Salmonella typhimurium Persists within Macrophages in the Mesenteric Lymph Nodes of Chronically Infected *Nramp1*$^{+/+}$ Mice and Can Be Reactivated by IFNγ Neutralization

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Abstract

Host-adapted strains of *Salmonella* are capable of establishing a persistent infection in their host often in the absence of clinical disease. The mouse model of *Salmonella* infection has primarily been used as a model for the acute systemic disease. Therefore, the sites of long-term *S. typhimurium* persistence in the mouse are not known nor are the mechanisms of persistent infection clearly understood. Here, we show that *S. typhimurium* can persist for as long as 1 yr in the mesenteric lymph nodes (MLNs) of 129sv *Nramp1*$^{+/+}$ (*Slc11a1*$^{+/+}$) mice despite the presence of high levels of anti–*S. typhimurium* antibody. Tissues from 129sv mice colonized for 60 d contain numerous inflammatory foci and lesions with features resembling *S. typhi* granulomas. Tissues from mice infected for 365 d have very few organized inflammatory lesions, but the bacteria continue to persist within macrophages in the MLN and the animals generally remain disease-free. Finally, chronically infected mice treated with an interferon-γ neutralizing antibody exhibited symptoms of acute systemic infection, with evidence of high levels of bacterial replication in most tissues and high levels of fecal shedding. Thus, interferon-γ, which may affect the level of macrophage activation, plays an essential role in the control of the persistent *S. typhimurium* infection in mice.

Key words: persistence • Slc11a1 • carriage • interferon-γ • monocytes

Introduction

Salmonella serovars are responsible for human diseases that range from gastroenteritis to systemic infections. *Salmonella typhi* causes human typhoid fever, whereas *Salmonella typhimurium* has a broad host range, causing disease in a variety of animals. Strains of *S. typhimurium* usually cause a self-limiting gastroenteritis in humans and a systemic typhoid-like disease in mice. Although *S. typhimurium* has been widely used as an experimental model for typhoid fever (1), there are significant genetic differences between *S. typhi* and *S. typhimurium* (2), which should be kept in mind when extrapolating between murine and human typhoid. *S. typhimurium* infection of mice and *S. typhi* infection of humans is characterized by inflammation at the site of bacterial entry, typically the Peyer's patches (3). After *Salmonella* penetrates the epithelial barrier, it preferentially infects phagocytes within the lamina propria. In *Salmonella* gastroenteritis, the infection is usually self-limiting and does not proceed beyond the lamina

propria. In host-adapted salmonellosis such as typhoid fever, the *Salmonella*-infected phagocytes gain access to the lymphatics and bloodstream permitting the bacteria to spread to the liver and the spleen (4).

S. typhi and *S. paratyphi* serovars are important human pathogens of immense public health and economic importance; moreover, they are endemic in regions of the world where drinking water quality and sewage treatment facilities are poor (5, 6). Infections remain difficult to treat by antibiotic therapy because the frequency of resistance to current antibiotics is increasing (7). A significant percentage (1–6%) of typhoid patients become chronic carriers of *S. typhi*, as do many people who have never had a clinical history of typhoid fever (8–10). These individuals shed bacteria in their stools or urine for a varying period of time ranging from 1 yr to a lifetime without any apparent signs of disease (11). Typhoid carriers are of special concern from a public health viewpoint because they are the reservoirs for the spread of infection and disease. *S. typhi* is carried for years,

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even in the presence of an immune response; chronic carriers of *S. typhi* have high levels of circulating serum antibodies to Vi and flagellar antigens, indicating that the organism has established a privileged niche that is sequestered from the host's immune defenses (5, 12). By investigating the chronic carrier state in salmonellosis, we hope to gain insight into bacterial strategies for survival as well as new clues for the potential treatment approaches for typhoid and other persistent microbial infections.

Mouse typhoid is similar to human typhoid in several ways, and *S. typhimurium* infections of laboratory mouse strains have been used successfully to study complex host–pathogen interactions. Different strains of mice show different levels of susceptibility to *Salmonella* infection (13). In the mouse, a significant component of the innate resistance/susceptibility to infections with *S. typhimurium* is controlled by the gene *Nramp1* (also called *Slc11a1*; references 14–16). Murine *Nramp1* expression is restricted to cells of the monocyte/macrophage lineage and affects the capacity of the host to control intracellular replication of microorganisms that reside in the phagolysosome (17). Thus, Nramp1 is involved in the control of the exponential growth of the bacteria in the reticuloendothelial organs during the early phase of infection (18). Susceptibility in mice is associated with a nonconservative amino acid substitution at position 169 (glycine^resistant to aspartic acid^sensitive), caused by a point mutation in the region encoding a putative transmembrane domain. Consequently, mice carrying two copies of the *Nramp1^Asp169* allele are significantly less resistant to lethal *S. typhimurium* infections compared with mice harboring the wild-type or *Nramp1^Gly169* allele (18). More recently, *Nramp1* has been implicated as being involved in influencing the clearance of *Salmonella enteritidis* during the acquired immunity phase of infection by an undetermined mechanism (19).

The control of *Salmonella* clearance during the late phase of infection appears to be influenced by various loci through their effects on the acquired immune response. The MHC of the mouse (also named H2) plays an important role in the clearance of *Salmonella* during the late phase of infection (13, 20). Likewise in humans, a genetic link between specific Class II and Class III MHC haplotypes and relative resistance to *S. typhi* has been demonstrated (21). Several studies have shown a requirement for both CD4+ and CD8+ T-lymphocytes for clearance of *Salmonella* infections (22–24). The humoral response is also involved in the clearance of *Salmonella*. Mice carrying a mutation in the *Btk* gene (or *xid* [X-linked immunodeficiency]) possess a defect in peripheral B cell activation that results in deficient antibody production and the inability to clear *Salmonella* infection (25, 26).

Most investigations of *Salmonella* pathogenesis have historically used *Salmonella*-sensitive mice. This reflects, in part, the convenience of death or pathology as an experimental endpoint that could be readily quantitated. Thus, acute *S. typhimurium* infections have been well characterized in this model. However, this model is not suitable for long-term carriage studies, as the mice either die rapidly from relatively low doses of *Salmonella* or clear the bacteria. Previous studies using mutant *S. typhimurium* strains in susceptible strains of mice have shown that these mutants can colonize mice for as long as 2 mo (27–29). Although these models are useful for understanding the development of protective immunity to *Salmonella*, they do not always contribute to an understanding of the pathogenesis of persistent *Salmonella* infections with wild-type bacteria.

We have explored a typhoid model of infection using mice carrying the wild-type *Nramp1* allele infected orally with a wild-type *S. typhimurium* strain. In this experimental model of infection, we have determined that *Salmonella* is carried chronically in the reticuloendothelial system (RES) as long as 1 yr after infection. We further found that bacteria persisting in the host were most often sequestered within macrophages of the mesenteric LNs (MLNs). We have shown that IFNγ is essential for the control of persistent *S. typhimurium* infection.

Materials and Methods

Bacterial Strains and Growth Conditions. SL1344, a wild-type *S. typhimurium* strain, was obtained from B.A.D. Stocker (Stanford University). SMB500 (SL1344*hha*::Tn5*lux*) was obtained from S. Burns (Stanford University). SMB500 contains the *luxCDABE* genes, which are required for the production of bioluminescent light, constitutively expressed from the prokaryotic EM7 promoter (InvivoGen). Bacteria were grown overnight with aeration in Luria broth before oral inoculation of mice. The bacteria were washed and resuspended with PBS to the appropriate concentration.

Mice and S. typhimurium Infections. 8–10-wk-old 129sv H-2^b *Nramp1^{+/+}* (Jackson Laboratories) and C57BL/6 H-2^b *Nramp1^{Asp169/Asp169}* (Jackson Laboratories) mice were kept under specific pathogen-free conditions in filter-top cages. Mice were provided with sterile water and food ad libitum.

Mice were deprived of food 14 h before orogastric inoculation. For LD$_{50}$ calculations, 35 129sv and 35 C57BL/6 mice were inoculated through a gastric tube, with serial 10-fold ($n = 5$ mice/inoculum) dilutions ranging from 1.38×10^4 to 1.38×10^{10} CFU in a volume of 0.1 ml. The health of the animals was monitored for 30 d after inoculation, and deaths were recorded. Mice were monitored for shedding of *S. typhimurium* in feces by collecting an average of 0.1 g of fresh fecal pellets from each individual mouse. The pellets were homogenized in PBS and 9/10 was plated directly, and 1/10 of the volume was incubated for 18 h in selenite broth. Both were plated on XLD (Oxoid) plates containing 200 μg/ml streptomycin to obtain CFU per gram of feces. For colonization experiments, mice were inoculated orogastrically with the indicated dose of wild-type *S. typhimurium* SL1344. Mice were bled before being killed on days 5 ($n = 3$), 60 ($n = 5$), 140 ($n = 4$), 180 ($n = 5$), 270 ($n = 5$), and 365 ($n = 13$) followed by dissection of cecum, PP, MLN, liver, gallbladder, and spleen. Tissues were homogenized and weighed, and dilutions were plated on Luria agar plates containing 200 μg/ml streptomycin to obtain CFU per gram of tissue.

Neutralization of IFNγ in Infected Mice. 10 mice were inoculated as described in the previous paragraph and kept in separate cages for 260 d. Mice were injected intraperitoneally with 200 μg

of either an anti-IFNγ neutralizing antibody or an isotype-matched control antibody (both provided by N.F. Landolfi, Protein Design Laboratories, Fremont, CA). The half-life of the anti-IFNγ and isotype-matched control antibodies is on the order of 14–18 d (Landolfi, N.F., personal communication). The mice were given two boosts of 200 μg of antibody on days 7 and 14 after the initial injection.

Anti-Salmonella Serum IgG Response. The serum IgG response to *S. typhimurium* was determined by ELISA using sonicated-killed *S. typhimurium* as coating antigen as described by Barrow et al. (30). The titer was determined as the highest dilution of serum to give an optical density reading above background.

In Vivo Bioluminescent Imaging. The group of mice that were infected with the SL1344::lux strain were monitored for bacterial counts at 7, 23, 40, and 80 d after infection with an in vivo imaging system (IVIS). Mice were anesthetized with 70 mg/kg pentobarbital and imaged as described previously (31). At the time of imaging, mice were placed in an IVIS equipped with a cooled CCD camera and lens (model Navitar f 0.9; Xenogen Corp.). A grayscale reference image was first taken under weak illumination followed by a 5-min image of the light that was transmitted through the animal's tissues. After photon collection, a pseudo-color representation of light intensity (red, most intense; blue, least intense) was superimposed over the grayscale body surface reference image. Data acquisition and analyses were performed using the LivingImage software (Xenogen Corp.) that runs as an overlay on the image analysis package (IgorPro; Wavemetrics).

Histology and Immunohistochemistry of Tissue Sections. For histological examinations on tissue sections, tissues were fixed in 10% buffered neutral formalin, embedded in paraffin, and serially sectioned. Some sections were stained with hematoxylin and eosin. MLN for immunohistochemistry was embedded in OCT and 15–20-μm frozen sections were cut on a cryostat. The sections were incubated in anti-*Salmonella* polyclonal rabbit antiserum (diluted 1:1,000) and a biotinylated pan-macrophage antibody MOMA-2 (diluted 1:10; Serotec), or a biotinylated neutrophil antibody (Gr-1; BD Biosciences), in PBS containing 3% BSA and 0.2% saponin. Next, tissue sections were incubated with anti–rabbit-Alexa488 antibody (Molecular Probes), streptavidin-Alexa594 (Molecular Probes), and either an anti–mouse IgG-Alexa660 antibody, a B cell antibody (B220) conjugated to Cy5, or ToTo-3 (Molecular Probes) to stain host cell nuclei. Coverslips were mounted over antiquench (Vector Laboratories) and sealed.

CLSM and Image Analysis. The images were collected on a microscope (model Optiphot-2; Nikon) attached to a confocal laser scanning microscope (model MRC1024; Bio-Rad Laboratories) using LaserSharp software (Bio-Rad Laboratories). The laser lines on the krypton/argon laser were 488 nm (Alexa488), 568 nm (Alexa594), and 647 nm (Alexa660 and TOTO3). The numerical aperture was 0.75 on the 60$\times$ oil objective. Volocity 2.0 (Improvision Inc.) was used for image analysis, and all images were based on maximum intensity projection.

Results

129sv Mice Are Colonized Systemically and Are Resistant to Death after Oral Inoculation with Wild-type S. typhimurium. Previous studies of *Salmonella* infections in mice carrying a wild-type *Nramp1* allele were performed by infecting mice either intravenously or intraperitoneally (18, 19, 32–36). In contrast, naturally acquired systemic *Salmonella* infections occur via the entry of a few bacteria from the gastrointestinal tract into tissue via systemic circulation (4, 37). Thus, we characterized the course of infection and colonization in *Nramp1* wild-type mice (129sv) after an oral inoculation with 10^6 CFU of a wild-type *S. typhimurium* strain (SL1344) and compared this with mice carrying a mutant *Nramp1* allele (C57BL/6). Both C57BL/6 and 129sv mice were colonized to similar levels in the cecum, PP, MLN, and spleen 5 d after oral inoculation (unpublished data), indicating *S. typhimurium* could establish the initial steps of a systemic infection in both mouse strains. However, by day 5, C57BL/6 mice showed signs of illness, such as ruffled fur and malaise, whereas the 129sv mice had no apparent signs of illness. We determined the oral LD$_{50}$ of both strains of mice and found that there were dramatic differences. The oral LD$_{50}$ in C57BL/6 mice was 2.4×10^5 CFU, whereas the oral LD$_{50}$ in 129sv mice was $>10^9$ CFU, as only two of the five mice died when the highest dose tested, 10^9 CFU, was administered.

Model of Chronic S. typhimurium Infection in 129sv Mice. Based on the aforementioned observations and work from a previous study (19), in which a strain of *S. enteritidis* was carried in the spleens of 129sv mice for 42 wk after an in-
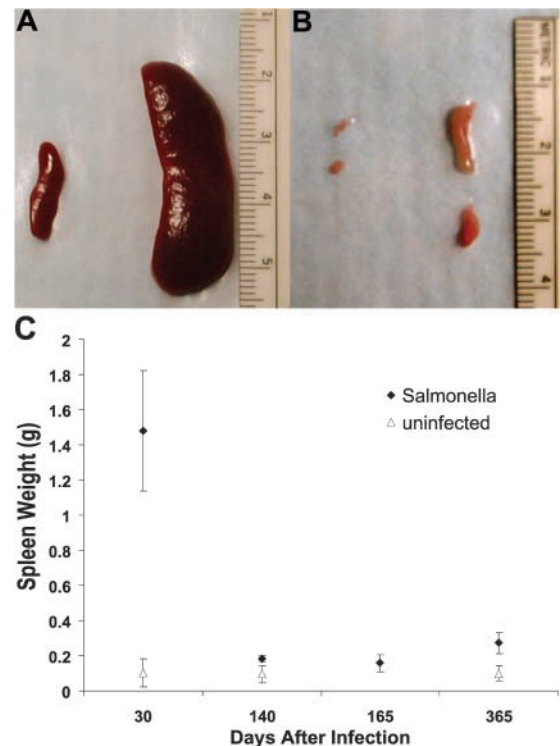

Figure 1. Systemic infections in 129sv mice induce splenomegaly and enlarged MLN. (A) The spleen from an uninfected age-matched 129sv mouse on the left is shown next to the spleen from a 129sv mouse (spleen on the right) infected with SL1344 for 60 d. (B) MLN from the same mice as in A (right, infected MLN). (C) The weights of the spleens are plotted on the y axis, with time from infection on the x axis. Infected spleens weighed significantly more than the uninfected control spleens. P < 0.0001 for day 30; P = 0.01 for day 140; P = 0.01 for day 365 (*n* = 5 for each day).
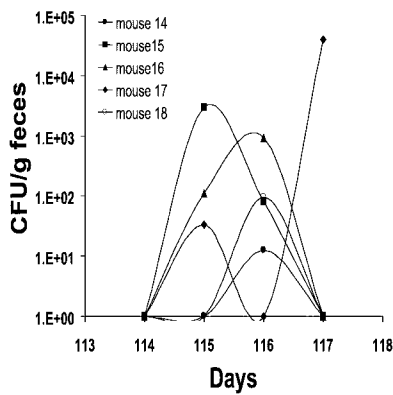
Figure 2. Shedding of *S. typhimurium* in feces from persistently infected 129sv mice. A mean of 0.1 g of feces was collected from five different mice on days 114–117 after infection with SL1344. The contents were homogenized and plated on XLD plates. The CFU per gram of feces varied from day-to-day in a given mouse.

Table I. *Log$_{10}$ CFU Per Gram of Tissues after Oral Infection of 129sv Mice with 10^8 SL1344*

Day	Mouse	Feces	Cecum	PP	MLN	Spleen	Liver	Gall bladder
60	1	–	–	–	2.18	2.31	0.75	–
	2	4.60	3.55	2.51	2.98	2.72	1.88	2.67
	3	5.10	1.18	–	2.94	2.37	1.25	1.84
	4	3.16	2.54	1.36	2.73	2.59	1.96	1.51
	5	–	–	–	3.69	–	–	–
140	1	–	–	–	3.88	–	–	–
	2	–	1.18	–	2.98	2.31	0.75	–
	3	–	–	–	3.56	–	–	–
	4	–	1.97	2.69	2.87	2.43	1.66	–
	5	–	–	–	2.19	1.96	1.31	2.40
180	1	–	2.38	–	3.86	1.18	1.16	–
	2	–	–	–	2.04	–	–	–
	3	–	–	–	2.35	2.11	2.10	2.52
	4	2.98	4.29	–	3.15	1.74	1.94	2.46
270	1	–	–	–	2.42	–	–	–
	2	–	–	–	–	–	–	–
	3	–	–	–	3.72	1.16	–	–
	4	–	–	–	3.22	–	–	–
	5	–	–	–	2.65	–	–	–
365	1	–	–	–	3.27	–	–	–
	2	–	–	–	4.42	–	–	–
	3	–	–	–	4.38	–	–	–
	4	–	–	–	2.41	–	–	–
	5	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–
	7	–	–	–	3.38	–	–	–
	8	–	–	–	–	–	–	–
	9[a]	4.16	4.89	4.81	4.50	1.16	2.43	3.73
	10	–	–	–	–	–	–	–
	11	–	–	–	2.97	2.94	2.01	–
	12	–	–	–	–	–	–	–
	13	–	–	–	2.54	2.93	1.80	2.07

[a]Mouse had signs of illness such as ruffled fur and malaise.

travenous inoculation with 10^3 organisms, we reasoned that this mouse line might prove extremely useful as a model to study the pathogenesis of long-term *S. typhimurium* infection in a mammalian host. Thus, we inoculated 35 129sv mice orally with 10^8 CFU of a wild-type *S. typhimurium* strain (SL1344) and monitored tissue colonization, fecal shedding, and tissue pathology over the course of 1 yr.

At early times after infection, most of the mice showed histopathological lesions characteristic of *S. typhimurium* in the spleen, liver, PP, and MLN, which was indicative of an acute infection. The number and severity of lesions and the weights of infected spleens decreased with time (Fig. 1 C), which was indicative of convalescence. 3 of the 35 mice died over the course of the experiment, on day 165, 280, and 295, respectively, after infection; at postmortem, these mice cultured positive in the cecum, MLN, spleen, and liver for *S. typhimurium* (not depicted).

S. typhimurium infection of 129sv mice induced pronounced splenomegaly (Fig. 1 A) and enlarged MLNs (Fig. 1 B), which peaked after 30–40 d and persisted for several weeks (Fig. 1 C). Spleen sizes slowly returned to a more normal size; however, there was a small, but significant increase in spleen weight compared with age-matched uninfected control spleens at 140 and 365 d after infection (Fig. 1 C). Despite the chronic presence of bacteria, most of the mice did not display any signs of acute illness such as malaise, weight loss, or ruffled fur. At 60 d, we found that the mice were still colonized at systemic sites (Table I). Although all five of the mice killed at 60 d were colonized, the bacterial burden per gram of tissue was considerably lower than mice infected for 5 d. In addition, we found some variation in the presence of *S. typhimurium* in different tissues: all of the mice were colonized in the MLN at 60 d; and two mice were colonized in all of the tissues tested. Three mice were shedding *S. typhimurium* in the feces on day 60; the presence of *S. typhimurium* in the feces correlated with gall bladder and cecal colonization (Table I). When we monitored additional mice for fecal shedding on four consecutive days (114–117 d after infection), we found that shedding varied from day to day in individual mice (Fig. 2), suggesting that shedding occurs in periodic waves as *Salmonella* replicates. This result is similar to what has been well documented for human typhoid carriers (38–40).

At 140 and 180 d after infection, there was no increase in the overall bacterial burden per gram of tissue (2–3 logs per gram of tissue; Table I); however, the number of mice that
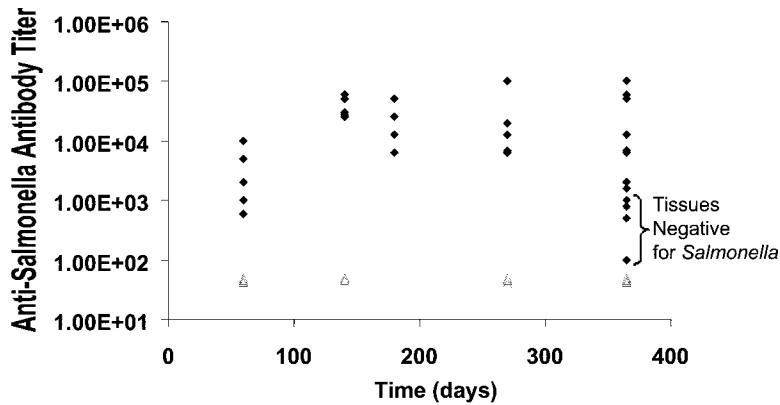
Figure 3. Mean anti–*S. typhimurium* IgG titers in persistently infected 129sv mice. *S. typhimurium*-infected ($n = 5$ for days 60 and 140; $n = 4$ for days 180 and 270; $n = 13$ for day 365) and uninfected age-matched control mice ($n = 3$, except for day 180). Antibody titers were determined by ELISA using killed whole *S. typhimurium* sonicate as antigen. (Closed diamonds) IgG titer in *S. typhimurium*-infected mice. (Open triangles) IgG titer in uninfected controls. Each serum was tested at least three times in triplicate.

were colonized in the intestinal sites, cecum, and PP, decreased. In contrast, all of the mice continued to be colonized in the RES, MLN, and/or spleen. Only one of the mice sampled at 180 d after infection was shedding; this animal was colonized in the cecum and gall bladder.

By 270 days, four of the five mice sampled were persistently infected in the MLN. One mouse, although it had been previously infected as indicated by the presence of *S. typhimurium* in the feces at 60 d after infection, appeared to have cleared the infection (Table I). The overall bacterial burden in the MLN did not change dramatically compared with the earlier time points. By 365 d, 5 of the remaining 13 mice had cleared the infection, although they had previously been shedding *S. typhimurium* at 60 d after infection, and 1 mouse at the time of sampling exhibited clear symptoms of illness and was colonized by *S. typhimurium* in all tissues tested (Table I). All eight of the persistently infected mice sampled 1 yr after infection were still infected in the MLN, with a range of 2–5 logs CFU per gram of tissue (Table I). These data indicate that after the early or acute phase of a systemic *Salmonella* infection, *S. typhimurium* persists predominately in the MLN and, to a lesser degree, in the spleen and liver of chronically infected mice.

Anti-Salmonella IgG Antibody Titers. To determine whether mice chronically infected with *S. typhimurium* produced antibodies to the organism, serum was collected from each mouse at the time of death, and anti-*Salmonella* IgG antibody titers were determined by ELISA. All of the infected mice had high antibody titers, with a 10-fold increase between 60 and 140 d (Fig. 3). At 365 d, the antibody titers of serum from infected mice ranged from 10^2 to 10^5, which were all significantly higher than the age-matched, uninfected control mice; however, the five mice that had apparently cleared the infection had lower anti-*Salmonella* antibody titers than the persistently infected mice (Fig. 3).

Histology of Chronically Infected Tissues. Histopathological studies were performed to examine the consequences of chronic *S. typhimurium* infection on tissue integrity, inflammation, and lesion formation. Histopathological lesions were more frequent in the spleen, liver, and MLNs at 60 d after oral inoculation than at 365 d (Fig. 4, A and B). The tissues from mice infected for 60 d contained typical foci of

necrosis, microgranulomas, or accumulations of PMNs (Fig. 4 C). At 140 d, discrete lesions were scarce in the liver and spleen. These rare inflammatory foci consisted predominantly of macrophages with minimal central necrosis (Fig. 4 D). By 365 d, the focal granulomas were not significantly different than 140 d, but they tended to have increased numbers of lymphocytes and large mononuclear
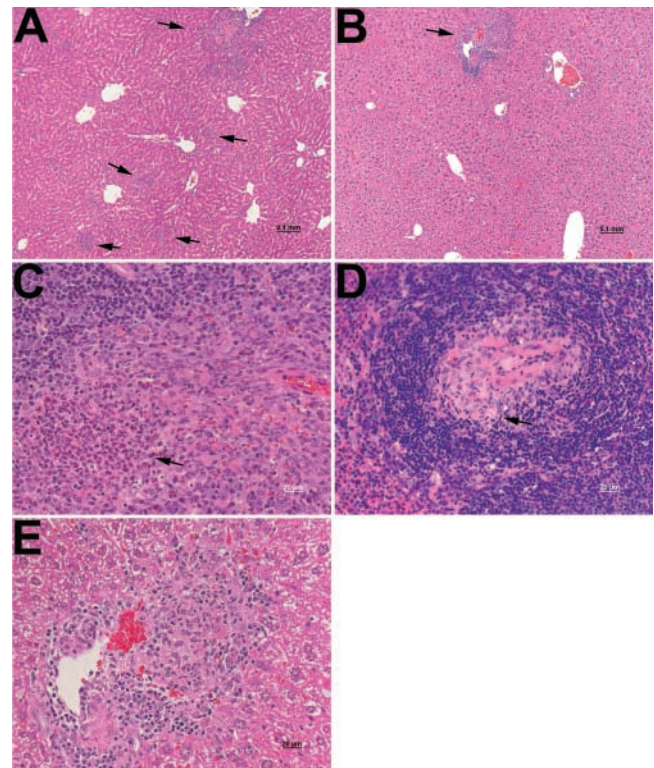

Figure 4. Histology of infected tissues from mice persistently infected with *S. typhimurium*. Liver and spleen sections were stained with hematoxylin and eosin. (A) Liver section from mouse infected for 60 d. Arrows point to multiple areas of inflammation and microgranulomas. (B) Liver section form mouse infected for 365 d. Arrow points to a focal granuloma. (C) Section of spleen infected for 60 d. Arrow shows accumulation of PMN in red pulp. (D) Section of spleen infected for 140 d. Arrow shows accumulation of macrophages. (E) Section of liver infected for 365 d. Bars: (A and B) 0.1 mm; (C–E) 20 μm.
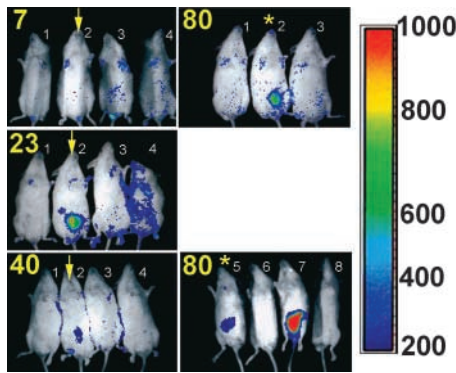
Figure 5. Monitoring 129sv mice persistently infected with *S. typhimurium* by an IVIS. 129sv mice were inoculated by oral administration of the bioluminescent labeled wild-type *S. typhimurium* strain, SL1344*hha::* Tn5*lux* at a dose of 10^8 CFU and monitored over 80 d. Days are indicated in the top left corner of each image. Light intensity is represented by a color scale in counts. Eight mice were inoculated and imaged on days 7, 23, 40, and 80. The arrow points to a mouse 2, which showed variable levels of signal over the 80 d. Mouse 4 died between 40 and 80 d. The asterisk indicates mice that were killed for analysis of MLN by confocal microscopy (e.g., mouse 2, 5, and 7).

cells surrounding central areas of tightly opposed macrophages (Fig. 4 E).

S. typhimurium Persist in MOMA-2⁺ MLN Macrophages. The tissue colonization data of 129sv mice orally infected with *S. typhimurium* indicated that during the initial stages of infection, bacteria were found at high levels at multiple sites throughout the mouse, but were beginning to be cleared between 60 and 270 d after infection (Table I). The bacteria persisted in a limited number of sites, predominately and invariably in the MLN, despite a strong antibody response.

We wished to determine the site of *S. typhimurium* persistence in the MLN. To ensure that MLNs were taken from mice still infected with wild-type *S. typhimurium*, we monitored the course of infection in 129sv mice using a noninvasive method that detects bacterial signal in live animals in a semi-quantitative manner, IVIS (31). Mice were infected with a wild-type strain of *S. typhimurium*, SMB500, which constitutively expresses the genes necessary for bioluminescent light production (41). This strain is as virulent as wild-type SL1344 in the mouse model and retains the bioluminescent genes in the mouse infection model (unpublished data). By following the course of infection in this noninvasive manner, we found that in a given mouse, the bacterial load varied over time. For example, Fig. 5 shows a mouse that has a higher bioluminescent signal at 23 d than at 40 d. 80 d after infection, the signal increased again. Three mice that were infected with *S. typhimurium* for 80 d and had elevated bioluminescent signals were killed, and the MLNs from each mouse were frozen and sectioned for immunohistochemistry.

To determine the host cells that *S. typhimurium* are associated with in persistently infected MLN, we stained frozen sections with several antibodies to host immune cells. Initially, we stained sections with an antibody that recognized neutrophils, Gr-1, an anti–*Salmonella* antibody, and an antibody that nonspecifically stains the host cells. We found that bacteria did not colocalize with neutrophils; rather, they resided within large host cells that were surrounded by neutrophils (Fig. 6 A). This finding and the results of our histological staining led us to examine the remaining sections with an antibody that stains the cytoplasm of all macrophages, MOMA-2. The bacteria were mainly found colocalized with MOMA-2⁺ macrophages in macrophage-rich areas of the lymph nodes within the subcapsular sinus
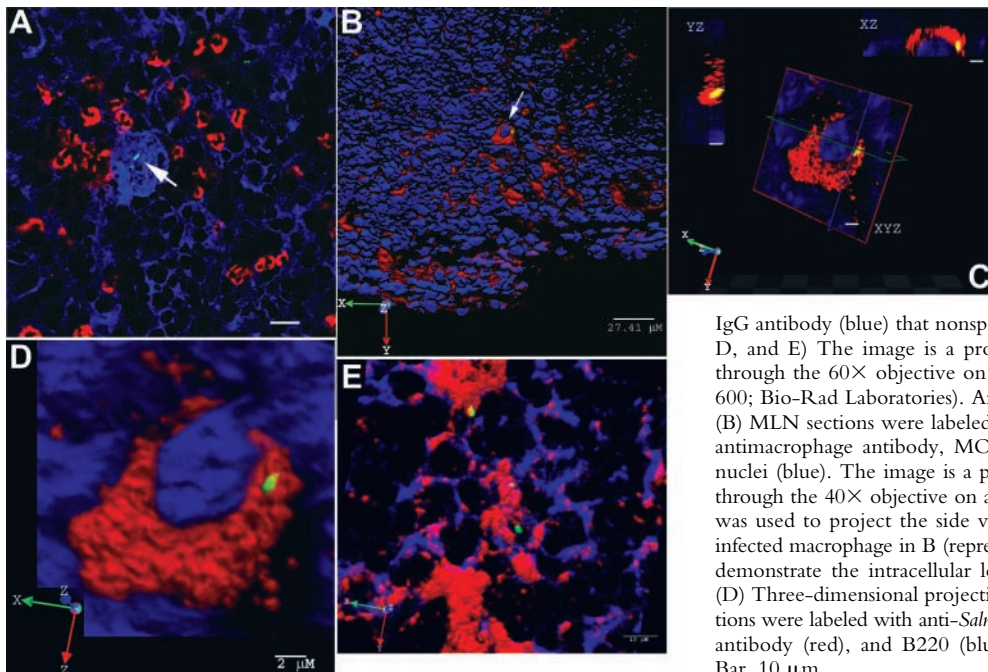

Figure 6. *S. typhimurium* persist inside of macrophages within the MLN of chronically infected mice. (A) MLN sections were labeled with anti–*Salmonella* antibody (green), antineutrophil antibody, Gr-1 (red), and an anti–mouse IgG antibody (blue) that nonspecifically stains other immune cells. (A, D, and E) The image is a projection of a 20-μm z-stack collected through the 60× objective on a confocal microscope (model MRC-600; Bio-Rad Laboratories). Arrow points to bacterium. Bar, 10 μm. (B) MLN sections were labeled with anti–*Salmonella* antibody (green), antimacrophage antibody, MOMA-2 (red), and ToTo-3 to stain all nuclei (blue). The image is a projection of a 15-μm z-stack collected through the 40× objective on a confocal microscope. (C) Volocity 2.0 was used to project the side view (yz) and bottom view (xz) of the infected macrophage in B (represented by the xy view), which together demonstrate the intracellular location of the bacterium. Bar, 5 μm. (D) Three-dimensional projection of macrophage in C. (E) MLN sections were labeled with anti–*Salmonella* antibody (green), antimacrophage antibody (red), and B220 (blue), the anti–B lymphocyte antibody. Bar, 10 μm.

Table II. *S. typhimurium Localize to Macrophages in MLN from Persistently Infected 129sv Mice*

Percentage of inside macrophages[a]	Mean No. bacteria/cell $\pm$ SE[b]
82.43 $\pm$ 1.19	3.89 $\pm$ 1.14

[a]The mean percentage of bacteria inside and standard error were calculated from serial sections stained with MOMA-2 antibody from three different mice. A minimum of 100 bacteria were counted from each mouse.
[b]The mean number of bacteria per 20-μm slice of a cell was calculated from serial sections stained with MOMA-2 antibody from three different mice.

and outer cortex (Fig. 6 B). Further analysis of the z-stacks obtained on the confocal microscope revealed that bacteria were inside these macrophages, as seen in the xz, yz, and three-dimensional projections (Fig. 6, C–E). To determine the frequency with which *S. typhimurium* were located inside macrophages, we examined every bacterium found within all sections and scored whether it was inside or outside a MOMA-2$^+$ host cell. 82% of the bacteria were clearly intracellular, whereas the remaining bacteria were either extracellular or their location was unclear (Table II). We noted at the same time the number of bacteria residing within the infected host cell and found that the mean number was between three and four bacteria per cell (Table II). We further characterized the host immune cells in the foci of bacterially infected macrophages and found that in addition to macrophages, the other immune cells in the vicinity of infected macrophages were predominantly B lymphocytes (Fig. 6 E). Thus, *S. typhimurium* persist intracellularly within macrophages of the MLN of 129sv mice.

IFNγ Neutralization Causes Reactivation of Systemic S. typhimurium Infection. The ability of *Salmonella* to survive and/or replicate inside macrophages is dependent on the activation state of the host cell, which can be affected by host cytokines such as IFNγ. We wished to test the role of IFNγ in the persistent *S. typhimurium* mouse infection. Mice that had been infected with *S. typhimurium* for 260 d, and were not shedding, were injected intraperitoneally with either an IFNγ neutralizing antibody or an isotype-matched control antibody. 3 wk after the initial injection of the IFNγ neutralizing antibody, three of the five mice in this group began to show signs of illness such as ruffled fur and malaise. At this time the mice were killed and dissected for tissue counts. The levels of detectable *S. typhimurium* in the PP, spleen, liver, and gall bladder were variable in the group of mice that received the neutralizing antibody; however, all five of the mice that received the neutralizing antibody were colonized in the MLN and cecum and were shedding high levels of *S. typhimurium* (2.3–7.8 logs per gram of feces; Fig. 7 A). In contrast, the mice that received the control antibody were colonized at low levels in the MLN, and only two were shedding lower levels of *S. typhimurium* (1.6–2.9 logs per gram of feces; Fig. 7 B). Furthermore, the tissues from mice that were
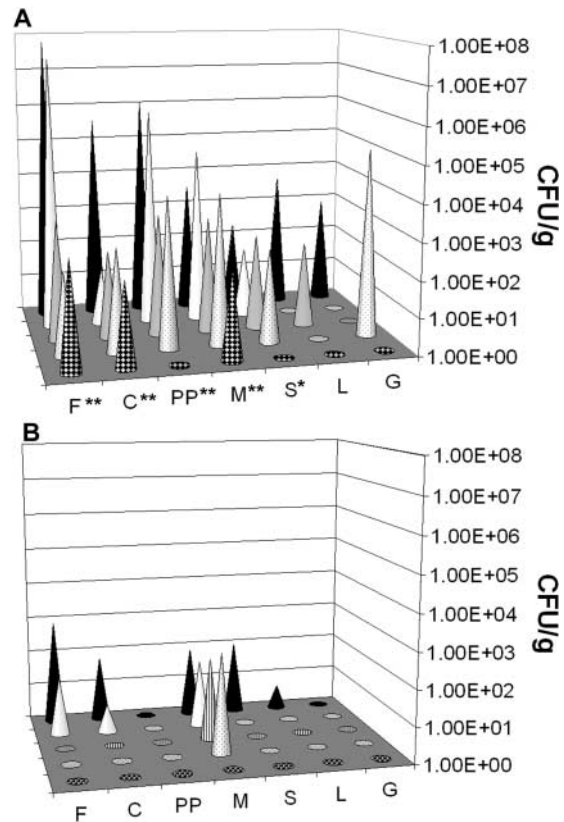

Figure 7. Anti-IFNγ neutralizing antibody reactivates *S. typhimurium* replication in persistently infected mice. (A) Bacterial counts in tissues from 129sv mice infected for 260 d and injected with IFNγ neutralizing antibody. (B) Bacterial counts in tissues from 129sv mice infected for 260 d and injected with isotype control antibody. The x axis is the tissue where F = feces, C = cecum, PP = Peyer's patches, M = mesenteric LNs, S = spleen, L = liver, G = gall bladder. The y axis denotes individual mice. The z axis is the CFU per gram of tissue. The Mann-Whitney U test was performed for statistical significance comparing the bacterial load from anti-IFNγ neutralizing antibody–treated mice with control antibody-treated mice. *, $P \leq 0.05$; **, $P \leq 0.01$.

treated with IFNγ neutralizing antibody contained lesions in the spleen and liver that are typical of *S. typhimurium* infection (Fig. 8 A). Surprisingly, the three mice that appeared ill had epicarditis/pericarditis and pleuritis (Fig. 8, B and C), and this inflammation was associated with the presence of *S. typhimurium* (Fig. 8, D and E). In contrast, the same tissues from mice treated with the control antibody did not contain inflammation in the heart or lungs (unpublished data). Thus, we were able to reactivate the chronically carried *S. typhimurium* to cause an acute infection even in the presence of anti–*Salmonella* antibody by inactivating the host cytokine IFNγ.

Discussion

Some bacterial pathogens are capable of establishing persistent infections in mammalian hosts despite activating inflammatory and antimicrobial responses (6, 42). For example, *Helicobacter pylori* inhabits the human gastric mucosa,
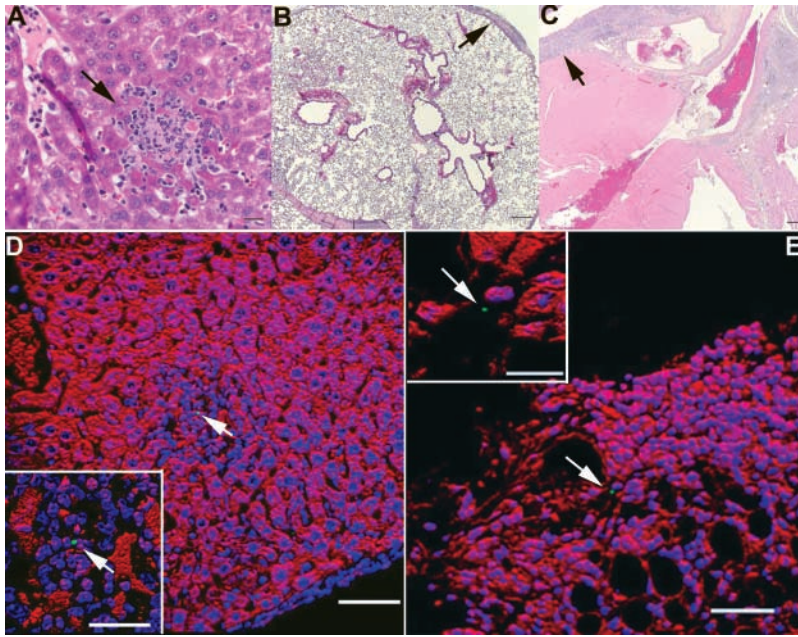
Figure 8. Histology of infected tissues from mice persistently infected with *S. typhimurium* and treated with IFNγ neutralizing antibody. (A) Liver, (B) lung, and (C) heart. Sections were stained with hematoxylin and eosin from a mouse infected for 260 d followed by 3 wk of IFNγ neutralizing antibody. (A) Arrow points to area of inflammation and microgranulomas. (B) Arrow points to inflammation in the pleura. (C) Arrow points to inflammation covering the epicardium. (D) Liver and (E) heart. Bacteria in sites of inflammation from serial sections were stained with antibody to *S. typhimurium* (green), actin was stained with phalloidin-Alexa594 (red), and nuclei of host cells were stained with Toto-3 (blue). (D) Arrow points to bacterium in the center of lesion. (Inset) Enlargement of the area of lesion containing the bacterium. (E) Arrow points to bacterium in the epicardium. (Inset) Enlargement of the area. Bars: (A) 20 μm; (B and C) 200 μm; (D and E) 100 μm.

and persistence can be lifelong (42). *Mycobacterium tuberculosis* persists inside alveolar macrophages within lung granulomas of infected humans for years and will sometimes reactivate to cause an acute infection (42). *Chlamydiae*, obligate intracellular bacterial pathogens, also cause persistent infections despite activating the host immune system (43). *S. typhi* causes systemic infection (typhoid fever) that involves colonization of the RES. Some *S. typhi*–infected individuals become lifelong carriers, periodically shedding high numbers of bacteria in their stools. Because *S. typhi* only colonizes humans, it has been suggested that these chronic carriers are the reservoir of *S. typhi* and that perhaps the carrier state is the ultimate and essential goal of *S. typhi* infection (44). Thus, we set out to establish a persistent *Salmonella* infection in mice and to characterize this model.

The laboratory mouse has been used as a model for acute systemic *Salmonella* infections, and early studies with classical, well-characterized inbred strains of mice have contributed substantially to our understanding of host–pathogen interactions. However, most of these studies have focused on infections of highly susceptible strains of mice, strains that carry a mutant *Nramp1* allele. *Nramp1* has been shown to be very important for controlling the exponential growth of *Salmonella* during the early phase of systemic infections; inbred strains of mice carrying mutant *Nramp1* alleles either die from low doses of *Salmonella* or clear the infection after ∼30 d (1, 18). Thus, it is difficult to study the pathogenesis of persistent *Salmonella* infection in these strains of mice. We describe here a model for persistent infection of a strain of mice that carries a wild-type *Nramp1* allele (129sv) using wild-type *S. typhimurium*. We have shown that, after oral infection of 129sv mice, which resulted in systemic infection, the period of recovery was characterized by long-term persistence of *S. typhimurium* in low numbers in the MLNs.

Some *Salmonella* serotypes such as *S. typhi*, *S. dublin*, and *S. pullorum* may, after clinical disease or asymptomatic infection, persist in the body for long periods of time. In the case of *S. typhi* and *S. dublin*, long-term excretion of the bacteria has classically been interpreted to represent localization to and chronic infection of the gall bladder (40, 45, 46). Even though humans that chronically carry *S. typhi* often have biliary tract disease, its presence is not an absolute requirement for development of the carrier state (10, 40). A previous study in primates demonstrated that *S. typhi* was carried exclusively in MLN at 50 d after an oral infection of chimpanzees (47). In the case of *S. pullorum*, it was recently shown that bacteria are carried in the spleen and reproductive tract, specifically the ovary and oviduct of hens (48). Our data suggest that the site of chronic carriage of *S. typhimurium* in mice is the MLN and not the gall bladder. However, we did find that all of the mice that were shedding *S. typhimurium* at the time of death (*n* = 5) were colonized in the gall bladder as well as the liver (Table I). Perhaps the true reservoir of persistent bacterial carriage even in humans is the MLN, and some change in host immune status leads to spread from the RES to the liver and gall bladder, which in turn leads to reseeding of the intestine and fecal shedding as is seen during a primary infection.

Although the site of *Salmonella* proliferation in susceptible strains of mice has been shown to be within macrophages in the spleen and liver (49, 50), the location of *S. typhimurium* persistence during chronic infection had not been determined previously. Our tissue colonization data indicated that viable bacteria were consistently isolated from MLN of persistently infected mice. We show here by microscopy of immunohistochemically stained tissue sections that 82% of the persisting bacteria reside inside macrophages within the MLN. We cannot say that all of the bacteria detected with our anti–*S. typhimurium* antibody are

viable; however, the large percentage of bacteria residing inside macrophages certainly suggests that this population serves as the persistent reservoir of *S. typhimurium*. Furthermore, bacteria were able to persist in the presence of high levels of circulating specific antibody against *S. typhimurium*. Thus, although an effective antibody response is generated and is likely to be important in clearing extracellular bacteria, the intracellular bacteria can survive the host's innate and adaptive immune defenses for as long as a year in 60% of the mice.

The fate of macrophages persistently infected with *S. typhimurium* is not known. It is possible that bacteria persist within macrophages for the lifetime of the host cell, and then infect a new macrophage. However, *S. typhimurium* is able to induce host cell death in vivo (49, 51). *S. typhimurium* mediates macrophage death by at least two mechanisms. One mechanism involves a very rapid macrophage death that requires the type III secretion system (TTSS) encoded on *Salmonella* pathogenicity island (SPI) 1, (52). *S. typhimurium* can also induce macrophage death that occurs ~18 h after infection. This delayed macrophage death requires a second TTSS that is encoded on SPI 2 and is used inside host cells (53, 54). Perhaps the dead or dying macrophages containing *S. typhimurium* are phagocytosed by macrophages that are recruited to the site of infection and serve as a safe haven for *Salmonella* to survive while avoiding extracellular host defenses.

The high antibody titer that we found at 365 d after infection in the mice where *S. typhimurium* was recovered indicates that the continued presence of bacteria is needed for this high sustained humoral response and that the immune response has not been totally abrogated. Perhaps the humoral response participates in keeping the number of bacteria inside each macrophage lower in our persistent *S. typhimurium* model compared with what has been reported in previous studies of acute infections (50). In this work, we did not determine whether antibodies to specific *S. typhimurium* antigens correlated with differences in those animals that eventually clear the infection as compared with those that continue to be persistently infected; this is one goal in our ongoing investigation. However, the adaptive immune response also provides a positive feedback to the innate immune system through the synthesis of cytokines that either increase effector cell numbers or activate these cells for an increased antibacterial performance.

Once activated T cells are present, they provide the macrophage-activating factor IFNγ. This type II interferon acts in concert with bacterial components (e.g., LPS) to generate macrophages with a maximal capacity to ingest and kill bacteria (55). IFNγ has been shown to be important for the maintenance of the latent state in the *M. tuberculosis* mouse model of infection as well as the latent state of *Chlamydia trachomatis* in cell culture (43, 56). Previous studies of the role of IFNγ in the acute *Salmonella* mouse infection have shown that IFNγ plays a role in controlling the early phase of *Salmonella* replication, but is not necessary for bacterial clearance (57–59). We tested the role of

IFNγ in the persistent *S. typhimurium* infection in mice by neutralizing this cytokine in chronically infected mice. Mice infected with *S. typhimurium* for 260 d showed no signs of illness before IFNγ neutralization. However, within 3 wk after treatment with the IFNγ neutralizing antibody, three out of five mice began to show signs of acute illness, and upon dissection for organ bacterial counts showed very high levels of *S. typhimurium* in some of the tissues compared with the group of mice that received the control antibody. Although the levels of bacteria isolated from the MLN, spleen, liver, and gall bladder of the mice injected with the neutralizing antibody were relatively low, all of these mice were colonized in the cecum, and four of the five mice were colonized in the PP. The role of IFNγ in colonization of the gastrointestinal tract relative to RES colonization is unclear. Perhaps the increased bacterial load in the gastrointestinal tract is due to a difference in the access of circulating antibodies within the RES compared with PP and cecum. Perhaps when intracellular bacteria are released they are killed by antibody-mediated killing, thus keeping the levels in the organs low. In contrast, the antibody-mediated killing does not occur in the intestine, allowing high levels of *S. typhimurium* replication. An alternate explanation for these data is that the high level of gastrointestinal colonization reflects reinfection of the PP and cecum upon ingestion of contaminated feces. We found that the levels of *S. typhimurium* recovered from the gall bladders of the anti–IFNγ-treated mice were very low. It is unclear why the classic explanation that fecal shedding represents contamination from the infected gall bladder does not seem to be borne out in our IFNγ neutralization results. We also found that three of the mice treated with the neutralizing antibody had pericarditis and pleuritis, which was associated with the presence of *S. typhimurium* in the thoracic exudates in these animals, likely indicating that the reactivation of a systemic infection led to colonization of distant sites in three of the treated mice. *Salmonella spp.* have been associated with cardiac infections in humans, typically occurring in people with underlying heart disease (60, 61); perhaps we are reproducing this situation in the treated mice. Our data indicate that IFNγ plays a role in maintaining the balance in the immune status of persistently infected mice, perhaps by stimulating the infected macrophage to suppress bacterial replication.

Previous studies have shown that *S. typhimurium* may limit the in vivo proliferation of CD4^{+} and CD8^{+} T cells despite their activated phenotype (36). Additionally, it has been shown that active *S. typhimurium* infection induces immunosuppression in mice and causes the production of large amounts of IL–10 and nitric oxide, which are both known immunosuppressive compounds (58, 62–64). We believe that *Salmonella* is not an innocent bystander in maintaining the balance between clearance and persistence. In this regard, *Salmonella* may play an active role in modulating or even directly manipulating the host responses, thereby preventing clearance of intracellular *S. typhimurium*.

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