IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

***Salmonella* Meningitis Associated with Monocyte Infiltration in Mice**

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In the current study, we examined the ability of *Salmonella enterica* serovar Typhimurium to infect the central nervous system and cause meningitis following the natural route of infection in mice. C57BL/6J mice are extremely susceptible to systemic infection by *Salmonella* Typhimurium because of loss-of-function mutations in *Nramp1* (SLC11A1), a phagosomal membrane protein that controls iron export from vacuoles and inhibits *Salmonella* growth in macrophages. Therefore, we assessed the ability of *Salmonella* to disseminate to the central nervous system (CNS) after oral infection in C57BL/6J mice expressing either wild-type (resistant) or mutant (susceptible) alleles of *Nramp1*. In both strains, oral infection resulted in focal meningitis and ventriculitis with recruitment of inflammatory monocytes to the CNS. In susceptible *Nramp1*^{-/-} mice, there was a direct correlation between bacteremia and the number of bacteria in the brain, which was not observed in resistant *Nramp1*^{+/+} mice. A small percentage of *Nramp1*^{+/+} mice developed severe ataxia, which was associated with high bacterial loads in the CNS as well as clear histopathology of necrotizing vasculitis and hemorrhage in the brain. Thus, *Nramp1* is not essential for *Salmonella* entry into the CNS or neuroinflammation, but may influence the mechanisms of CNS entry as well as the severity of meningitis. (*Am J Pathol* 2017, 187: 187–199; <http://dx.doi.org/10.1016/j.ajpath.2016.09.002>)

Meningitis caused by *Salmonella enterica* serovars is a life-threatening disease with high fatality rates and frequent long-term neuropsychological sequelae.¹ Although this is a rare disease in most parts of the world, in sub-Saharan Africa nontyphoidal *S. enterica*, particularly serovar Typhimurium (*Salmonella* Typhimurium), is now one of the most common causes of bacterial meningitis.^{2,3} In severe cases, *Salmonella* meningitis can lead to ventriculitis, hydrocephalus, or cerebral abscess.^{4–7}

Animal models are essential to study of the mechanisms involved in bacterial meningitis, particularly entry into the central nervous system (CNS) and induction of meningeal inflammation. Recently, *Salmonella* Typhimurium was shown to be able to infect the CNS after oral inoculation in mice.^{8,9} In addition, a few animals developed clinical signs of rolling that may be indicative of *Salmonella*-induced neurological damage.⁹ These studies indicate that mice may be a useful model to study *Salmonella* meningitis after the natural oral route of infection. A natural route of

infection would allow analysis of the relative rate of *Salmonella* infection in the CNS, the correlation of brain infection with peripheral infection, and the association of an inflammatory response with infection in the brain. Furthermore, a natural route model would allow for the analysis of how the peripheral host immune response influences the ability of *Salmonella* to gain access to the brain as well as the development of bacterial meningitis.

In mice, a significant component of innate resistance to *Salmonella* Typhimurium is because of the natural resistance-associated macrophage protein 1 (*Nramp1*; alias *Slc11a1*), a divalent cation transporter expressed in cells of

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the monocyte/macrophage lineage. NRAMP1 limits the amount of iron within phagosomes and thus restricts replication of vacuolar pathogens, such as *Salmonella*, within macrophages.¹⁰ During the early phase of infection, NRAMP1 restricts the intracellular replication of *Salmonella* in the reticuloendothelial organs.¹¹ Murine strains with homozygous loss-of-function mutations in *Nramp1* (eg, BALB/c and C57BL/6) develop lethal acute infections, whereas mice with functional *Nramp1* (eg, Sv129S6) develop a chronic or sustained infection. Resistant mice often show no clinical disease, although *Salmonella* Typhimurium can persist for long periods within macrophages in the mesenteric lymph nodes.^{12–15} Interestingly, *Salmonella* Typhimurium has been detected in the CNS of both *Nramp1*^{+/+} and *Nramp1*^{-/-} strains of mice.^{8,9}

To directly examine the influence of *Nramp1* on dissemination to the CNS and subsequent meningitis, we infected wild-type C57BL/6J (*Nramp1*^{-/-}) and *Nramp1*-reconstituted C57BL/6 (*Nramp1*^{+/+}) mice with *Salmonella* Typhimurium by oral gavage. *Nramp1*^{+/+} C57BL/6 are more resistant than wild-type C57BL/6 mice¹³ and provide a model to analyze how a stronger innate immune response may influence *Salmonella* infection of and damage to the brain. We examined the time course of *Salmonella* Typhimurium infection in the brain, the localization of bacteria within the CNS, and the neuroinflammatory response to infection. Almost all mice had detectable *Salmonella* in the CNS and developed mild foci of inflammation in the meninges, although the time course of infection and the histopathology differed between *Nramp1*^{-/-} and *Nramp1*^{+/+} strains. In addition, a few *Nramp1*^{+/+} mice developed a more severe CNS infection with clinical signs of neurological disease and histological features consistent with *Salmonella*-induced meningitis in humans, including ventriculitis and abscesses.

Materials and Methods

Ethics Statement

All animal work was performed in accordance with the Animal Welfare Act and Public Health Service Policy. Animal experiments were performed at the Rocky Mountain Laboratories (Hamilton, MT) or the University of Colorado at Boulder under protocols approved by the Institutional Animal Care and Use Committees (protocol numbers: RML 2014-028, RML 2014-070, UCB 1307.02).

Mice

C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 129SvEvTac mice (Taconic Laboratories, Hudson, NY), and C57BL/6 *Nramp1*^{G169D} mice homozygous for the wild-type *Nramp1* (*Slc11a1*) allele derived from Sv129 mice¹⁶ (Dr. Ferric Fang and Dr. Steve Libby, University of Washington, Seattle) were used for these studies. Mice were

kept in pathogen-free conditions with free access to food and water, except where noted.

Bacterial Infections

Salmonella enterica serovar Typhimurium wild-type strain SL1344¹⁷ was grown to stationary phase for 18 hours at 37°C with shaking in Luria-Bertani–Miller broth supplemented with streptomycin (100 µg/mL). Bacteria were pelleted and diluted to the appropriate concentration based on the OD at 600 nm. The actual inoculum size was verified by plating on Luria-Bertani plates for colony-forming units (CFUs). Adult 6- to 8-week-old 129SvEvTac (Taconic Laboratories), C57BL/6 *Nramp1*^{G169D} (*Nramp1*^{-/-}), and C57BL/6 *Nramp1*^{Gly169} (*Nramp1*^{+/+}) mice were without food and water for 2 hours before oral inoculation (oral gavage) with *Salmonella*. The inoculum was administered in 100 µL of sterile buffered pharmaceutical grade saline (Mediatech, Manassas, VA) using a 20-gauge gavage needle. Food and water were restored 1 hour after inoculation. Mice were monitored daily for signs of clinical illness (ie, ruffled fur, hunched posture, or ataxia). Under anesthesia, blood was collected via cardiac puncture before transcardial perfusion with 5 mL of heparin sterile buffered pharmaceutical grade saline (100 U/mL). Tissues (brain, spleen, and liver) were collected in preweighed tubes containing 500 µL sterile buffered pharmaceutical grade saline, homogenized with a Precellys 24 homogenizer, and adjusted for concentration (based on weight) before plating and enumeration of CFUs. For most studies, the brain was divided into two sagittal sections, one half used for CFU counts and the other used for immunohistochemistry or flow cytometry.

Flow Cytometry

Brain tissue was homogenized using a 7-mL dounce homogenizer in 2 mL phosphate-buffered saline (PBS). Homogenate was strained using a 70 µmol/L cell strainer (Bioexpress, Kaysville, UT). The microglia fraction was collected at the 70%/30% interface of a 70%/30%/0% Percoll gradient after centrifugation. Cells were further processed for flow cytometry staining. Fc receptors were blocked using CD16/CD32 Fcγ III/II (clone 2.4G2; BD Biosciences, San Jose, CA). Cells were stained using a combination of the following antibodies: CD11c-PE/Cy7 (clone HL3; BD Biosciences), F4/80-e450 (clone BM8; eBiosciences), CD45- PE (clone 30-F11; BD Biosciences), Ly6C-AF700 (clone AL-21; BD Biosciences), Ly6G-APC/Cy7 (clone 1A8; BD Biosciences), CD4-APC/Cy7 (clone GK1.5; BD Biosciences), CD8-PE (clone 53-6.7; BD Biosciences), NK1.1-AF700 (clone PK136; BD Biosciences), and CD45R/B220 (clone RA3-6B2; BD Biosciences), for 1 hour on ice, followed by a wash step and fixation in 2% paraformaldehyde for 20 minutes at room temperature. Cells were washed, resuspended in PBS, and processed for flow cytometry analysis. All flow analysis was done on a LSR II cytometer (BD Biosciences) and analyzed using FloJo software version 10.0.7 (FlowJo, Ashland, OR).

Histopathology

Tissues were fixed in 10% neutral buffered formalin (Leica Biosystems, Buffalo Grove, IL) for a minimum of 7 days before being placed in cassettes and processed with a Leica RM2265 microtome. Embedded tissues are divided into sections (5 μ m thick) and dried overnight at 42°C before staining. Samples were deparaffinized and rehydrated using a graded series of xylene, ethanol (100%, 95%, 70%), and PBS. Antigen retrieval was performed in a citrate buffer using a Decloaking Chamber (Biocare Medical, Concord, CA) at 120°C for 20 minutes under high pressure. After PBS wash and blocking (2% donkey serum, 1% bovine serum albumin, 0.1% Triton X-100, 0.05% Tween-20 in PBS), the tissues were stained using rabbit anti-Iba1 (1:200; Wako, Richmond, VA), rabbit anti-glia fibrillary acidic protein (1:500; Agilent, Santa Clara, CA), rabbit anti-CD3 (1:100; Dako), and goat anti-*Salmonella* common structural antigen (1:500; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) primary antibodies. Secondary antibodies used were Alexa Fluor 488–conjugated chicken anti-rabbit and Alexa Fluor 594–conjugated chicken anti-goat (1:300; Life Technologies, Carlsbad, CA). Samples were mounted with ProLong Gold with DAPI (Life Technologies).

Pathology Scoring

All samples were scored blindly (D.S.) using the following criteria to assess for infiltration of inflammatory cells (ie, neutrophils) and presence of meningitis and/or encephalitis. Score 0, no lesion; 1, focal infiltration of the meninges and/or gray matter by small numbers of lymphocytes, macrophages, and rare neutrophils; 2, focal to multifocal foci in the meninges and gray matter or olfactory bulb that is infiltrated by small numbers of lymphocytes, macrophages, and few neutrophils, may be minimal hemorrhage; 3, multifocal areas of encephalomalacia and meningitis with infiltration by moderate numbers of neutrophils, macrophages, and fewer lymphocytes, multifocal vasculitis with or without fibrin thrombi, multifocal mild hemorrhage, and numerous extracellular bacilli; and 4, coalescing areas of encephalomalacia and meningitis with infiltration by moderate numbers of neutrophils, macrophages, and fewer lymphocytes, multifocal vasculitis with or without fibrin thrombi, multifocal moderate hemorrhage, and numerous extracellular bacilli.

Imaging

Confocal images were captured using ZEN software version 8.1.10 (Carl Zeiss, Thornwood, NY) on a Carl Zeiss LSM710 confocal laser scanning microscope with either a Plan APO-CHROMAT 63 $\times$ /1.4 numerical aperture (NA) or a 20 $\times$ /0.8 NA objective. Wide field images were acquired using Nikon Elements software version 3.2 on a Nikon Eclipse 55i microscope with either a 40 $\times$ /0.75 NA or a 20 $\times$ /0.50 NA objective and a Nikon DS-Ri1 digital camera (Nikon, Melville, NY). Slides were also imaged using ScanScope software version 102.0.0.33 (Leica) on either an Aperio ScanScope Fluorescent slide scanner or an Aperio XT Brightfield slide scanner,

both equipped with a UPLSAPO 20 $\times$ /0.75 NA objective (Leica). All image analysis was done with ImageJ (version 2.0.0 W.S. Rasband; NIH, Bethesda, MD) and Adobe Photoshop CS5.1 (Adobe, San Jose, CA).

Statistical Analysis

Graphpad software Statmate version 2.00 (GraphPad, La Jolla, CA) was used to determine group sizes before experimentation, and Graphpad Prism software version 7 was used for all statistical analyses. The tests are indicated in the figure legends.

Results

Detection of *Salmonella* in the Brain after Oral Infection in *Nramp1*^{−/−} and *Nramp1*^{+/+} Mice

Wild-type strains of *Salmonella* Typhimurium can disseminate to the brain and cause meningoencephalitis in susceptible mice, but it is not clear how this correlates with systemic infection and whether there is a dose-dependent response.^{8,9} To investigate these issues, we infected C57BL/6 (*Nramp1*^{−/−}) mice by oral gavage with doses ranging from 10⁴ to 10⁹ CFUs. Mice were monitored daily and euthanized when they developed clinical signs of salmonellosis (hunched posture and ruffled fur). No mice showed signs of neurological disease (ataxia, rolling, jumping, twirling, and loss of motor control). To avoid blood contamination of tissues, particularly the brain, transcardial perfusion was performed on all mice immediately before removing tissues. Bacterial loads were examined in the brain, blood, spleen, and liver by plating (Figure 1A). Irrespective of the inoculating dose, all mice ($n = 10$) with clinical bacteremia had high bacterial loads in the liver (10⁶ to 10⁸ CFUs/g), spleen (10⁷ to 10⁸ CFUs/g), and blood (10⁴ to 10⁷ CFUs/mL). Bacteria (10⁴ to 10⁶ CFUs/g) were also recovered from the brains of all of the symptomatic mice, although at lower levels than those found in the spleen or liver. Thus, bacteremia in C57BL/6 *Nramp1*^{−/−} mice correlated with infection of the CNS and did not show a dose dependence.

Although C57BL/6 mice are widely used as a model for systemic salmonellosis, their inability to control *Salmonella* Typhimurium during the early phase of infection means that they succumb to infection within 2 weeks (Figure 2). In contrast, *Nramp1*-reconstituted (*Nramp1*^{+/+}) C57BL/6 mice control early infection, providing a useful model to study how the immune response may influence *Salmonella* infection of the CNS. To establish a baseline for the meningitis studies, we first compared the course of infection in *Nramp1*^{−/−} and *Nramp1*^{+/+} C57BL/6 mice after oral gavage with 10⁸ or 10⁹ CFUs, respectively. These doses were selected based on initial experiments and on published studies so that 100% of the mice should develop systemic infection.¹³ Under these conditions, <30% of *Nramp1*^{+/+} mice developed clinical signs of disease by day 21, whereas all of the *Nramp1*^{−/−} mice developed clinical signs of

Figure 1 *Salmonella* infection and dissemination in *Nramp1*^{-/-} and *Nramp1*^{+/+} mice. **A**: Organ colonization of *Nramp1*^{-/-} mice with clinical salmonellosis. Mice were infected orally with 10⁴, 10⁶, 10⁷, or 10⁸ *Salmonella*. Samples were collected on development of clinical disease, up to 10 days postinfection (dpi). **B**: Percentage survival of *Nramp1*^{-/-} and *Nramp1*^{+/+} mice infected with 10⁸ or 10⁹ colony-forming units (CFUs) *Salmonella*, respectively. **C**: Bacterial loads in *Nramp1*^{-/-} mice infected with 10⁸ *Salmonella*. **D**: Bacterial loads in *Nramp1*^{+/+} mice infected with 10⁹ *Salmonella*. Mice with clinical ataxia or rolling disease are shown separately. **E** and **F**: Relationship between bacterial loads in the blood and brain of *Nramp1*^{-/-} (**E**) or *Nramp1*^{+/+} (**F**) mice at 6 or 21 days postinfection. Data were fitted using a linear regression model of transformed log-log CFU data. Mice with ataxia are indicated. Data represent means ± SD (**C** and **D**). *n* = 3 (**A**, 10⁴ and 10⁷ *Salmonella*); *n* = 2 (**A**, 10⁶ and 10⁸ *Salmonella*); *n* = 50 (**B**, *Nramp1*^{-/-}); *n* = 54 (**B**, *Nramp1*^{+/+}); *n* = 3 to 14 mice per time point (**C**); *n* = 2 to 20 mice per time point (**D**).

salmonellosis and were euthanized by day 10 (Figure 1B). The difference between these strains is consistent with previous findings¹³ and provides a model to study the effect of the peripheral immune response on *Salmonella* infection of the CNS.

To examine when *Salmonella* colonizes the CNS, and how *Nramp1* influences this process, we did a time course analysis of infection in *Nramp1*^{+/+} and *Nramp1*^{-/-} mice. Bacterial loads were determined daily, up to day 6, in *Nramp1*^{-/-} mice and on days 2, 7, 14, and 21 in *Nramp1*^{+/+} mice (Figure 1, C and D, and Supplemental Figures S1 and S2). Bacteria were recovered from the liver and spleen of most mice within 1 to 2 days of infection, although, as expected, the levels of bacteria did not peak until after 7 days in the *Nramp1*^{+/+} mice. Bacteria were recovered from the blood as early as 2 days postinfection (dpi) in *Nramp1*^{-/-} mice and 7 dpi in *Nramp1*^{+/+} mice. Infection of the brain was slightly delayed compared to blood and peripheral tissues, with >50% of mice having recoverable bacteria in the brain at 4 dpi in *Nramp1*^{-/-} mice (Supplemental Figure S1) and 14 dpi in *Nramp1*^{+/+} mice (Supplemental Figure S2). The bacterial load in the brain, which ranged from undetectable to 10⁶, was consistently lower than that in the spleen or liver for both *Nramp1*^{-/-} and *Nramp1*^{+/+} mice (Figure 1, C and D), with the exception of

three *Nramp1*^{+/+} mice that had clinical signs of ataxia (Table 1). Thus, bacterial infection of the CNS was observed in most animals for both *Nramp1*^{-/-} and *Nramp1*^{+/+} mice after oral inoculation.

Salmonella Colonization of the Brain Correlates with Bacteremia in *Nramp1*^{-/-} but Not *Nramp1*^{+/+} Mice

The kinetics of bacterial loads in the brain followed the same trend as bacterial loads in the blood for both *Nramp1*^{-/-} and *Nramp1*^{+/+} mice (Figure 1, C and D). Therefore, to investigate whether there was a direct correlation, we compared bacterial loads in the blood and brain for individual mice. A direct correlation was observed in *Nramp1*^{-/-} mice (*R*² = 0.9450) (Figure 1E); however, no correlation was observed in *Nramp1*^{+/+} mice (*R*² = 0.4744) (Figure 1F). This suggests that *Salmonella* levels in the blood may be more important for neuroinvasion in *Nramp1*^{-/-} mice, compared to *Nramp1*^{+/+} mice.

Infection of the CNS in Mice Resistant to *Salmonella* Bacteremia

Nramp1 is only one of the genes that influences susceptibility to *Salmonella* infection in mice.^{13,18} 129S6 mice are

Figure 2 *Salmonella* colonization of the brain of 129S6 (129SvEv) mice. Mice were orally infected with 10^9 *Salmonella*. Samples were collected 2 weeks after infection. Each symbol represents one mouse. Means and SEM are shown. $n = 6$. CFU, colony-forming unit.

more resistant to *Salmonella* infection than the reconstituted *Nrap1*^{+/+} C57BL/6 mice.¹³ To determine whether other genetic factors may also influence *Salmonella* infection in the brain, we examined 129S6 mice. However, even in the more resistant 129S6 mice, *Salmonella* could be readily detected in the brain (Figure 2), suggesting that even restriction factors that prevent bacteremia were not sufficient to prevent *Salmonella* from entering the CNS.

Salmonella Infection Results in Inflammatory Monocyte Recruitment to the Brain

A characteristic feature of bacterial meningitis, in humans and animal models, is the recruitment of inflammatory cells, including monocytes, macrophages, and neutrophils.^{19,20} To assess which inflammatory cells are recruited during *Salmonella* infection, we isolated cells from the brain by

Percoll gradient and analyzed them for different cell markers by flow cytometry. We selected 4 and 6 dpi for *Nrap1*^{-/-} mice and 7 and 14 dpi for *Nrap1*^{+/+} mice based on the bacterial loads in the brain at those time points (Figure 1). For each mouse, half of the brain was used for flow cytometry, whereas the other half was used to determine the bacterial load. Infiltrating cells were separated from resident microglia and other brain cells by CD45^{hi} expression (Figure 3A). Cells were then defined as dendritic cells (CD11c⁺), T cells (CD3⁺), B cells (CD45R/B220⁺), natural killer cells (NK1.1⁺), or myeloid cells (CD11b⁺). Myeloid cells were further characterized as neutrophils (Ly6G⁺), inflammatory M1 monocytes (Ly6C^{hi} and Ly6G⁻), or nonclassic M2 monocytes (Ly6C^{lo} and Ly6G⁻) (Figure 3A).

Infiltrating (CD45^{hi}) cells were observed in both strains, relative to mock-infected controls (Figure 3B). Mice with detectable CFUs in the CNS generally had a higher percentage of infiltrating cells relative to infected mice without detectable CFUs (Figure 3B). In both strains, the infiltrating cells were composed primarily of M1 and M2 monocytes with lower levels of neutrophils, dendritic cells, T cells, B cells, and natural killer cells (Figure 3B and Supplemental Figure S3). Thus, *Salmonella* infection resulted in recruitment of inflammatory cells to the brain, primarily M1 and M2 monocytes, in both *Nrap1*^{+/+} and *Nrap1*^{-/-} mice. Interestingly, the presence of NRAMP1, which inhibits the ability of *Salmonella* to replicate in monocytes, did not appear to substantially affect the cell types in the CNS. Similar to monocyte infiltrate in the brain, we also found increased M1 and M2 monocytes in the blood of both *Nrap1*^{+/+} and *Nrap1*^{-/-} mice (Supplemental Figure S4). Neutrophils were also increased in *Nrap1*^{+/+} mice, whereas an increase in natural killer cells was observed in *Nrap1*^{-/-} mice. The increase in both monocyte populations in the blood correlates with the detection of these cell types in the brain and indicates a proliferation of monocytes from the bone marrow in response to *Salmonella* infection in both *Nrap1*^{+/+} and *Nrap1*^{-/-} mice.

Histological Analysis Demonstrates More Severe Meningitis and Encephalitis in *Nrap1*^{+/+} Mice Compared to *Nrap1*^{-/-} Mice

In both *Nrap1*^{-/-} and *Nrap1*^{+/+} mice, we observed consistent bacterial infection and inflammatory cell recruitment to the brain after *Salmonella* infection (Figures 1 and 3), which are indicative of meningitis or encephalitis. Next, hematoxylin and eosin–stained sections of brain tissue were analyzed in a blinded manner (D.S.) by a pathologist using a scoring system of 0 to 4 to rank meningitis and encephalitis (Tables 1 and 2). Only 1 of 27 (approximately 4%) *Nrap1*^{-/-} mice scored positive for meningitis, whereas 10 of 20 (50%) *Nrap1*^{+/+} mice scored positive for meningitis and/or encephalitis. Of the mice that scored positive by histology, four were

Table 1 Comparison of Clinical Signs and Histopathology

Variable	Clinical diagnosis*		Histopathology positive†	
	<i>Nrap1</i> ^{-/-}	<i>Nrap1</i> ^{+/+}	<i>Nrap1</i> ^{-/-}	<i>Nrap1</i> ^{+/+}
Total analyzed	27	20	1	10
Asymptomatic	19	9	1	3
Salmonellosis	8	6	0	3
Roller	0	2	0	1
Ataxic	0	3	0	3

*Mice analyzed include animals at earlier time points (Figure 1C) that had not progressed to clinical disease but were analyzed for histological changes.

†Number of animals from Clinical Diagnosis columns that scored positive for clear signs of meningitis or encephalitis by blinded histological examination (D.S.) of hematoxylin and eosin sections.

A **B**

Figure 3 Infiltration of immune cells in the brains of infected *Nramp1*^{-/-} and *Nramp1*^{+/+} mice. Mice were orally infected with *Salmonella*, and samples were collected on day 6 (*Nramp1*^{-/-}) and day 14 (*Nramp1*^{+/+}) after infection. **A:** Example of gated analysis used for determining M1 and M2 cell phenotypes. **B:** Percentage of infiltrating immune cells in the brains of infected *Nramp1*^{-/-} (red symbols) and *Nramp1*^{+/+} (blue symbols) mice. Half of the brain was used to estimate the bacterial load [colony-forming units (CFUs)], whereas the other half was processed for flow cytometry. Infected mice, where no CFUs were detected, are indicated for *Nramp1*^{-/-} (red squares) and *Nramp1*^{+/+} (blue squares). Mock-infected *Nramp1*^{-/-} (white diamonds) and *Nramp1*^{+/+} (white triangles) mice were used as controls. Each symbol represents one mouse. *n* = 8 (B, infected and mock-infected *Nramp1*^{-/-} mice); *n* = 6 (B, infected and mock-infected *Nramp1*^{+/+} mice). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. NK, natural killer.

Table 2 Individual Pathology Scores of Mice

Mouse no.	<i>Nramp1</i>	Clinical diagnosis	CFU/g	Meningitis*	Encephalitis†
51-2	—/—	Asymptomatic	2.00×10^4	1	2
92-4	+/+	Asymptomatic	1.40×10^4	1	0
126-2	+/+	Asymptomatic	4.60×10^3	2	0
130-1	+/+	Asymptomatic	1.10×10^3	0	2
92-3	+/+	Salmonellosis	8.80×10^4	1	0
135-2	+/+	Salmonellosis	3.60×10^5	0	2
149-1	+/+	Salmonellosis	4.70×10^3	0	1
155-1	+/+	Roller	3.40×10^3	1	0
93-3	+/+	Ataxic	1.90×10^8	4	4
126-1	+/+	Ataxic	2.50×10^8	2	4
135-1	+/-	Ataxic	1.30×10^8	4	4

*Tissue was scored for meningitis based on a ranking of 0 to 4, as described in [Materials and Methods](#).

†Tissue was scored for encephalitis based on a ranking of 0 to 4, as described in [Materials and Methods](#).

CFU, colony-forming unit; +, positive; —, negative.

asymptomatic, three had salmonellosis, one showed signs of rolling, and three had signs of ataxia ([Table 2](#)); all had detectable CFUs in the brain. Thus, meningitis and encephalitis were observed in a greater percentage of *Nramp1*^{+/+} mice compared to *Nramp1*^{-/-} mice and were observed in both asymptomatic and symptomatic mice. The three *Nramp1*^{+/+} mice with ataxia had the highest bacterial load in their brains (10^8 CFUs/g) as well as the highest scores for meningitis (4, 2, 4) and encephalitis (4, 4, 4).

Immunohistochemistry Analysis Shows Focal Areas of Inflammation in the Meninges and Ventricles of Both *Nramp1*^{-/-} and *Nramp1*^{+/+} Mice

To determine the localization of *Salmonella* and inflammation in the brain of infected mice, we used immunohistochemistry. Tissue sections were stained with anti-Iba1 to detect microglia and monocytes, anti-glial fibrillary acidic protein to detect astrocytes, anti-CD31 to detect

Figure 4 Meningitis in *Nramp1*^{-/-} mice after *Salmonella* infection. Brain tissue of *Nramp1*^{-/-} mice orally infected with *Salmonella* was fixed and immunostained up to 10 days after infection. **A** and **B**: Extended representative images of uninfected (**A**) and infected (**B**) brain tissue immunostained for Iba1 (green), *Salmonella* (red), and DAPI (blue). **Arrowheads** indicate regions of meningitis. **C–F**: High-magnification representative images of uninfected (**C** and **E**) and infected (**D** and **F**) brain tissue immunostained for *Salmonella* (red), DAPI (blue), and either glial fibrillary acidic protein (GFAP) or Iba1 (green). Scale bars = 40 μ m (**A–F**).

Figure 5 Meningitis in *Nrampl1*^{+/+} mice after *Salmonella* infection. Brain tissue of *Nrampl1*^{+/+} mice orally infected with *Salmonella* was fixed and immunostained up to 21 days after infection. **A** and **B**: Extended representative images of uninfected (**A**) and infected (**B**) brain tissue immunostained for Iba1 (green), *Salmonella* (red), and DAPI (blue). **Arrowheads** indicate regions of meningitis. **C–F**: High-magnification representative images of uninfected (**C** and **E**) and infected (**D** and **F**) brain tissue immunostained for *Salmonella* (red), DAPI (blue), and either glial fibrillary acidic protein (GFAP) or Iba1 (green). Scale bars: 40 μ m (**A** and **B**); 20 μ m (**C–F**).

endothelial cells, and anti-lipopolysaccharide to detect *Salmonella*. Focal areas of inflammation consisting of Iba1⁺ monocytes/macrophages (green fluorescence) and *Salmonella* were observed in the meninges and ventricles in most mice with detectable CFUs in the brain (Figures 4 and 5). These mice included animals that were scored negative for meningitis and encephalitis, possibly because of the focal nature of the areas of inflammation. Interestingly, *Salmonella* in these focal areas was generally more intense in *Nrampl1*^{-/-} mice (Figure 4) than in *Nrampl1*^{+/+} mice (Figure 5). These focal areas of infection were associated with increased glial fibrillary acidic protein staining at the meningeal layer (Figures 4 and 5), indicating astrocyte activation in response to infection. *Salmonella* was often found within Iba1⁺ monocytes in the meningeal layer (Figure 4, Figure 6, A and B, and Figure 7), but extracellular bacteria were occasionally found within the brain parenchyma (Figure 6C and Figure 7) in both *Nrampl1*^{-/-} and *Nrampl1*^{+/+} mice. Focal areas of meningitis and ventriculitis were observed in both strains of mice, consisting of *Salmonella*, *Salmonella*-infected monocytes, and other inflammatory cells. These immunohistochemistry studies correlated with the detection of *Salmonella* CFUs in the brain of both *Nrampl1*^{-/-} and *Nrampl1*^{+/+} mice (Figure 1), as well as the increase in monocytes (Figure 3).

Pathology Correlates with Neurological Signs of Ataxia in *Nrampl1*^{+/+} Mice

Of the 53 *Nrampl1*^{+/+} mice infected with *Salmonella*, 5 (10%) developed clinical signs of disease clearly distinguishable from systemic salmonellosis (hunched posture and ruffled fur). These fell into two distinct groups. Two mice displayed barrel rolling and spinning behaviors, whereas three displayed classic signs of ataxia, including hind limb tremors, failure to splay legs, grasping of paws, and altered gait. Comparison of the bacterial burdens of these two groups (Figure 1D) revealed that the ataxic mice had much higher bacterial loads in the brain (>10⁸ CFUs/g), whereas the roller mice had loads similar to asymptomatic mice (10³ CFUs/g). Remarkably, although the bacterial burden in the brains of ataxic mice was 1000- to 10,000-fold higher than all other mice, the bacterial levels in other organs remained comparable (Figure 1D). Thus, the three *Nrampl1*^{+/+} animals that developed signs of neurological disease were associated with high bacterial levels in the brain.

Imaging of brain tissue from the three ataxic animals revealed multiple areas of necrotizing vasculitis often occluded by fibrin thrombi (Figure 8). Brains from these three mice contained periventricular malacia associated with infiltration of large numbers of intact and degenerate neutrophils,

Figure 6 *Salmonella* intracellular infection of the ventricle, meninges, and parenchyma. Brain tissue of *Nramp1*^{-/-} mice orally infected with *Salmonella* was fixed and immunostained up to 10 days after infection. **A:** Representative image of infected ventricular brain tissue immunostained for Iba1 (green), *Salmonella* (red), and DAPI (blue). **Arrowheads** indicate examples of intracellular bacteria. **B** and **C:** XYZ projections illustrating presence of intracellular and extravascular bacteria in the meninges (**B**) and parenchyma (**C**) immunostained for *Salmonella* (red), DAPI (blue), and either Iba1 (green; **B**) or CD31 (green; **C**). XZ or YZ images were acquired in the XY plane with slices taken along the Z-axis. Scale bars: 20 μm (**A**); 10 μm (**B** and **C**).

which were not observed in uninfected controls (Figure 8). Similar signs of inflammation were observed in the lateral and third ventricles (Figure 8). Thus, ataxic mice had histological changes characteristic of Gram-negative bacillary meningoencephalitis.²¹ Fluorescence immunohistochemistry and confocal microscopy revealed that the severe histological changes in the ataxic *Nramp1*^{+/+} mice were associated with areas of bacterial infection. *Salmonella* was observed in the olfactory bulb (Figure 9A), periventricular areas (Figure 9B), ventricles (Figure 9, C and D), and meninges (Figure 9, E and F). Most *Salmonella* was found in the areas of

periventricular malacia and neutrophil infiltration (Figure 9, B–D), but was not directly observed around blood vessels associated with necrotizing vasculitis (areas of red blood cell infiltration as shown by white cells) (Figure 9B). Thus, *Salmonella* infection was widespread in ataxic mice, although primarily localized to the ventricles, periventricular areas, and meninges.

Immunohistochemistry of brain tissue sections from the two roller mice did not show significant bacteria (Supplemental Figure S5A), consistent with the lack of severe neurological pathology (Table 1) and the low CFU

Figure 7 *Salmonella* infection of the brain parenchyma in *Nramp1*^{+/+} neurological mice. Brain tissue of *Nramp1*^{+/+} mice orally infected with *Salmonella* was fixed and immunostained at 5, 9, and 14 days after infection. XYZ projections illustrating presence of intracellular and extravascular bacteria in the parenchyma immunostained for *Salmonella* (red), DAPI (blue), and either Iba1 (green; **A**) or CD31 (green; **B**). XZ and YZ images were acquired in the XY plane with slices taken along the Z-axis. Scale bar = 10 μm (**A** and **B**).

Figure 8 Neutrophil infiltration, focal hemorrhaging, and ventriculitis in the brains of ataxic *Nrampl1*^{+/+} mice. Brains from mice with clinical signs of ataxia after infection with *Salmonella* were stained with hematoxylin and eosin. **A:** Low-magnification image showing neutrophil infiltration and hemorrhaging (**boxed area**), magnified in **B**. Malacia with neutrophil infiltrate and focal hemorrhaging in the corpus callosum (**B** and **C**) and lateral ventricle (**D**). **E:** Image of hippocampus and corpus callosum from an uninfected mouse for comparison. **F** and **G:** Infiltration and vascular thrombosis. **Black arrowheads** indicate regions of hemorrhage; **yellow arrowheads**, regions of tissue clearing; and **white arrowheads**, regions of cellular infiltrate. Scale bars: 2 mm (**A**); 200 μ m (**B**); 500 μ m (**C–E**); 50 μ m (**F** and **G**).

counts (Figure 1D). Because signs of spinning and barrel rolling can be indicative of otitis media (ear infection), we analyzed one of the roller mice for *Salmonella* infection in the inner ear. Bacterial infection was readily detected in the ear, as determined by fluorescent labeling for *Salmonella* (Supplemental Figure S5, B, C, and E). Histological examination revealed monocytes in the ear canal (Supplemental Figure S5, D and F), as confirmed by immunohistochemistry staining for Iba1 (Supplemental Figure S5E). Thus, the clinical sign of barrel rolling was associated with an acute inner ear infection, rather than CNS infection.

Discussion

Gram-negative meningitis is a serious health concern, with high mortality and long-term sequelae. However, there are

only a few animal models that reconstitute the natural route of infection of the CNS, making it difficult to fully understand the progression of the disease. In the current study, we compared the development of meningitis in highly susceptible *Nrampl1*^{-/-} and more resistant *Nrampl1*^{+/+} mice following a natural route of *Salmonella* infection. In both strains, meningitis was observed with detectable levels of *Salmonella* in the CNS and an influx of monocytes and other immune cells in the brain. In addition, a small number of *Nrampl1*^{+/+} animals developed clinical neurological disease characterized by ataxia. The pathology observed in these mice was similar to that reported in human neurological disease cases of *Salmonella* meningitis, including ventriculitis and hemorrhage.^{6,22} Thus, oral infection of *Salmonella* results in meningitis in both susceptible and resistant strains of mice, with a more severe form of meningitis occurring infrequently in resistant mice. The

Figure 9 Bacterial accumulation and macrophage infiltration in the brains of ataxic *Nrampl*^{+/+} mice. Brains from mice with clinical signs of ataxia after infection with *Salmonella* were immunostained. **A:** Low-magnification image of infected brain tissue immunostained for glial fibrillary acidic protein (GFAP; green), *Salmonella* (red), and DAPI (blue). **Boxed regions** illustrate bacteria in the corpus callosum, olfactory bulb, and meninges and are magnified in **B**, **C**, and **F**, respectively. For orientation purposes, the olfactory bulb (OB) and cerebellum (CB) are labeled. Representative image of *Salmonella* in the corpus callosum (**B**), olfactory bulb (**C**), ventricle (**D** and **E**), and cerebellar meninges (**F**) of a neurological mouse immunostained for either Iba1 or GFAP (green), *Salmonella* (red), and DAPI (blue). **Arrowheads** represent intracellular bacteria present in a monocyte (**D**) and presence of bacterial meningitis (**F**). Scale bars: 2 mm (**A**); 100 μ m (**B–D**); 200 μ m (**E** and **F**).

development of the more severe form of meningitis could be linked to the persistent infection in *Nrampl*^{+/+} mice, which may allow more opportunity for *Salmonella* to cross the blood-brain or blood–cerebrospinal fluid (CSF) barriers and gain better access to the CSF. Once *Salmonella* infects the CSF, it may be able to evade the immune system and replicate to high enough levels to then induce the severe damage observed in the brains in the ataxic *Nrampl*^{+/+} mice. Future studies on how *Salmonella* gains access to the CSF and whether the CSF environment allows for uncontrolled replication of *Salmonella* remain important to answer questions that need to be addressed.

Both *Salmonella* and *Escherichia coli* K1, the most common cause of neonatal Gram-negative bacillary meningitis in the United States, are enteropathogens that enter the CNS through hematogenous spread. The mechanism of bacterial entry into the CNS is not well understood. Experimental models for Gram-negative meningitis are limited. In most cases, adult animals do not reliably develop meningitis unless the pathogen is inoculated directly into the CSF or by intracranial installation. Although these models have yielded important information about the factors leading to, and the effects of, meningeal inflammation, they bypass the natural dissemination of the bacteria from the vascular system to the central nervous system.

An important aspect of the current study was the ability to follow bacterial dissemination from the gastrointestinal tract via the blood into the CNS. Our studies suggest that macrophages may play an important role in this process for *Salmonella*. One of the primary host factors that restrict intracellular survival of *Salmonella* in macrophages is *Nrampl*. We found that bacteremia levels correlated strongly with brain CFUs in *Nrampl*^{−/−} mice, but not in *Nrampl*^{+/+} mice. *Salmonella*-infected monocytes were also readily detected in the brains of *Nrampl*^{−/−} mice, consistent with the inability of *Nrampl*^{−/−} monocytes to effectively suppress *Salmonella* growth.^{16,23} These *Salmonella*-infected monocytes were not as readily detected in the brains of *Nrampl*^{+/+} mice, despite higher numbers of cells in the CNS. Thus, *Nrampl*^{−/−} and *Nrampl*^{+/+} mice provide two distinct models of *Salmonella* meningitis, with strong differences in the ability of monocytes to control the *Salmonella* infection. Comparison of these two models will provide a better understanding of how monocytes mediate meningitis in immunocompetent and immunocompromised hosts.

Studies in humans and animal models for *E. coli* K1 have indicated that a high degree of bacteremia is a prerequisite for invasion of the CNS,^{24,25} with at least 10⁵ CFUs/mL required in adult animals.²⁶ However, herein, brain neuroinvasion of *Salmonella* was observed when the blood levels

were at least 2 logs lower, approximately 10^3 CFUs/mL in *Nramp1*^{-/-} mice and $<10^2$ CFUs/mL in *Nramp1*^{+/+} mice. Thus, in this model, *Salmonella* may have a lower bacteremia threshold for entry into the CNS. Whether or not this lower requirement is because of a role for monocyte/macrophage trafficking of bacteria into the CNS remains to be determined. In our model, *Nramp1*^{+/+} mice, which have lower bacteremia levels, had greater infiltration of monocytes in the brain than *Nramp1*^{-/-} mice. Thus, there was no direct correlation between monocytes in the CNS and bacteremia with *Salmonella* infection. However, it is unclear if there is a percentage of monocytes infected with *Salmonella* that differs between *Nramp1*^{+/+} and *Nramp1*^{-/-} mice, which could influence the contribution of infected monocytes to *Salmonella* infection in the brain.

Multiple myeloid cell types have been shown to contribute to inflammation and pathogenesis in the brain, including resident microglia, M1 inflammatory monocytes, M2 nonclassical monocytes, and resident brain macrophages. In this model, both M1 and M2 infiltrating monocytes were increased in both strains of mice, even in animals with almost undetectable bacteria in the brain. M1 and M2 monocyte populations may have distinct functions in meningitis, as M1 monocytes are considered to be more proinflammatory, whereas M2 monocytes are considered to be anti-inflammatory, as evidenced by their production of anti-inflammatory cytokines, such as IL-10.²⁷ In *E. coli* K1 studies, depletion of circulating M1 monocytes resulted in more severe meningitis, but this was because of peripheral control of bacteremia rather than a direct effect within the CNS.²⁸ As *Nramp1*^{-/-} monocytes have limited ability to clear bacterial infections, the role of these cells in *Salmonella* meningitis may be mostly damaging. In contrast, *Nramp1*^{+/+} monocytes may have both protective and pathogenic roles in the disease process.

In conclusion, we found that oral inoculation of *Salmonella*, the natural route of infection, results in meningitis in both resistant and susceptible strains of mice. This meningitis includes the presence of *Salmonella* in the CNS as well as the recruitment of inflammatory cells to the brain. This provides a unique opportunity to dissect out these processes and gain a better understanding of the underlying mechanisms of disease progression that leads to Gram-negative bacterial meningitis.

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Supplemental Data

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