Humanized nonobese diabetic-*scid IL2ry^{null}* mice are susceptible to lethal *Salmonella* Typhi infection

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Salmonella enterica serovar Typhi, the cause of typhoid fever, is host-adapted to humans and unable to cause disease in mice. Here, we show that *S. Typhi* can replicate in vivo in nonobese diabetic (NOD)-*scid IL2ry^{null}* mice engrafted with human hematopoietic stem cells (hu-SRC-SCID mice) to cause a lethal infection with pathological and inflammatory cytokine responses resembling human typhoid. In contrast, *S. Typhi* does not exhibit net replication or cause illness in nonengrafted or immunocompetent control animals. Screening of transposon pools in hu-SRC-SCID mice revealed both known and previously unknown *Salmonella* virulence determinants, including *Salmonella* Pathogenicity Islands 1, 2, 3, 4, and 6. Our observations indicate that the presence of human immune cells allows the in vivo replication of *S. Typhi* in mice. The hu-SRC-SCID mouse provides an unprecedented opportunity to gain insights into *S. Typhi* pathogenesis and devise strategies for the prevention of typhoid fever.

pathogenesis | typhoid | virulence | animal model | bacterial infections

Typhoid fever is a disease of antiquity that continues to exact a considerable human toll today. The World Health Organization estimates that more than 16,000,000 new cases of typhoid each year result in ~600,000 deaths (1). Typhoid is caused by *Salmonella enterica* serovar Typhi (*S. Typhi*), a Gram-negative bacterium transmitted through contaminated food or water. Humans are the only known reservoir for *S. Typhi*. Multidrug-resistant strains have created new challenges for typhoid treatment (2). Neither vaccine currently available for typhoid prevention is completely effective, with protection rates varying from 60% to 80% (3). The Vi parenteral vaccine has a high incidence of local adverse reactions, fails to induce mucosal responses, does not elicit a booster effect, and induces relatively short-lived protection. The Ty21a oral vaccine can elicit durable responses (4) but does not withstand storage under adverse conditions (5) and requires multiple doses.

Much has been learned about *Salmonella* pathogenesis in recent decades (6). *Salmonella* is distinguished from other enteric bacteria principally on the basis of acquired DNA in the form of genomic islands, smaller islets, plasmids, and bacteriophages. In particular, a type III secretory system (T3SS) encoded by *Salmonella* Pathogenicity Island-1 (SPI-1) allows *Salmonella* to invade host epithelial cells, induce intestinal inflammation, and cause macrophage death (7, 8); the SPI-2–encoded T3SS expressed in the intracellular environment interferes with vesicular trafficking and promotes bacterial survival (9–11), and SPI-7 encodes a capsule that enables *S. Typhi* to resist phagocytosis and complement killing (12) and suppresses innate inflammatory responses (13, 14). *Salmonella* virulence factors are controlled by a plethora of regulators, including two component systems (PhoQ-PhoP and SsrA-SsrB), counter silencers (SlyA), alternative sigma factors (σ^E and σ^S), and nucleoid-associated proteins (H-NS) (15–18).

Most current understanding of *Salmonella* pathogenesis comes from studies of *S. Typhimurium* in mice. *S. Typhimurium*–murine interactions are often stated to mimic *S. Typhi*–human interactions

(19), but murine typhoid and human typhoid differ in a number of important respects. *S. Typhi* possesses virulence factors (e.g., Vi capsular polysaccharide and the CdtB cytolethal distending toxin) not shared with *S. Typhimurium* (20, 21) and likewise, has lost numerous *S. Typhimurium* genetic loci by genomic decay (22). Although *aro* and *phoP* mutations attenuate virulence in both *S. Typhimurium* and *S. Typhi* (23–27), other mutations that attenuate virulence in *S. Typhimurium* (e.g., *galE* and *cya-crp*) fail to attenuate the ability of *S. Typhi* to cause bacteremia and typhoid-like symptoms in humans (3, 28, 29). This complicates attempts to construct improved live attenuated typhoid vaccines on the basis of observations in mice infected with *S. Typhimurium*. Even more importantly, determinants of innate immunity to *S. Typhimurium* and *S. Typhi* are fundamentally distinct. Studies in mice have shown that IFN γ /IL-12 signaling and the NADPH phagocyte oxidase play a critical role in innate immunity to *S. Typhimurium* (30, 31). However, humans deficient in these host defenses exhibit enhanced susceptibility to *S. Typhimurium* but not *S. Typhi* (32–34). Similarly, mice lacking CD4⁺ T cells are highly susceptible to *S. Typhimurium* (30) as are humans with low CD4 T cell counts caused by HIV infection (35). However, HIV infection does not confer a risk of increased incidence or severity of typhoid because of *S. Typhi* infection (36). This indicates that *S. Typhimurium* and *S. Typhi* have qualitatively different interactions with innate and adaptive immunity, and the murine *S. Typhimurium* infection model fails to recapitulate essential aspects of human typhoid.

S. Typhi is highly adapted to humans and fails to cause progressive infection in normal mice. Only the coadministration of hog gastric mucin to overwhelm host phagocytes or the use of massive bacterial inocula renders *S. Typhi* lethal for mice (37, 38); however, such models provide limited insights into typhoid pathogenesis and have not been found to correlate well with human typhoid (39), because they are drastically removed from physiological host–pathogen interactions. The lack of a small-animal model has been a major impediment in understanding mechanisms of *S. Typhi* virulence. Here, we report the development of a humanized small-animal model for the study of human typhoid fever.

Immunocompromised mouse strains, including mice lacking a respiratory burst and inducible nitric oxide synthase (31), or sublethally irradiated mice, with impaired cell-mediated immunity (37), fail to support productive infection of *S. Typhi*, suggesting that *S. Typhi* replication in mice is not restricted by murine innate immunity but rather, that infection of human immune cells is re-

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quired for *S. Typhi* replication in vivo. This is consistent with studies indicating that human macrophage cell lines are better able than murine cells to support *S. Typhi* replication in vitro (40), although more substantial differences in intracellular survival of *S. Typhimurium* and *S. Typhi* are observed when mouse macrophages are infected in vivo (41).

Recent technological advances have permitted the development of humanized mice engrafted with human hematopoietic stem cells that generate human immune systems. This has been facilitated by lineages of immunodeficient mice, most notably NOD (nonobese diabetic)-*scid* *IL2r^{null}* mice that lack the IL-2 receptor common γ -chain (42). These mice exhibit multiple defects in innate immunity, lack adaptive immune function, and support heightened human hematolymphoid engraftment. The IL-2 γ -chain is required not only for IL-2 high-affinity ligand binding and intracellular signaling but also for IL-4, IL-7, IL-9, IL-15, and IL-21 binding and signaling (42), and its absence results in a complete block of mature T cell, B cell, and NK (natural killer) cell development. Sublethally irradiated newborn NOD-*scid* *IL2r^{null}* mice engrafted with CD34⁺ hematopoietic stem cells (HSC) from T cell-depleted human umbilical cord blood (hu-SRC-SCID or human-SCID repopulating cell-SCID mice) develop into mice with chimeric hematopoietic systems containing human immune cells in the immunodeficient mouse environment (42), including human B cells, CD4⁺ and CD8⁺ T cells, NK cells, monocytes, and myeloid and plasmacytoid dendritic cells (43, 44).

Results

Course of *Salmonella Typhi* Infection in hu-SRC-SCID Mice. I.p. injection of $\sim 10^5$ cfu of *S. Typhi* strain Ty2 (Vi-antigen positive) resulted in progressive lethal infection of hu-SRC-SCID mice within 2–3 d (Fig. 1A). NOD immunocompetent and nonengrafted NOD-*scid* *IL2r^{null}* animals infected in parallel as controls survived and seemed well throughout the course of the experiment, confirming that even severely immunocompromised NOD-*scid* *IL2r^{null}* mice are resistant to *S. Typhi*. At necropsy, the organism burden in livers and spleens of hu-SRC-SCID mice exceeded the initial inoculum by more than 10-fold (Fig. 1B), indicating the occurrence of net *S. Typhi* replication in vivo. This contrasts with the absence of net *S. Typhi* replication observed in immunocompromised mice treated with inhibitors of inducible nitric oxide synthase or the NADPH phagocyte oxidase (Fig. S1) or in nonengrafted NOD-*scid* *IL2r^{null}* animals (Fig. 1B). Infected hu-SRC-SCID mice contained 10- to 100-fold higher organism burdens in the liver and spleen compared with nonengrafted NOD-*scid* *IL2r^{null}* animals, with statistically significant differences observed in the livers ($P = 0.0317$) and a nonsignificant trend observed in the spleens ($P = 0.1905$).

Pathology of the hu-SRC-SCID Model. Pathological changes in infected hu-SRC-SCID mice included evidence of central lobular hepatocellular injury with vesiculation and cytoplasmic hyaline changes, Kupffer cell swelling, effacement of normal splenic architecture with lymphocyte depletion, and the presence of large splenic granulomas with palisading epithelioid macrophages and multinucleated giant cells (Fig. 2). Human mononuclear cells could be visualized in the livers and spleens of infected hu-SRC-SCID mice by immunohistochemical staining (Fig. 3). Cell death in the spleens of infected animals was confirmed by TUNEL staining (Fig. 2G and H). In contrast, few inflammatory changes were observed in infected but nonengrafted NOD-*scid* *IL2r^{null}* control animals.

Human and Murine Cytokine Production. Blood samples obtained from *S. Typhi*-infected NOD-*scid* *IL2r^{null}* and hu-SRC-SCID mice euthanized 56–72 h after inoculation were analyzed for cytokine levels using a commercial bead array capable of distinguishing cytokines of murine and human origin (BD Biosciences). Elevated levels of IL-6 and monocyte chemoattractant protein-1 (MCP-1) of

Fig. 1. *S. Typhi* virulence in hu-SRC-SCID mice. (A) Survival of engrafted hu-SRC-SCID mice after i.p. injection of 5.5×10^4 – 3.2×10^5 cfu (low, $n = 11$) or 3×10^6 cfu (high, $n = 8$). *S. Typhi* Ty2 is shown as a Kaplan–Meier plot. Survival is compared with that of parental nonengrafted NOD-*scid* *IL2r^{null}* ($n = 6$) or immunocompetent NOD^{+/+} ($n = 6$) mice receiving i.p. injection of 5.5×10^4 – 1.8×10^5 cfu. Aggregate data include mice receiving Ty2 and Ty2-derived transposon pools. (B) Organism burden was quantified in livers and spleens of nonengrafted NOD-*scid* *IL2r^{null}* and engrafted hu-SRC-SCID mice 48 h after i.p. inoculation of 1 – 3×10^5 cfu *S. Typhi* Ty2. Horizontal lines indicate medians. Asterisk denotes significant difference by Wilcoxon rank-sum test.

both human and murine origin were observed, along with IFN γ and TNF α of predominantly human origin and IL-10 of predominantly murine origin (Fig. 4). Only modest elevations in murine IL-6 and MCP-1 were observed in *S. Typhi*-infected nonengrafted NOD-*scid* *IL2r^{null}* mice, indicating that the failure of *S. Typhi* to replicate in these mice is not a consequence of a heightened innate immune response. The elevated production of Th1 cytokines IFN γ and TNF α was more pronounced in *S. Typhi*-infected hu-SRC-SCID mice than in control C57BL/6 *iNOS* mice challenged with wild-type *S. Typhimurium* (Fig. S2), and elevated levels of MCP-1 were consistent with the observed infiltration of infected tissues with mononuclear inflammatory cells (Fig. 2).

Microarray-Based Screen to Identify *S. Typhi* Virulence Determinants in hu-SRC-SCID Mice. A microarray-based strategy was used to determine the effects of transposon insertion mutations on the in vivo competitive fitness of *S. Typhi* in individual hu-SRC-SCID mice. Inocula of 3×10^6 cfu of *S. Typhi* Ty2 pools each carrying $\sim 1,500$ –

Fig. 2. Pathology of typhoid in hu-SRC-SCID mice. (A) Granulomatous inflammation with mononuclear cell infiltration in the spleen of an infected hu-SRC-SCID mouse after 48–72 h. (B) Multinucleated giant cell (arrow) in the spleen of an infected hu-SRC-SCID mouse. (C) Central lobular hepatocellular changes in the liver of an infected hu-SRC-SCID mouse. (D) Kupffer cell swelling (arrow) in the liver of an infected hu-SRC-SCID mouse. (E) Mild hepatocellular changes with normal-appearing Kupffer cells (arrow) in the liver of an infected control NOD-*scid* IL2 γ ^{null} mouse. (F) Pyknotic lymphocytes (arrow) with cytoplasmic shrinkage in the spleen of an infected hu-SRC-SCID mouse. (G) Low background levels of cell death (arrow) visualized by TUNEL staining in the spleen of an infected control NOD-*scid* IL2 γ ^{null} mouse. (H) Increased cell death (arrows) visualized by TUNEL staining in the spleen of an infected hu-SRC-SCID mouse. (Magnification: A, 100 $\times$; B–H, 400 $\times$; scale bar: A, 100 μ m; B–H, 10 μ m.)

2,000 random EZ-Tn5 transposon insertions (Epicentre Biotechnologies) were injected intraperitoneally into hu-SRC-SCID mice. After 30 h, livers and spleens were harvested and homogenized with aliquots removed to determine cfu per organ, with the remainder added to broth and grown overnight at 37 °C. DNA was extracted to represent the output DNA sample. DNA from input and output pools was hybridized to NimbleGen whole-genome tiling arrays (45) (Roche NimbleGen). From two independent transposon pools containing insertions in 1,953 loci (7,820 total) in the *S. Typhi* genome, 4.8% of loci contained transposon insertions that were underrepresented in at least one output pool (Tables S1 and S2).

Fig. 3. Visualization of human CD45⁺ cells in infected hu-SRC-SCID mice. Sections were obtained from NOD-*scid* IL2r^{null} or hu-SRC-SCID mice infected as in Fig. 2 and stained with H&E (Left). Engrafted hematopoietic cells expressing human CD45 are stained brown and counterstained with hematoxylin (Right).

Discussion

Humanized mice provide an unprecedented opportunity to investigate the pathogenesis of infections caused by microbes that are specifically adapted to humans. The present study shows that the presence of human immune cells allows the replication of the typhoid bacillus in mice. In contrast, neither immunocompetent NOD mice nor immunocompromised nonengrafted NOD-*scid* *IL2 γ* ^{null} mice were able to support *S. Typhi* replication. The hu-SRC-SCID mouse represents a tractable small-animal model that reproduces a number of important pathological and inflammatory features of human typhoid fever. The pathological abnormalities provide experimental correlates of the hepatocellular toxicity, splenic typhoid nodules, Kupffer cell swelling, and granulomatous inflammation with multinucleated giant cells observed in human typhoid (46–52). Hepatic centrilobular microvesicular changes and splenic lymphocyte apoptosis (53, 54), general systemic responses to lipopolysaccharide in both normal and humanized mice, were also evident. Elevated serum concentrations of IL-6, IFN- γ , and TNF- α , as measured in the hu-SRC-SCID mice, have also been observed in patients with typhoid fever (55, 56). The marked inflammatory response may be an important contributor to the mortality of *S. Typhi* infection in hu-SRC-SCID mice, because elevated IL-6 and TNF- α levels correlate with severity of illness in children with typhoid (57).

S. Typhi mutants carrying transposons in loci implicated in stress resistance, DNA repair, and iron acquisition as well as in known *Salmonella* virulence determinants exhibited reduced competitive fitness in hu-SRC-SCID mice (Table S1). The *rseP* protease identified in this screen has been recently shown to be required for *Salmonella* survival in macrophages (58). RcsC is part of a regulatory system shown to modulate the expression of invasion proteins, flagellin, and Vi capsular antigen in *S. Typhi* (59). The *ampD* locus was also identified, and this gene is required for *S. Typhimurium*

Fig. 4. Serum cytokine levels in *S. Typhi*-infected hu-SRC-SCID mice. Serum concentrations of murine (M) and human (H) IL-6, IL-10, MCP-1, IFN γ , TNF α , and IL12p70 were assayed by cytometric bead array in nonengrafted NOD-scid IL2r γ ^{null} or engrafted hu-SRC-SCID mice 56–72 h after infection with *S. Typhi* Ty2 ($n = 5$ per group, error bars = SEM).

virulence in mice (60). The *entF* locus encoding a serine-activating enzyme involved in siderophore synthesis is of interest, because *S. Typhi* mutants deficient in enterobactin-mediated iron uptake have been found to exhibit reduced growth in human mononuclear cells (61). Moreover, sera from patients with typhoid fever react with FepA, the enterobactin receptor (62), showing that *S. Typhi* uses enterobactin to obtain iron during human typhoid fever. MgtC is a known virulence determinant in *S. Typhimurium* encoded within the SPI-3 pathogenicity island (63). MgtC has been implicated in the ability of *S. Typhimurium* to survive in cultured macrophages, and recent studies have shown that MgtC also promotes *S. Typhi* survival in human cells (64). Several loci belonging to the SPI-1 and SPI-2 Pathogenicity Islands were also identified in our transposon screen (Table S1), and both SPI-1 and SPI-2 are expressed when *S. Typhi* is internalized by human macrophages (65). SPI-2 mutations have been incorporated in Ty2-based human typhoid vaccines to reduce virulence (66). It was of particular interest to obtain multiple insertions in SPI-6, which encodes a putative type VI secretion system (67). Type VI secretion has been implicated in the virulence of *Pseudomonas aeruginosa* and other pathogenic bacteria (68), and the present study provides evidence of a role in *Salmonella* virulence.

Some important limitations of this model should be recognized. The individual engraftment of newborn mice is an expensive and labor-intensive process. Significant subject to subject variation may be seen as a result of the genetic heterogeneity of donors and degrees of engraftment. The rapid progression to death observed in this model after i.p. inoculation may enable the recognition of host and bacterial factors involved in acute typhoid septicemia but cannot be used to study the pathogenesis of subacute or persistent typhoid infections or the complex interactions between *S. Typhi* and the host gastrointestinal tract. Finally, a chimeric immune system in which murine and human hematopoietic cells coexist may create artifactual interactions. However, new generation models under development will address these issues (69, 70). The limitations notwithstanding, the hu-SRC-SCID mouse model created by the engraftment of NOD-scid IL2r γ ^{null} mice with human

umbilical cord-derived hematopoietic stem cells represents a small-animal model in which *S. Typhi* causes lethal infection and recapitulates important pathological features of human typhoid. This model promises to provide insights into typhoid pathogenesis, identify typhoid vaccine candidates, and lead to improved strategies for the prevention of *S. Typhi* infections.

Materials and Methods

Bacterial Strains and Growth Conditions. The Ty2 strain of *S. Typhi* (JSG624) used in this study was provided by John Gunn (Ohio State University, Columbus, OH). Bacteria were cultivated in Terrific Broth (12 g Bacto Tryptone, 24 g yeast extract, and 4 mL 100% glycerol per 1 L with 100 mL 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ added after autoclaving). Liquid cultures were grown at 37 °C with vigorous shaking. Kanamycin 50 μ g·mL⁻¹ was used for selection as indicated. Cfus were enumerated by serial dilution in PBS and plating onto Terrific Broth agar followed by incubation at 37 °C overnight.

EZ-Tn5 <KAN-2> Transposon Library Construction. An *S. Typhi* Ty2 transposon library was constructed using the EZ-Tn5 <KAN-2> Promoter Insertion Kit from Epicentre. Log-phase *S. Typhi* Ty2 grown in 400 mL Terrific Broth (TB) broth was centrifuged, and the pellet was washed three times with cold 10% glycerol before concentration in 1.2 mL 10% glycerol. Two mixtures containing 1 μ L TypeOne restriction inhibitor (Epicentre), 1 μ L glycerol, 2 μ L EZ-Tn5 transposase, and 2 μ L EZ-Tn5 <KAN-2> were incubated for 3 h before electroporation of 1 μ L into aliquots of 100 μ L of *S. Typhi* suspended in 10% glycerol. After electroporation, 1 mL Luria-Bertani (LB) broth was added to each aliquot, and the cells were incubated at 37 °C with agitation for 1 h. After joining the electroporation mixtures, an aliquot was plated onto LB-kanamycin (50 μ g·mL⁻¹) agar, with the remainder used to inoculate 400 mL TB with 50 μ g·mL⁻¹ kanamycin, and incubated overnight, with aliquots subsequently frozen in glycerol at -80 °C.

Hu-SRC-SCID Mouse Construction. A complete description of the construction of humanized mice used in this study is published elsewhere (43, 44). Briefly, newborn NOD-scid IL2r γ ^{null} pups from 24 to 48 h of age were irradiated with 100 cGy from a ¹³⁷Cs source. After irradiation, pups were injected with a suspension of T cell-depleted human umbilical cord blood containing 3×10^4 CD34⁺ cells through intracardiac injection. The pups were returned to their mothers and weaned after 21–24 d. Successful engraftment was documented by assaying peripheral blood by flow cytometry (Fig. S3) to detect the presence of human CD45⁺, CD3⁺, and CD20⁺ cells. The mice used in this study contained an average of $28\% \pm 15\%$ (range = 8–58%) CD45⁺ human hematopoietic cells in their peripheral blood 10 wk after transplantation, and an average of $26\% \pm 23\%$ of these cells were CD3⁺ T cells by flow cytometry.

Mouse Infection and Histology. NOD/LtJ (NOD), NOD.CB17-Prkdc^{scid} (NOD-scid), NOD-scid IL2r γ ^{tm1Wjl}/SzJ (NOD-scid IL2r γ ^{null}), and human hematopoietic stem cell-engrafted NOD-scid IL2r γ ^{null} (hu-SRC-SCID) mice were housed in a specific pathogen-free (SPF) vivarium and maintained on medicated water (0.168 mg·mL⁻¹ trimethoprim-sulfamethoxazole, 0.015 mg·mL⁻¹ voriconazole) until 3 d before infection. Overnight cultures of *S. Typhi* were grown in TB with kanamycin and diluted into PBS to desired concentrations for inoculation with cfu as indicated. *S. Typhi* was administered by i.p. injection with 500 μ L bacterial suspension in PBS. Mice were closely monitored, and moribund animals were euthanized by CO₂ asphyxiation at designated time points. Livers and spleens were removed aseptically from infected mice, and blood was collected by cardiac puncture. Blood was allowed to clot and then was centrifuged with removal of serum and storage at -80 °C. One half of each liver and spleen was placed in 20 mL 10% PBS-buffered formalin, and the other one-half was placed in 1 mL sterile PBS. Tissues were homogenized, and aliquots were serially diluted and plated onto TB agar to determine cfu per organ. Significance was determined by the Wilcoxon rank-sum (Mann-Whitney U) test. Organs were removed from formalin after 2 d and placed into 70% ethanol. Organs were embedded in paraffin, and sections were stained with H&E. TUNEL staining to detect cell death was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore). Immunohistochemical staining was performed with mAb specific for human CD45 (clone 2B11 + PD7/26; Dako) using a DakoCytomation EnVision Dual Link system implemented on a Dako Autostainer Universal Staining System. Sections were counterstained with hematoxylin.

Measurement of Cytokine Production. Production of inflammatory cytokines IL-6, IL-10, MCP-1, IFN γ , TNF α , and IL-12p70 was measured using a Cytometric Bead Array (CBA-Flex Kit; BD Biosciences). Measurement of human and murine

cytokine production was performed on samples from the hu-SRC-SCID or control nonengrafted NOD-*scid* IL2 γ^{null} and C57BL/6 mice according to manufacturer's instructions. As a positive biological control for murine cytokines, sera were obtained from C57BL/6 *iNOS*^{-/-} mice challenged 96 h earlier with 10³ cfu wild-type *S. Typhimurium* 14028s. Reagents for cytokine measurements were provided with the kits. Standards for individual cytokines were purchased for the generation of standard curves. FACS analysis was performed using BD FACScan or BD FACSCanto, and data were analyzed with FlowJo (TreeStar) or FCAP Array (BD Biosciences) software at the University of Washington Department of Immunology Cell Analysis Facility.

Analysis of Transposon Pools for in Vivo Fitness. Mice were injected intraperitoneally as above with 3×10^6 cfu *S. Typhi* transposon pools, each containing ~1,500–2,000 unique EZ-Tn5 insertions. Mice were euthanized 30 h after infection. Liver and spleen homogenates were used to inoculate 20 mL TB in a 125-mL Erlenmeyer flask containing 50 μ g·mL⁻¹ kanamycin and incubated for 18 h at 37 °C with vigorous shaking. The culture-homogenate mixture was transferred to a 50-mL conical tube, and particulate matter was allowed to settle for 1 h. Supernatant was removed for DNA purification, and a portion was archived by the addition of sterile glycerol before storage at -80 °C. DNA representing the output pool was purified using MasterPure reagents from Epicentre according to manufacturer's instructions. Total DNA representing the input pool was also purified from overnight cultures of *S. Typhi* used to infect mice. Samples were labeled and prepared for hybridization to NimbleGen *Salmonella* whole-genome tiling arrays (Roche NimbleGen) as described (45). Briefly, the DNA was fragmented by sonication and then, poly-A tailed; fragments containing Tn5-derived P₁₇ were

subsequently amplified and in vitro transcribed (AmpliScribe T7 transcription kit; Epicentre). Cy-dye labels were incorporated during in vitro transcription, and purified RNA was hybridized to tiling arrays containing ~387,000 oligos. Arrays were designed based on the *S. Typhimurium* LT2 genome, representing 4.14 Mb (86.4%) of the *S. Typhi* Ty2 genome at >95% accuracy, but not including Typhi-specific regions such as SPI-7. Normalized signal intensities were compared from input and output pools to identify transposon insertions counterselected during infection of hu-SRC-SCID mice. Transposon peaks were automatically detected by considering oligos exhibiting the top 2.5% of all intensities and comparing relative intensity with the next overlapping oligo. Analysis used one-half (background), median (within array), and quantile (between array) normalization, and transposon detection and analysis tools were implemented in WebarrayDB (www.webarraydb.org). Transposons were considered potentially changed in abundance if the median *P* value of the difference between input and output samples for oligonucleotides around the peak was *P* < 0.05. The detailed array platform and hybridization results are Minimal Information About a Microarray Experiment (MIAME)-compliantly deposited at www.webarraydb.org under MPMD ID 168.

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