed significantly higher adhesion of the examined *S.* Infantis strains compared with *S.* Typhimurium SL1344 (Supplementary Figure 2). One exception to this observation was found for the 335-3 strain in the IPI-2I cells that presented an adhesion phenotype similar to that of *S.* Typhimurium. Nonetheless, in sharp contrast to the adhesion

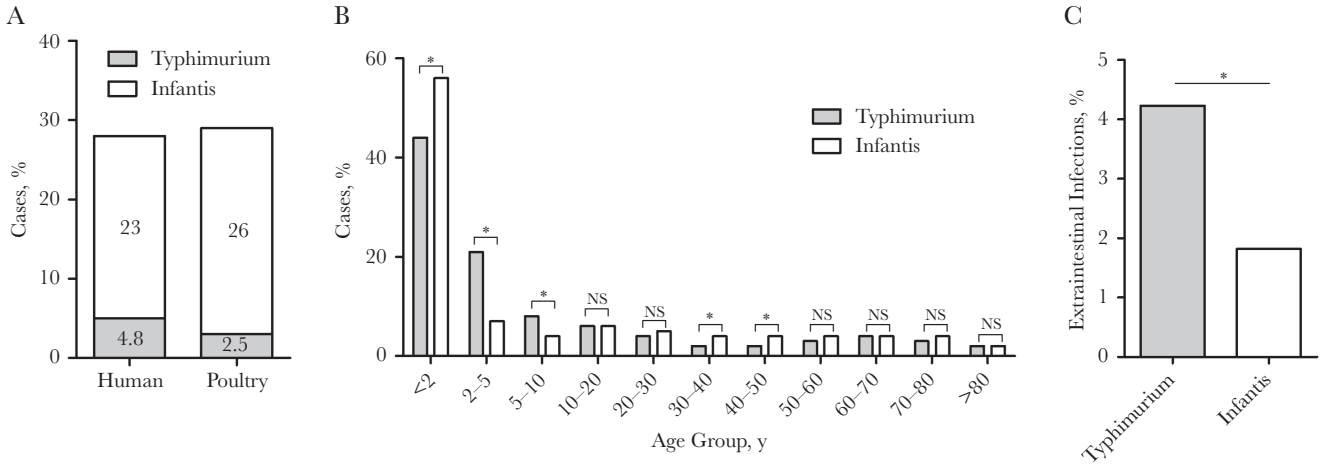

Figure 1. *Salmonella* serovar Infantis (*S.* Infantis) is more frequently associated with infant infections and causes lower rates of invasive salmonellosis than serovar Typhimurium (*S.* Typhimurium). *A,* The occurrence of *S.* Typhimurium and *S.* Infantis among human and poultry samples was determined in 33 924 clinical and 29 342 poultry *Salmonella* isolates, which were reported to and serotyped by the National *Salmonella* Reference Center between 2006 and 2017. *B,* Frequencies of infections caused by *S.* Typhimurium and *S.* Infantis according to patient age, shown as percentage of all infections caused by *S.* Typhimurium (n = 1654) or *S.* Infantis (n = 7838). Statistical significance was calculated based on the *z* score test for 2 population proportions with 2-tailed hypothesis. *C,* Frequencies of extraintestinal infections caused by *S.* Typhimurium and *S.* Infantis, shown as proportion of all infections caused by the serovar. Statistical significance was calculated as in *B.* Abbreviation: NS, not significant. **P* < .001.

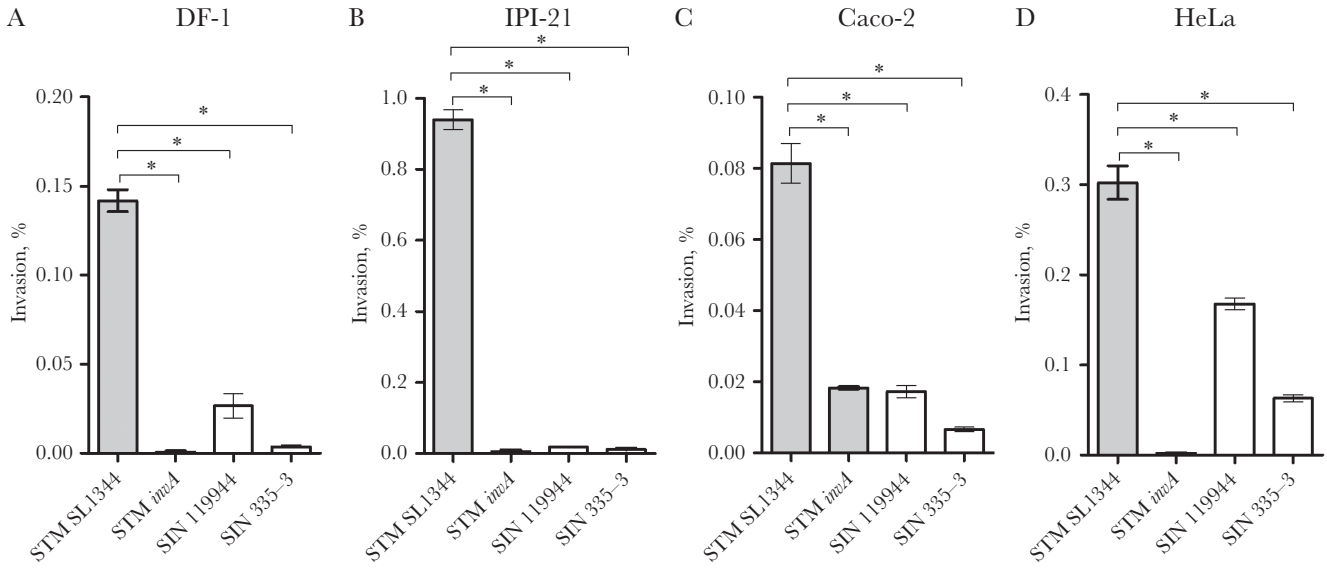

Figure 2. *Salmonella* serovar Infantis (*S.* Infantis) is less invasive in host cells than serovar Typhimurium (*S.* Typhimurium). *S.* Typhimurium SL1344 and *S.* Infantis strains 119944 (pESI positive) and 335-3 (pESI negative) were grown in Luria-Bertani (LB) broth at 37°C to the late logarithmic phase and used to infect chicken fibroblasts DF-1 (*A*), porcine small intestines epithelial cells IPI-2I (*B*), human colonic epithelial Caco-2 cells (*C*), and human epithelial HeLa cells (*D*). Invasion was determined using the gentamicin protection assay and is calculated as the percentage of intracellular bacteria (colony-forming units [CFUs]) recovered at 2 hours after infection from the total number of CFUs in the initial inoculum used to infect the cells. Graph bars represent the mean and standard error of the mean of 4–8 biological replicates. Analysis of variance with Dunnett multiple-comparison test was used to determine differences between data sets. *$P < .001$.

phenotype, in all of these cell lines, *S.* Typhimurium exhibited significantly higher levels of host cell invasion than *S.* Infantis strains (Figure 2).

To confirm the lower invasion of *S.* Infantis compared with *S.* Typhimurium, we studied the invasion of these strains into polarized Caco-2 cells and compared the TEER 1.5 hours after infection. *S.* Typhimurium infection induced a 3.5-fold stronger decrease in TEER than infection with either *S.* Infantis strain, indicating significantly milder loss of epithelial polarity by *S.* Infantis (Figure 3A). Staining of zonula occludens 1 protein, found in tight junctions, demonstrated higher disruption of tight junctions in cells infected with *S.* Typhimurium than in those infected with *S.* Infantis 119944 or *S.* Infantis 335-3 (Figure 3B). Collectively, these results indicate that both pESI-positive and pESI-negative *S.* Infantis strains are less disruptive to the integrity of the epithelium than *S.* Typhimurium in vitro.

Comparison of Virulence In Vivo

To investigate possible differences in virulence between *S.* Infantis and *S.* Typhimurium, we implemented the colitis mouse model, and infected groups of C57BL/6 mice with 3×10^6 CFUs of *S.* Typhimurium SL1344 and with *S.* Infantis 335-3 and 119944 strains. Interestingly, similar levels of colonization by the *S.* Infantis and *S.* Typhimurium strains were observed in the cecum, colon, and ileum 1 day after infection (Supplementary Figure 3); however, at 4 days after infection, the bacterial burden of *S.* Typhimurium was 3–5 orders of magnitude higher than the *S.* Infantis 119944 strain at these sites (Figure 4A). Similarly, although systemic organs, including spleen, liver and mesenteric lymph nodes, were highly colonized by *S.* Typhimurium, they were only poorly colonized by *S.* Infantis (Figure 4B). These results indicate that initial colonization of mouse intestines is similar for *S.* Infantis and *S.* Typhimurium. However, during later stages of infection, *S.* Infantis colonization is compromised and *S.* Infantis bacteria are cleared. In contrast, *S.* Typhimurium can establish stable colonization, followed by bacterial dissemination to systemic sites.

Subsequently, we assessed the histopathological changes in the ceca of mice 4 days after infection with these *Salmonella* strains. Tissues infected with *S.* Typhimurium were highly inflamed, demonstrating severe submucosal edema, crypt destruction and crypt abscesses, epithelial desquamation and ulceration, and massive inflammatory cell infiltrates in the mucosa and submucosa. In contrast, *S.* Infantis 119944 induced milder inflammation with less severe submucosal edema, less epithelial destruction, and fewer invading inflammatory cells. As expected owing to the absence of pESI, infection with *S.* Infantis 335-3 resulted in very mild disease, characterized by mild epithelial damage and only a few infiltrating immune cells (Figure 4C and 4D).

Higher inflammation is also associated with shrinking of the cecum and therefore lower cecum weight. Although mice infected with *S.* Infantis had only a small (335-3 strain) or moderate (119944 strain) decrease in cecum weight compared with uninfected mice, the cecum weight loss was significantly more severe in mice infected with *S.* Typhimurium (Supplementary Figure 4).

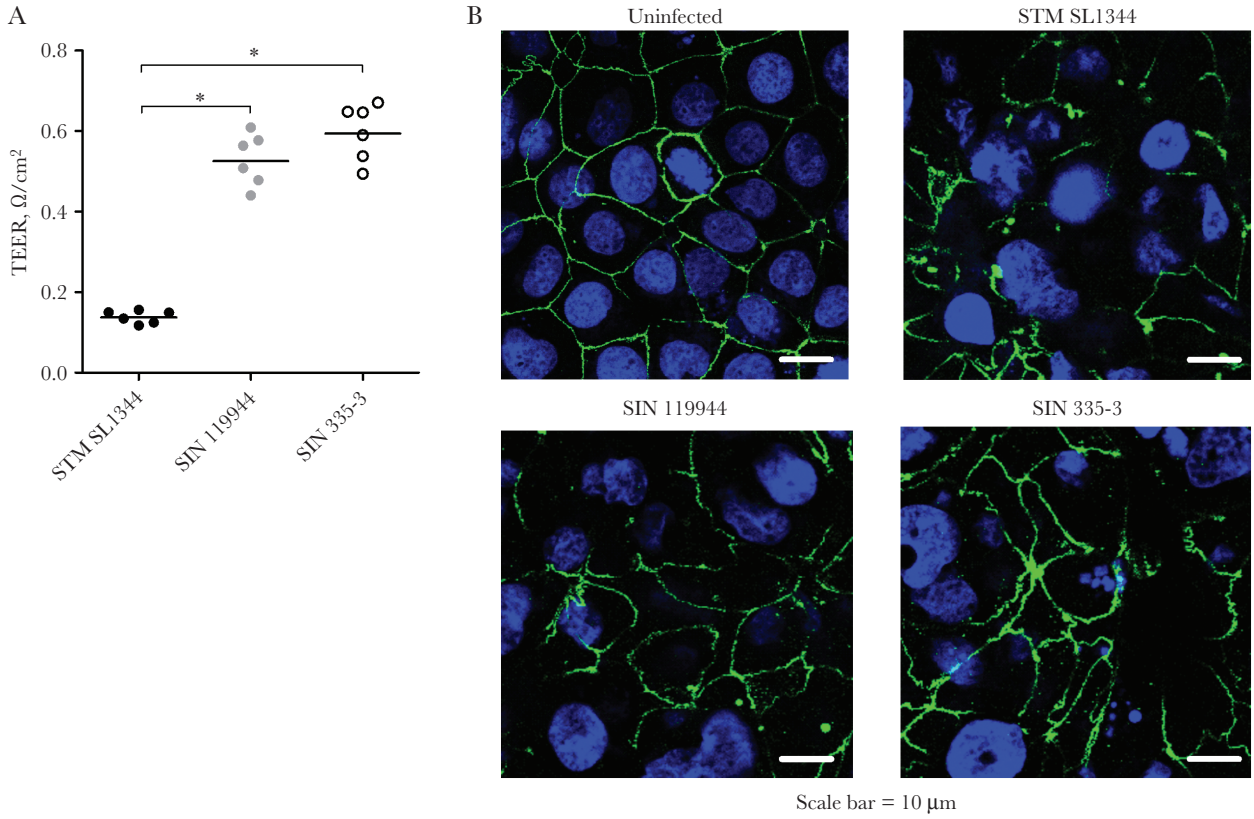

Figure 3. *Salmonella* serovar Infantis (*S.* Infantis) disrupts epithelial cell polarity to a lesser extent than serovar Typhimurium (*S.* Typhimurium). A, *S.* Typhimurium SL1344 and *S.* Infantis strains 119944 and 335-3 were grown in Luria-Bertani (LB) medium to the midlogarithmic phase and were used to infect polarized Caco-2 cells at a multiplicity of infection of 10. The integrity of the epithelial monolayer was determined 1.5 hours after infection and is shown as the change in transepithelial electrical resistance (TEER) from the time of infection. *$P < .001$. *B*, Uninfected and *Salmonella*-infected polarized Caco-2 cells were stained with anti–zonula occludens 1 (ZO-1) antibodies and Alexa Fluor 488–labeled secondary antibodies (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Note the disruption of the ZO-1 staining in *S.* Typhimurium–infected cells compared with the milder disruption in cells infected with either *S.* Infantis strain. (Original magnification, ×630; scale bar, 10 μm.)

To investigate the level of inflammation caused by *S.* Typhimurium and *S.* Infantis in the mouse model, the expression of the proinflammatory cytokines were determined in the cecum of uninfected and infected mice at day 4 after infection. Noticeably, the transcription of *Tnfa* (Figure 5A), *Ifng* (Figure 5B), *Il6* (Figure 5C), and *Ccl2* (Supplementary Figure 5) was higher in the ceca of mice infected with *S.* Typhimurium than in those infected with either *S.* Infantis strain, indicating much higher levels of inflammation with serovar Typhimurium than with Infantis.

A histopathological hallmark of NTS-induced inflammation is the early influx of neutrophils to the site of infection [25]. Thus, we used staining for myeloperoxidase to identify neutrophils in the cecum of mice. Although we observed a massive infiltration of neutrophils in the ceca of mice infected with *S.* Typhimurium, only a few myeloperoxidase-positive cells could be found in the ceca of mice infected with *S.* Infantis 119944 and none in the ceca of mice infected with *S.* Infantis 335-3 (Figure 5D). Cumulatively, the data presented in Figures 4 and 5 clearly show that *S.* Typhimurium infection in the mouse induces more severe disease, characterized by higher pathology, and greater inflammatory response than *S.* Infantis.

Expression Levels of SPI-1 Genes

Because the T3SS-1–translocated effectors are known to play key roles in *Salmonella* invasion and intestinal inflammation [4, 26], we compared in silico the distribution of all known T3SS-1–translocated effectors, involved in *Salmonella* invasion and inflammatory response (AvrA, SipA, SipB, SipC, SipD, SptP, SopA, SopB/SigD, SopD, SopE, SopE2, SteA, and SlrP) in *S.* Infantis versus *S.* Typhimurium. With the exception of SopE, which is not encoded in the *S.* Infantis genome but is also absent from the genome of non-SL1344 virulent *S.* Typhimurium strains (eg, 14028S), all other SPI-1 effector genes were found to be intact and highly conserved between serovars Typhimurium and Infantis (Supplementary Table 3).

Given the similar SPI-1 effectors repertoire, we speculated that possible variations in their expression might contribute to the differences in virulence between *S.* Infantis and *S.* Typhimurium. To test this possibility experimentally, we used

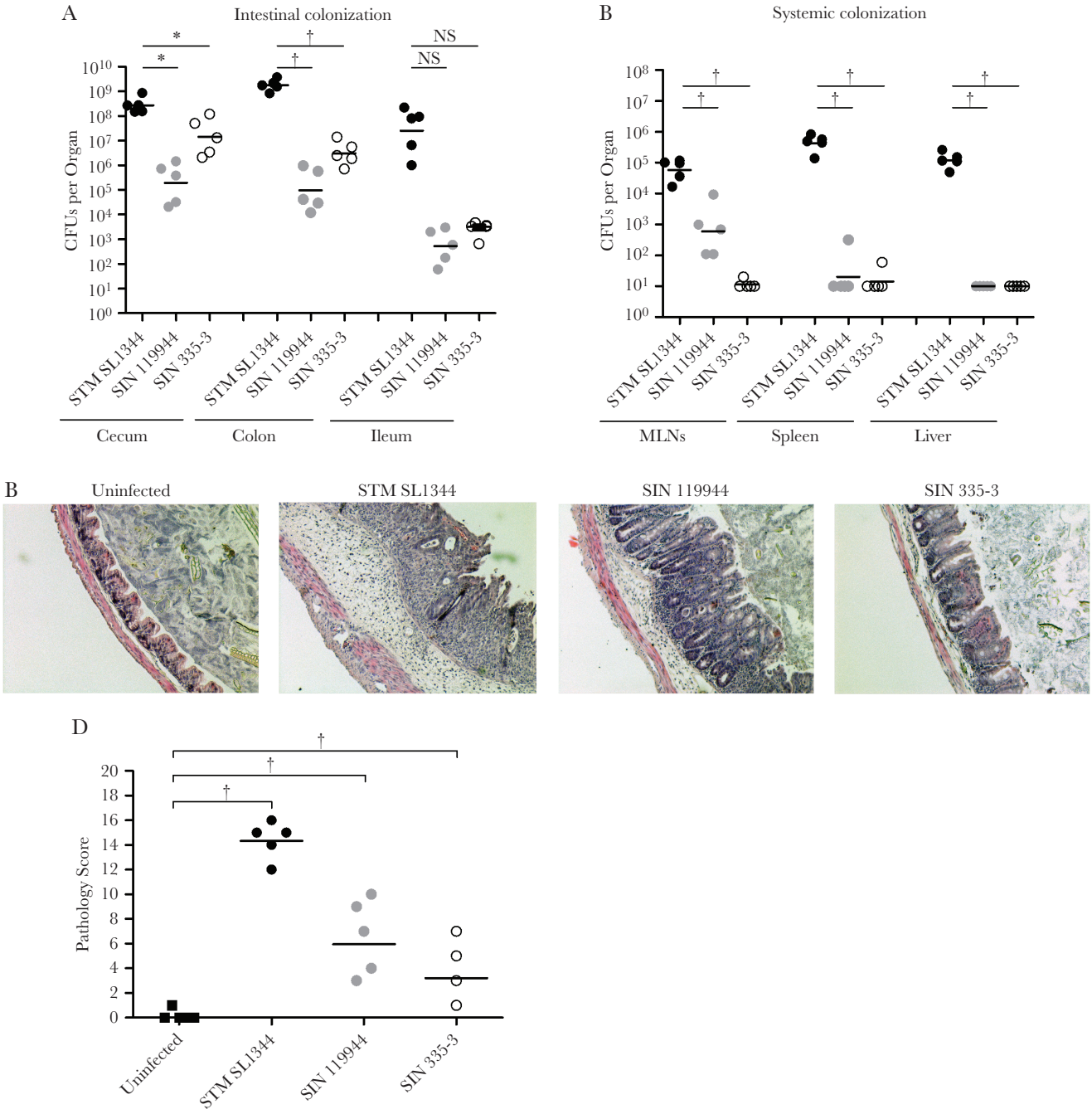

Figure 4. *Salmonella* serovar Infantis (*S.* Infantis) causes milder disease than serovar Typhimurium (*S.* Typhimurium) in mice. Groups of 8-week old female C57BL/6 mice were treated with streptomycin 1 day before infection and inoculated orally with 3×10^6 CFUs of *S.* Typhimurium SL1344 (*black circles*), *S.* Infantis 119944 (*gray circles*) and *S.* Infantis 335-3 (*open circles*). At day 4 after infection, mice were euthanized and *Salmonella* colonization of intestinal organs (*A*) and systemic sites (*B*) was determined. *C,* Hematoxylin-eosin–stained cecal sections were analyzed for histopathological changes. Representative micrographs at an original magnification of ×100 are shown. *D,* Histopathological scoring (0–25) of cecum sections of uninfected and *Salmonella*-infected mice. The geometric mean is shown as a horizontal line, and analysis of variance with Dunnett multiple-comparison test was used to determine differences between *S.* Typhimurium and *S.* Infantis infections. Abbreviation: MLNs, mesenteric lymph nodes; NS, not significant. *$P < .05$; †$P < .001$.

semiquantitative reverse-transcription PCR (RT-PCR) to determine the expression of 3 SPI-1 regulatory genes, *hilA*, *invF*, and *invH*, and the transcription of 2 SPI-1–translocated effectors, *sipB* and *sopB*, in *S.* Typhimurium SL1344 and *S.* Infantis 119944 and 335-3 strains. As shown in Figure 6A, all tested SPI-1 genes had lower expression levels in *S.* Infantis than in *S.* Typhimurium. The transcription levels of *rpoD*, encoding the vegetative sigma factor ($\sigma^{D/70}$) of *Salmonella* that was included as a SPI-1–independent control, showed similar levels of expression in all strains. In agreement with these results, quantitative

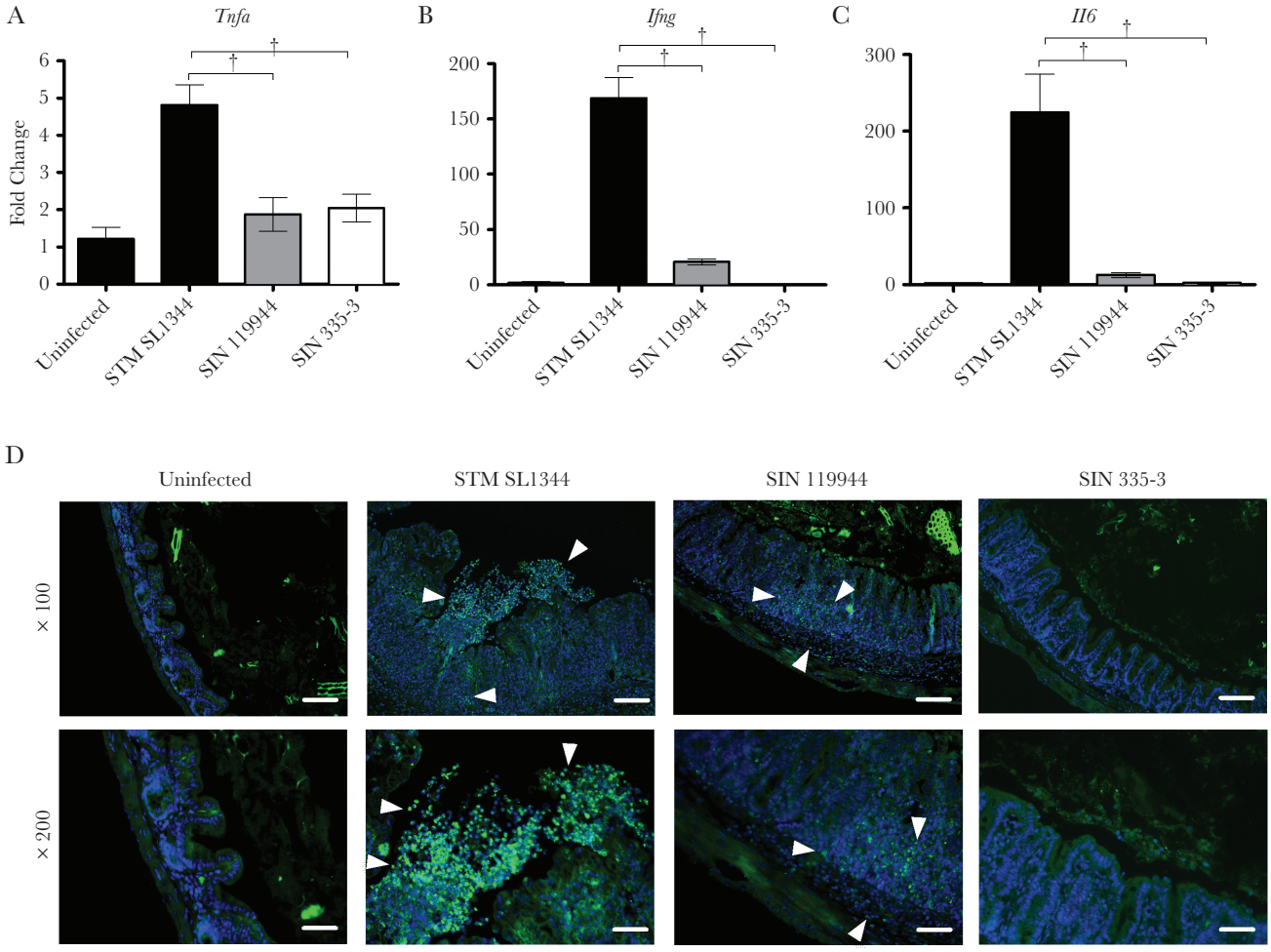

Figure 5. *Salmonella* serovar Infantis (*S.* Infantis) infection provokes lower expression of proinflammatory cytokines in vivo than serovar Typhimurium (*S.* Typhimurium). RNA was isolated from cecum tissues of mice infected for 4 days. Extracted RNA was reverse-transcribed, and expression of cytokines *Tnfa* (*A*), *Ifng* (*B*), and *Il6* (*C*) was analyzed by means of quantitative reverse-transcription polymerase chain reaction (RT-PCR) and normalized to the housekeeping gene *Gapdh*. Note the strong induction of these cytokines by *S.* Typhimurium infection compared with the much milder induction by infection with the *S.* Infantis strains (n = 5 mice per group). Data were analyzed using 1-way analysis of variance with Tukey posttest. **P* < .01; †*P* < .001. *D,* Cecum tissue sections were stained with antibodies against myeloperoxidase (MPO) and secondary Alexa Fluor 488–labeled antibodies to visualize neutrophils (*green*). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). Note the massive infiltration of neutrophils (shown by arrowheads) in the ceca of mice infected with *S.* Typhimurium, whereas only few MPO-positive cells could be found in ceca of mice infected with *S.* Infantis 119944 and none were found in the ceca of mice infected with *S.* Infantis 335-3 (n = 5 mice per group). One representative image for each group is shown. Scale bars represent 100 µm (*upper row*) and 50 µm (*lower row*).

real-time RT-PCR demonstrated that the expression of these 5 SPI-1 genes was 20–100-fold lower in the *S.* Infantis strains than in *S.* Typhimurium (Figure 6B).

To further examine whether the lower levels of SPI-1 gene expression in *S.* Infantis are not specific to the examined strains and represent differences on the serovar level, we determined the expression of *hilA*, encoding the master regulator of SPI-1 genes [27]. This analysis included 2 *S.* Typhimurium laboratory strains (SL1344 and 14028S) and 12 low-passage clinical isolates that were compared with 14 clinical *S.* Infantis isolates (Supplementary Table 1). As seen in Figure 6C, although intraserovar variation in *hilA* transcription exists (mainly among *S.* Infantis strains), the mean expression of *hilA* in the *S.* Infantis strains was significantly (19-fold) lower than its expression in *S.* Typhimurium. This was especially pronounced in the clinical isolates of *S.* Typhimurium that expressed even higher *hilA* levels than the laboratory strains SL1344 and 14028S (Figure 6C). These results suggested that serovars Infantis and Typhimurium differ in their native expression levels of SPI-1 genes.

Next, we investigated whether the differences in *hilA* expression between *S.* Typhimurium and *S.* Infantis also occur in the context of in vivo infection. Two groups of C57BL/6 mice were infected with 3×10^6 CFUs of *S.* Typhimurium SL1344 and *S.* Infantis 119944. One day after infection, the mice were euthanized, and total RNA was isolated from their ceca. Quantitative RT-PCR was subsequently used to determine *hilA* expression relative to the house-keeping gene *rpoD*. Concurring with the in vitro data, we

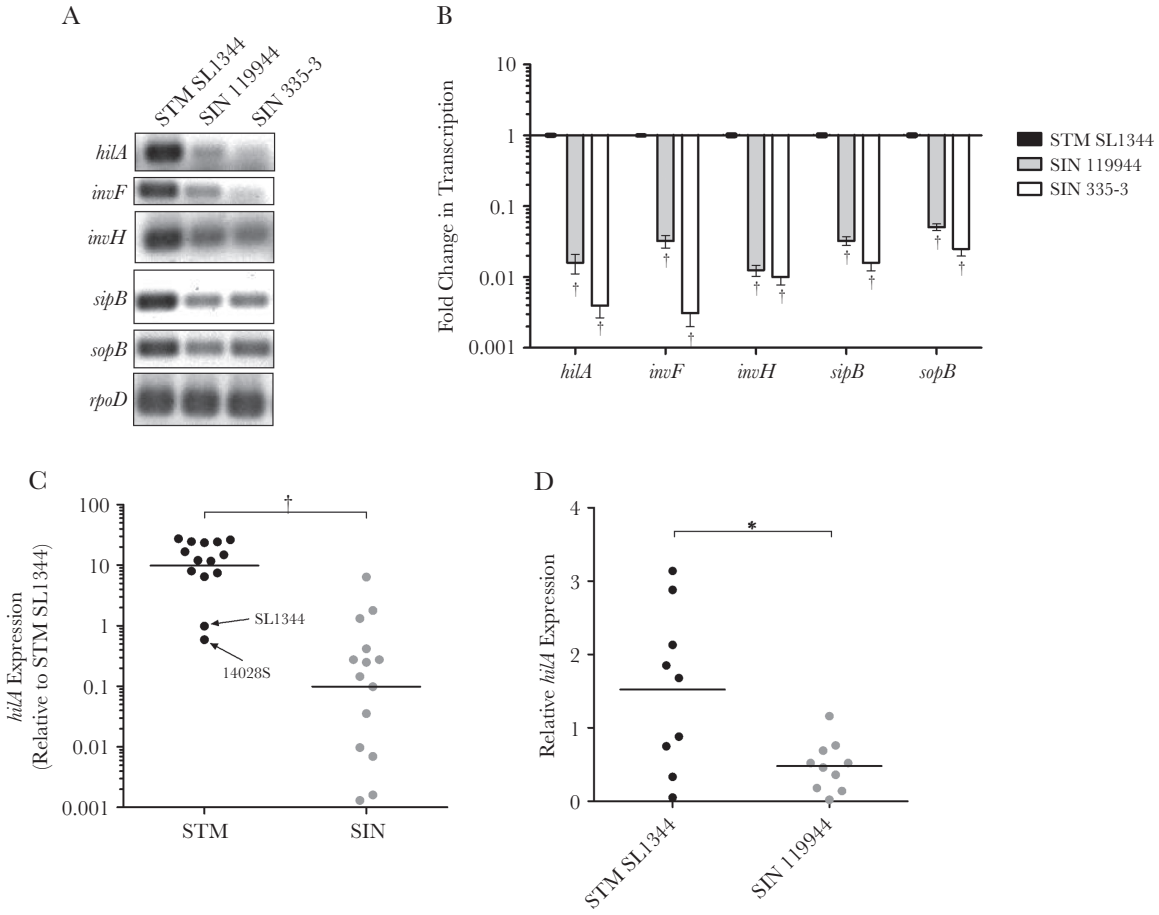

Figure 6. *Salmonella* serovar Infantis *(S.* Infantis) expresses lower levels of *Salmonella* pathogenicity island (SPI) 1 genes than serovar Typhimurium (*S.* Typhimurium). *A,* RNA was harvested from *S.* Typhimurium SL1344 and from *S.* Infantis strains 119944 and 335-3 cultures grown aerobically to the late logarithmic phase at 37°C in Luria-Bertani (LB) medium (SPI-1–inducing conditions). *Salmonella* purified RNA was subjected to semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) for 5 SPI-1 genes (*hilA, invF, invH, sipB,* and *sopB*) and *rpoD* as a SPI-1–unrelated control. *B,* Quantitative RT-PCR was conducted to determine the fold change in the expression of SPI-1 gene transcripts (normalized to *rpoD*) in *S.* Infantis strains relative to their expression in *S.* Typhimurium. The indicated values represent the mean and the standard error of the mean of 9 RT-PCR replicates from 2 independent RNA extractions. *C,* The expression of *hilA* in 14 *S.* Typhimurium isolates (the laboratory strains SL1344 and 14028S, and the clinical isolates 93561, 88359, 98001, 130100, 115043, 96172, 129377, 138741, 74701, 129307, 143789, and 144578), and 14 *S.* Infantis clinical isolates (119944, 335-3, 15467, 153959, 119815, 154136, 121102, 90206, 1126–71233, 120100, 90205, 120029, 120314, and 121135) was determined relative to its expression in *S.* Typhimurium SL1344 (normalized to *rpoD*). Each dot shows the mean of 3 RT-PCR reactions of 1 isolate, and the geometric mean of each serovar is shown by the horizontal line. Arrows indicate the 2 laboratory strains of *S.* Typhimurium (SL1344 and 14028S). *D,* Total RNA was extracted from the cecum of 10 mice infected with 3×10^6 CFUs of *S.* Typhimurium SL1344 and *S.* Infantis 119944 at 1 day after infection. Expression of *hilA* was quantified by quantitative RT-PCR (normalized to *rpoD* and 16S ribosomal RNA genes). Statistical significance was calculated using 2-tailed *t* tests. **P* < .05; †*P* < .001.

found significantly higher expression of *hilA* in the mice infected with *S.* Typhimurium than in those infected with *S.* Infantis (Figure 6D). These results demonstrated that *S.* Infantis strains exhibit lower expression of *hilA* and other SPI-1 genes than *S.* Typhimurium strains and that these differences might contribute to the lower invasion of *S.* Infantis into host cells.

To further examine this hypothesis, we cloned the *hilA* gene under an arabinose-inducible promoter and studied SPI-1 gene expression and *S.* Infantis invasion into HeLa cells. Remarkably, in the presence of 50 mmol/L arabinose, SPI-1 gene expression was increased 50–430-fold in *S.* Infantis carrying this construct (Figure 7A). Accordingly, under SPI-1–directed induction, the invasion of *S.* Infantis into HeLa cells increased up to 23-fold in a dose-dependent manner (Figure 7B), whereas induction of *hilA* in *S.* Typhimurium only slightly improved its invasion (Figure 7C). These results support the notion that suboptimal SPI-1 gene expression in *S.* Infantis contributes to the lower host cell invasion of this serovar in vitro and may play a role in the milder virulence of *S.* Infantis in vivo.

DISCUSSION

By using a systematic comparison with *S. enterica* serovar Typhimurium, we established that *S.* Typhimurium and *S.* Infantis differ in their pathogenicity in vitro and in vivo and in the expression of at least 1 major virulence regulon. Additional epidemiological and clinical differences found between serovars Typhimurium and Infantis revealed that *S.* Infantis is more

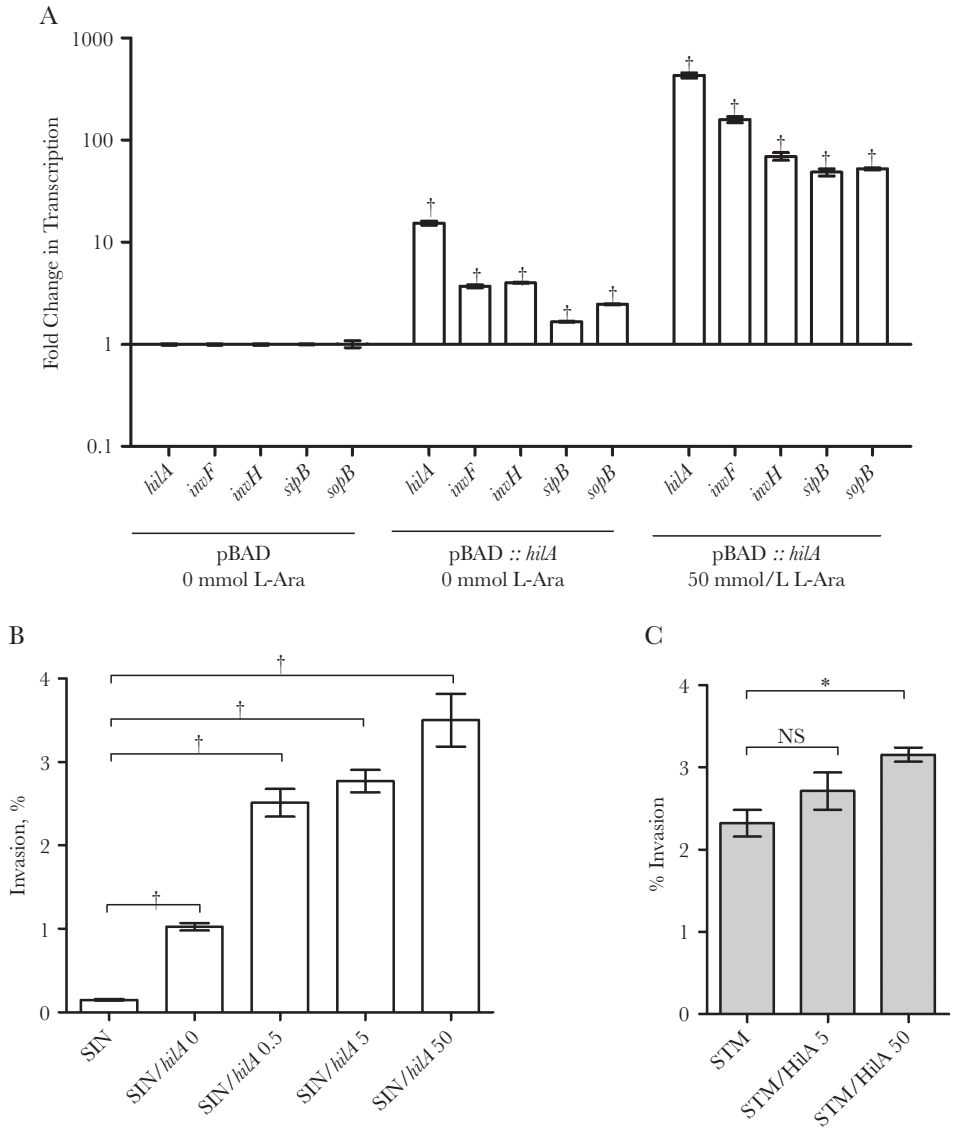

Figure 7. Induction of *Salmonella* pathogenicity island (SPI) 1 genes in *Salmonella* serovar Infantis (*S.* Infantis) increases its invasion to host cells. *A,* Total RNA was harvested from *S.* Infantis 119944 carrying the empty vector pBAD18 or pBAD::*hilA* grown in Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin in the presence or absence of 50 mmol/L L-arabinose (L-Ara) to the late logarithmic phase and was subjected to quantitative reverse-transcription polymerase chain reaction (RT-PCR). The fold change in the abundance of SPI-1 genes (*hilA, invF, invH, sipB,* and *sopB*) transcripts (normalized to *rpoD*) in *S.* Infantis harboring pBAD::*hilA* is shown relative to their expression in *S.* Infantis carrying the empty vector pBAD18. The indicated values represent the mean and the standard error of the mean (SEM) of 3 independent RT-PCR experiments. *B, S.* Infantis 119944 (SIN) and this strain carrying pBAD::*hilA* were grown to the late log phase in LB medium supplemented with 0, 0.5, 5, or 50 mmol/L arabinose and used to infect HeLa cells. Percentage of *Salmonella* invasion at 2 hours after infection from the infected inoculum is shown. *C, Salmonella* serovar Typhimurium (*S.* Typhimurium) SL1344 (STM) and this strain carrying pBAD::*hilA* were grown to the late log phase in LB medium supplemented with 5 or 50 mmol/L arabinose and used to infect HeLa cells. The percentage of *Salmonella* invasion 2 hours after infection from the infected inoculum is shown. Analysis of variance with Dunnett multiple-comparison test was implemented to compare the invasion of the different strains. The results show the mean and SEM of ≥4 biological replicates in 1 of 3 representative experiment. Abbreviation: NS, not significant. *$P < .05$; †$P < .001$.

frequently associated with infections in infants <2 years old and prone to cause less invasive disease than serovar Typhimurium. *S. enterica* is a highly diverse species containing >2600 defined serovars. Clinically, these serovars are divided into typhoidal serovars and NTSs; however, this distinction may represent an oversimplification of the ecological and clinical diversity of this bacterial species. Marzel et al [28] showed elsewhere that several NTSs, including 9,12:l,v:-, cause extraintestinal infections more often than others. Similarly, serovars Dublin and Choleraesuis, which are considered host-adapted serovars to cattle and swine, respectively, are known to cause bacteremia in humans more frequently than other NTS serovars [29, 30]. Taken together, these data demonstrate that NTSs differ in their clinical and epidemiological characteristics despite their high genetic similarity.

One possible source of variation that can affect virulence and clinical associated phenotypes of *Salmonella* serovars

is the expression pattern of virulence pathways such as SPI-1. Down-regulation of SPI-1 encoded genes and the motility regulon have been shown to play a key role in the noninflammatory nature of both *S.* Typhi [31–33] and *S.* Paratyphi A [20, 34] infections. Moreover, it was shown that differences in invasiveness of *S.* Typhimurium strains are also associated with heterogeneity in SPI-1 gene expression [35], suggesting that the variable expression of SPI-1 genes may contribute to the diverse invasive phenotype presented by different salmonellae.

Considering the fundamental role of SPI-1 in host-cell invasion and *Salmonella*-mediated inflammation [36, 37], it is expected that serovars or strains that naturally express suboptimal levels of SPI-1 effectors will induce milder inflammation and will cause less invasive disease. In the current analysis, we showed that serovars Infantis and Typhimurium carry a similar repertoire of SPI-1 effectors, yet the former expresses significantly lower levels of SPI-1 genes. Associated with this difference, we showed that *S.* Infantis is less invasive, whereas directed induction of SPI-1 expression greatly improved the ability of *S.* Infantis to enter nonphagocytic host cells. Moreover, using the colitis mouse model, we clearly demonstrated that although *S.* Infantis and *S.* Typhimurium colonize the mouse intestines to a similar extent at an early time point after infection, *S.* Infantis causes much milder disease and elicits a much lower inflammatory response than *S.* Typhimurium. This was exhibited by lower expression of several important proinflammatory cytokines and poorer recruitment of neutrophils in *S.* Infantis infected mice.

Taken together, our data suggest that lower expression of SPI-1 genes by *S.* Infantis than by *S.* Typhimurium results in less efficient host cell invasion and is likely to contribute to a milder inflammatory response and lower infiltration of neutrophils and other inflammatory cells required for *Salmonella* dissemination to the blood system. Thus, *S.* Infantis is expected to cause fewer extraintestinal infections. Indeed, this model is in good agreement with our new clinical findings showing less invasive infections caused by *S.* Infantis than by *S.* Typhimurium in humans. In a broader perspective, our results suggest that serovar-dependent variation in the expression of the SPI-1 regulon, which has been overlooked so far, may contribute to the epidemiological and clinical differences seen between genetically similar *S. enterica* serovars.

SUPPLEMENTARY DATA

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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