

# ***Salmonella* Pathogenicity Island 2 Mediates Protection of Intracellular *Salmonella* from Reactive Nitrogen Intermediates**

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## **Abstract**

*Salmonella typhimurium* causes an invasive disease in mice that has similarities to human typhoid. A type III protein secretion system encoded by *Salmonella* pathogenicity island 2 (SPI2) is essential for virulence in mice, as well as survival and multiplication within macrophages. Reactive nitrogen intermediates (RNI) synthesized by inducible nitric oxide synthase (iNOS) are involved in the control of intracellular pathogens, including *S. typhimurium*. We studied the effect of *Salmonella* infection on iNOS activity in macrophages. Immunofluorescence microscopy demonstrated efficient colocalization of iNOS with bacteria deficient in SPI2 but not wild-type *Salmonella*, and suggests that the SPI2 system interferes with the localization of iNOS and *Salmonella*. Furthermore, localization of nitrotyrosine residues in the proximity was observed for SPI2 mutant strains but not wild-type *Salmonella*, indicating that peroxynitrite, a potent antimicrobial compound, is excluded from *Salmonella*-containing vacuoles by action of SPI2. Altered colocalization of iNOS with intracellular *Salmonella* required the function of the SPI2-encoded type III secretion system, but not of an individual “*Salmonella* translocated effector.” Inhibition of iNOS increased intracellular proliferation of SPI2 mutant bacteria and, to a lesser extent, of wild-type *Salmonella*. The defect in systemic infection of a SPI2 mutant strain was partially restored in iNOS<sup>-/-</sup> mice. In addition to various strategies to detoxify RNI or repair damage due to RNI, avoidance of colocalization with RNI is important in adaptation of a pathogen to an intracellular life style.

Key words: *Salmonella typhimurium* • SPI2 • iNOS • peroxynitrite • innate immunity

## **Introduction**

Phagocytes employ an array of enzymatic activities to inactivate and degrade phagocytosed organisms. Among other antimicrobial effector mechanisms, phagocytes possess enzyme systems that are involved in the production of reactive oxygen intermediates (ROI)\* and reactive nitrogen intermediates (RNI) with activities against a wide array of pathogens. Infection experiments with knockout mice defective in gp91<sup>phox</sup> (ROI production), inducible nitric oxide synthase (iNOS; or NOS2, RNI production), and both enzymes in-

dicated that these enzymes contribute to the control of bacterial infections (1). The combined activity of gp91<sup>phox</sup> and iNOS is required for the generation of peroxynitrite, one of the most potent antimicrobial effectors of macrophages (2). Although RNI- and ROI-generating pathways are usually highly efficient and result in the killing of phagocytosed bacteria, several intracellular pathogens, such as *Salmonella enterica*, have evolved strategies to circumvent the action of these bactericidal activities of their host cells.

Infections with *S. enterica* constitute a significant public health problem in most countries. *S. enterica* causes self-limited, acute intestinal inflammations, manifesting clinically with diarrhea and vomiting. However, infections with certain serovars of *S. enterica* can lead to systemic diseases with significant lethality such as typhoid fever. Two hallmarks of pathogenesis of *Salmonella* infections are the invasion of non-phagocytic cells and the survival and replication within host cells, for example macrophages. Many events during pathogenesis of systemic *Salmonella* infections have been studied in detail in the model system of murine salmonellosis, a ty-

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\*Abbreviations used in this paper: aa, amino acid(s); DPI, diphenyliodonium chloride; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; L-NMMA, L-monomethylarginine; MOI, multiplicity of infection; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; SCV, *Salmonella*-containing vesicle; SPI, *Salmonella* pathogenicity island; STE, *Salmonella*-translocated effector; TTSS, type III secretion system.

phoid fever-like infection caused by *S. enterica* serotype Typhimurium (*Salmonella typhimurium*) in susceptible mice.

Type III secretion systems (TTSS) are a family of highly evolved protein secretion systems in Gram-negative pathogens. TTSS translocate, in a contact-dependent manner, effector proteins into the eukaryotic host cell (3). The function of a TTSS encoded by *Salmonella* pathogenicity island 1 (SPI1) is important for interactions of *S. typhimurium* with the epithelial mucosa, such as invasion of enterocytes (4) and the induction of diarrhea (5). During systemic infections macrophages represent an important site for replication of *S. typhimurium* (6, 7). Mutant strains of *S. typhimurium* unable to survive and replicate within macrophages are also severely attenuated in virulence in the animal model of infection (8). Survival and replication of *S. typhimurium* in macrophages requires the function of a large number of proteins. These functions range from biosynthesis of macromolecules that are limited in the phagosomal environment, enzyme systems involved in the repair of damages due to antimicrobial activities of the host cells, to virulence factors that actively modulate host cell functions (9, 10). *Salmonella* pathogenicity island 2 (SPI2) harbors genes encoding a second TTSS for virulence proteins in *S. enterica* (for a review, see reference 11). The function of SPI2 genes is absolutely required for the progression of systemic infections by *S. typhimurium* in the model of murine salmonellosis (12) and the proliferation within infected organs such as liver and spleen (13). Furthermore, the role of SPI2 in intracellular replication and survival has been demonstrated (14, 15, 16). On the cellular level, the function of SPI2 proteins affects vesicle trafficking in host cells (17). SPI2 is also required to prevent the localization of NADPH-oxidase to *Salmonella*-containing vesicles (SCVs) of the infected host cell, an activity leading to the evasion of intracellular *Salmonella* from oxidative damage (18, 19).

Infections with *Salmonella* induce iNOS expression (for a review, see reference 20) and recent studies demonstrated that the function of iNOS is required to control the proliferation of *S. typhimurium* in infected organs (21) and within infected macrophages (22). As the function of SPI2 is also required for both phenotypes, we asked if SPI2 is involved in the control of iNOS expression or activity, or may control the production of RNI and their effects on intracellular *S. typhimurium*. To investigate the cellular microbiology of this antimicrobial effector mechanism in more detail, we established an in vitro system to study the effects of *S. typhimurium* on expression of iNOS and production of RNI. In this study we report that intracellular *S. typhimurium* employ the TTSS of SPI2 to prevent the localization of iNOS with SCVs. To our knowledge, this is the first observation that a pathogen can alter the cellular localization of iNOS to protect itself from the damaging effects of RNI and the combined effects of RNI and ROI.

## Materials and Methods

**Bacterial Strains.** Bacterial strains used in this study are listed in Table I. *Salmonella enterica* serovar Typhimurium (*S. typhimu-*

*rium*) NCTC 12023 was used as wild-type strain and all mutant strains were constructed in the *S. typhimurium* 12023 background. For colocalization experiments *S. typhimurium* strains were transformed with plasmid pFPV25.1 constitutively expressing green fluorescent protein (GFP; reference 23).

**Construction of Mutations in *Salmonella*-translocated Effector Genes.** The method of one-step inactivation of chromosomal genes (24) was applied. Briefly, primers with 56 to 65 bases length including 20 bases priming sequences for the template pKD4 and 36 to 45 bases homologous to the respective flanking regions of the gene of interest have been constructed. PCR reactions were performed with the Expand High Fidelity PCR system (Roche) using pKD4 as template. PCR products were purified, digested with DpnI, and gel purified. The PCR products were transformed into *S. typhimurium* cells harboring pKD46 and grown in the presence of arabinose to induce the Red recombinase. Recombinant clones were selected by plating on Luria-Bertani (LB)-agar containing 50  $\mu$ g/ml kanamycin. Kanamycin-resistant colonies were further checked for chromosomal insertion of the antibiotic resistance cassette by PCR using primers k1 and k2 (24). The proper insertions of the antibiotic resistance cassettes within the various *Salmonella*-translocated effector (STE) genes were confirmed by DNA sequencing. Finally, the mutant alleles were moved into a fresh strain background by P22 transduction according to standard procedures (25).

**Cell Culture.** The murine macrophage cell line RAW267.4 was obtained from American Type Culture Collection, and maintained in DMEM containing 10% heat-inactivated FCS (Invitrogen/Life Technologies) at 37°C under 5% CO<sub>2</sub>. The cells were washed gently with PBS and removed from the flasks by scraping. The cells were suspended in 24-well plates for experiments at the density of 5  $\times$  10<sup>4</sup> cells per well. Peritoneal macrophages were harvested from thioglycollate-elicited peritoneal lavages from C57BL/6 mice and plated in DMEM supplemented

**Table I.** Bacterial Strains Used in This Study

Strain designation	Relevant characteristics	Remarks/reference
<i>S. typhimurium</i> NCTC 12023	Wild-type	Identical to ATCC 14028
P8G12	<i>ssrB::mTn5</i>	13
P2D6	<i>ssaV::mTn5</i>	13
P11D10	<i>sscA::mTn5</i>	13
P7G2	<i>ssaC::mTn5</i>	13
HH101	<i>sseA::aphT</i>	15
HH102	<i>sseB::aphT</i>	15
HH104	<i>sseC::aphT</i>	15
P3H6	<i>sifA::mTn5</i>	44
P7B12	<i>sipC::mTn5</i>	45
MvP374	$\Delta$ <i>sifB::aph</i>	This study
MvP375	$\Delta$ <i>sspH1::aph</i>	This study
MvP376	$\Delta$ <i>sspH2::aph</i>	This study
MvP377	$\Delta$ <i>sseJ::aph</i>	This study
MvP378	$\Delta$ <i>sseI::aph</i>	This study
MvP379	$\Delta$ <i>slrP::aph</i>	This study

with 10% FCS. After 2 h at 37°C, the cells were washed twice with PBS and the adherent cells were incubated overnight in DMEM plus 10% FCS. Cells were always cultured in antibiotic-free media.

**Survival of *S. typhimurium* After In Vitro Exposure to Nitrosative Stress.** Overnight cultures of wild-type *S. typhimurium* and the *ssaV* mutant strain were diluted to OD<sub>600</sub> of 0.2. S-nitrosoglutathione (GSNO; Calbiochem) and sodium nitrite (NaNO<sub>2</sub>; Sigma-Aldrich) were prepared freshly in distilled water, adjusted to pH 5.0, and added to 1 ml LB broth at various concentrations. SIN-1 (Calbiochem) was dissolved in distilled water and added to 1 ml LB at various concentrations. Subsequently, 100 μl of the diluted culture was added to each tube. The tubes were incubated at 37°C under shaking conditions. After 3 and 9 h of exposure serial dilutions were made and plated onto Mueller-Hinton agar for the enumeration of surviving bacteria.

**Infection of Macrophages.** Bacteria were grown overnight in LB broth in glass test tubes with aeration in a “roller drum” (New Brunswick Scientific). The OD at 600 nm of the cultures was adjusted to 0.2 and 1 ml of the bacterial suspension was centrifuged, washed twice with PBS, and resuspended in DMEM. 10 μl of the bacterial suspension was further added to 1 ml of DMEM. 100 μl of the bacterial suspension was added to the wells at a multiplicity of infection (MOI) of 10 and the plates were centrifuged at 1,200 rpm for 5 min to synchronize the infection. The plates were incubated at 37°C, 5% CO<sub>2</sub> for 25 min. Subsequently, the plates were washed three times with PBS and incubated with media containing 100 μg/ml gentamicin for 1 h, followed by media containing 10 μg/ml of gentamicin for the rest of the experiment. For infection of peritoneal macrophages, bacteria were opsonized for 30 min at 37°C with 10% normal mouse serum before infection. Heat-killed bacteria were obtained by incubation at 80°C for 10 min. This procedure did not affect the integrity of the LPS.

**Assessment of Intracellular Growth of Bacteria.** Intracellular replication was assessed by means of the standard gentamicin assay (26). After completion of the infection period, the cells were incubated with 100 μg/ml gentamicin for 1 h to kill the extracellular bacteria and subsequently with 10 μg/ml gentamicin for the rest of the time period. 2 and 16 h after infection, the supernatant was removed and cells were washed three times with DMEM (without serum) and lysed with 0.1% Triton X-100. Serial dilutions of the lysates were plated onto LB agar to enumerate intracellular bacteria.

For investigation of intracellular replication by microscopy, the cells were grown on glass coverslips and infected with *S. typhimurium* strains constitutively expressing GFP. The gentamicin protection was performed as described above, and cells were fixed with 3% para-formaldehyde (PFA).

The inhibitors L-NMMA (L-monomethylarginine; Sigma-Aldrich) or DPI (diphenyleneiodonium chloride) were added after 1 h of gentamicin treatment to avoid the possible effect of the inhibitors on the uptake of bacteria.

**Infection Studies.** C57BL/6 and iNOS<sup>-/-</sup> mice were bred and maintained under specific pathogen-free conditions in accordance with national guidelines for animal studies. Groups of 6–7 mice were infected by intraperitoneal injection of an inoculum consisting of a mixture of 500 cfu wild-type *S. typhimurium* (Nal<sup>R</sup>, Km<sup>S</sup>) and 500 cfu of SPI2 mutant strain HH104 (Nal<sup>S</sup>, Km<sup>R</sup>). At various time points after infection, mice were killed by cervical dislocation and livers and spleens were removed aseptically. Organs were homogenized in 1 ml of PBS and the organ burdens of wild-type *S. typhimurium* and the SPI2 mutant strain were deter-

mined by plating serial dilutions on LB-agar containing 50 μg/ml nalidixic acid or 50 μg/ml kanamycin.

**Determination of NO Production.** NO was measured as its end product nitrite using the Griess reagent as described previously (27). Culture supernatants (50 μl) were mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% orthophosphoric acid). After 10 min, absorbance at 570 nm was measured in a microplate ELISA reader (Bio-Rad Laboratories). The concentration of nitrite in the culture supernatant was determined with reference to the standard curve using sodium nitrite. All data represent the mean value of triplicates ± SD.

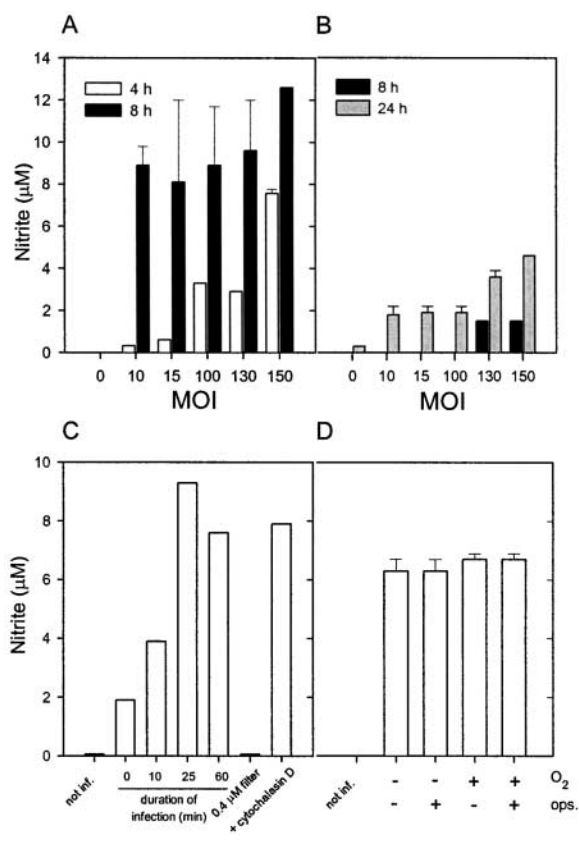
**Immunoblotting.** RAW267.4 cells were seeded in 6-well plates (4 × 10<sup>5</sup> cells/well) and infected. Cells were lysed in SDS-PAGE sample buffer and boiled for 5 min at 100°C. Aliquots containing equal amounts of protein (20 μg) were loaded onto a 6% gradient polyacrylamide gel, run under reducing conditions, and transferred onto nitrocellulose membranes. The membranes were treated with 5% bovine serum albumin for 1 h to block nonspecific binding, rinsed, and incubated with rabbit polyclonal antibodies against mouse iNOS (raised against peptide amino acids (aa) 1125–1144, BioTrend) for 1 h. The blots were further treated with goat anti rabbit IgG-horseradish peroxidase conjugate (AP-Biotech; dilution 1:3,000) for 1 h. The immune complex on the blots was detected with enhanced chemiluminescence substrate (AP-Biotech) and exposed to Eastman Kodak Co. XAR x-ray film.

**Immunofluorescent Staining of iNOS and Peroxynitrite.** RAW 267.4 cells were seeded onto glass coverslips and infected as described before. The antibodies were diluted in PBS containing 5% goat serum, 2% BSA, and 0.1% saponin. For immunostaining of iNOS, cells were incubated with 1:500 dilutions of rabbit antisera raised against aa 1125–1144 (BioTrend) or aa 1137–1144 of mouse iNOS (donated by Dr. C. Bogdan, University of Erlangen, Erlangen, Germany). For detection of peroxynitrite formation, cells were incubated with a 1:500 dilution of a rabbit antiserum raised against nitrotyrosine (Upstate Biotechnology) for 1 h. After washing with PBS, the cells were incubated with Cy3-conjugated goat anti-rabbit IgG antibody (Dianova) for 30 min. After washing, the cells were inspected for localization of iNOS or peroxynitrite using a fluorescence microscope or a confocal laser-scanning microscope (Leica) using filter sets for FITC (510 nm excitation, 530 nm emission) and TRITC (566 nm excitation, 590 nm emission). Images were further processed using the Leica TCS software package.

## Results

**Induction of Nitrite Production by RAW267.4 Macrophages Requires Live Salmonella but Does Not Require Bacterial Internalization.** The role of RNI during experimental salmonellosis in murine hosts has recently been demonstrated (21, 22). To elucidate the role of RNI in the cellular microbiology of *Salmonella*, we first investigated how a cultured macrophage-like cell line reacts to infection with *S. typhimurium*. RAW267.4 macrophages were infected with *S. typhimurium* wild-type and the NO response as measured by nitrite accumulation was studied. An increase in the MOI resulted in a gradual increase of the nitrite production by the RAW267.4 cells at early time after infection (Fig. 1 A). 8 h after infection, no significant difference in nitrite

production was observed after infection at various MOI in the range of 10 to 150. In contrast, macrophages infected with heat-killed bacteria did not exhibit significant nitrite accumulation after 8 h (Fig. 1 B). After 24 h, accumulation of nitrite was detectable but lower than that observed with live *Salmonella*. This residual nitrite accumulation may be due to LPS, which is a prime inducer of NO response (28). No nitrite accumulation was detected in macrophage cultures separated from the *Salmonella* inoculum by a membrane, indicating that contact was required for induction of NO synthesis (Fig. 1 C). However, phagocytosis per se was not required for the NO production, as inhibition of phagocytosis by cytochalasin D did not inhibit the induction

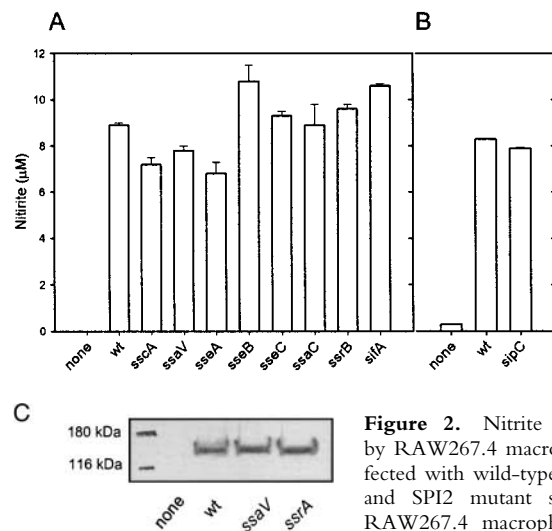


**Figure 1.** Induction of iNOS by RAW267.4 macrophages requires live *Salmonella*. (A) RAW267.4 cells were infected with wild-type *Salmonella* at various MOI and nitrite in the supernatant was measured by the Griess reaction 4 and 8 h after infection. (B) RAW267.4 macrophages were infected with heat-killed *Salmonella* and the nitrite was measured 8 and 24 h after infection. The data represent the mean  $\pm$  SD of three independent experiments done in triplicate. (C) RAW267.4 macrophages were infected with wild-type *Salmonella* at a MOI of 10 under various experimental conditions as indicated, and nitrite production was measured 8 h after infection. After adding the bacteria to the macrophages and centrifugation, infection took place for 0, 10, 25, or 60 min. In further assays, macrophages were infected for 25 min, but were separated from the bacteria by a 0.4  $\mu$ M filter, or cytochalasin D was added to the macrophages at 1  $\mu$ g/ml before infection. not inf., not infected. (D) Effect of bacterial culture conditions on nitrite production. Wild-type *S. typhimurium* was grown for 16 h in LB media without aeration as standing cultures or with aeration in test tubes in a roller drum. If indicated, the bacteria were opsonized by incubation with 10% normal mouse serum for 30 min. Infection was performed at a MOI of 10 and nitrite production was assayed after 16 h.

of nitrite production by *Salmonella* (Fig. 1 C). Infection with bacteria grown with aeration to stationary phase or bacteria cultured under O<sub>2</sub> limitation resulted in similar levels of NO production (Fig. 1 D). Also, opsonization of the inoculum did not affect the induction of NO production (Fig. 1 D). Phagocytosis of latex beads alone did not result in nitrite production by RAW267.4 cells (data not shown), implying that live *Salmonella* set a specific signal for NO induction early after infection.

*Wild-type S. typhimurium and Mutant Strains in SPI2 Induce Similar NO Responses in Macrophages.* The requirement of bacterial viability led us to investigate whether gene products required for intracellular proliferation of *S. typhimurium* are affecting the NO production. Here, we focused on the role of SPI2 genes. Our experiments revealed no significant difference between the NO response induced by infection with wild-type *S. typhimurium* and various mutants in the SPI2 locus (Fig. 2 A). The SPI2 mutants induced as high levels of NO accumulation as wild-type *Salmonella*. Furthermore, no difference in the cellular amounts of iNOS protein was observed in macrophages infected with wild-type *S. typhimurium* or various SPI2 mutants (Fig. 2 C). These observations indicate that SPI2 function is not directly affecting the NO production or the cellular levels of iNOS.

The induction of iNOS expression by effector proteins of SPI1, such as SipC, has been reported recently (29). We observed similar levels of NO synthesis after infection with wild-type *Salmonella* and different strains deficient in SPI1, such as a *sipC* strain (Fig. 2 B). SPI1-mediated invasion of macrophages by *Salmonella* has cytotoxic effects on macrophages (for a review, see reference 30). Under the assay conditions applied in our study, bacteria were grown to



**Figure 2.** Nitrite production by RAW267.4 macrophages infected with wild-type *Salmonella* and SPI2 mutant strains. (A) RAW267.4 macrophages were infected at a MOI of 10 with different SPI2 mutants and the nitrite was measured in the supernatant after 8 h. (B) Infection was performed as before with wild-type *S. typhimurium* and an isogenic strain deficient in *sipC* encoded by SPI1. wt, wild-type. (C) Western blot analysis of iNOS expression in noninfected RAW267.4 macrophages, and macrophages infected with wild-type *S. typhimurium* or strains harboring mutations in SPI2 genes *ssaV* or *ssaA*.

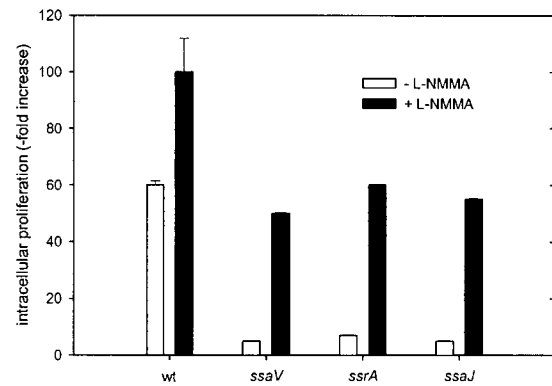
**Table II.** Induction of Nitrite Production by RAW267.4 Macrophages

L-NMMA	Nitrite	
	Noninfected	Infected
$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$
0	0	$8.9 \pm 0.9$
5	0	$2.4 \pm 0.3$
10	0	$0.4 \pm 0.6$
100	0	$0.0 \pm 0.0$

RAW267.4 macrophages were infected with wild-type *S. typhimurium* at an MOI of 10. Noninternalized bacteria were removed by washing followed by killing with gentamicin. After infection L-NMMA was added as indicated and 8 h after infection, nitrite levels in the culture supernatant were assayed by the Griess reaction.

stationary phase, thus reduced in expression of SPI1, non-invasive and noncytotoxic. Therefore, in our experimental system mutations in the secretion system of SPI1 or effector proteins of SPI1 are unlikely to affect iNOS expression.

*Inhibition of iNOS Enhances Intracellular Proliferation of S. typhimurium and Rescues Mutants Deficient in SPI2.* As iNOS has been recently shown to be one of the potent candidate molecules in controlling the *Salmonella* infection (22), we further looked at the intracellular growth of wild-type *S. typhimurium* and SPI2 mutant strains. Irrespective of the high nitrite production, wild-type *Salmonella* proliferated rapidly inside host cells, while SPI2 mutant strains had reduced rates of intracellular proliferation. Determination of intracellular bacteria in RAW267.4 macrophages at 2 and 16 h after infection indicated a 60-fold increase for *S. typhimurium* wild-type compared with a 5 to 7-fold increase for mutants in SPI2 genes. L-NMMA, a competitive inhibitor of NO synthases, dose dependently inhibited the nitrite production by macrophages infected with wild type *S. typhimurium* (Table II). Inhibition of iNOS by L-NMMA in-

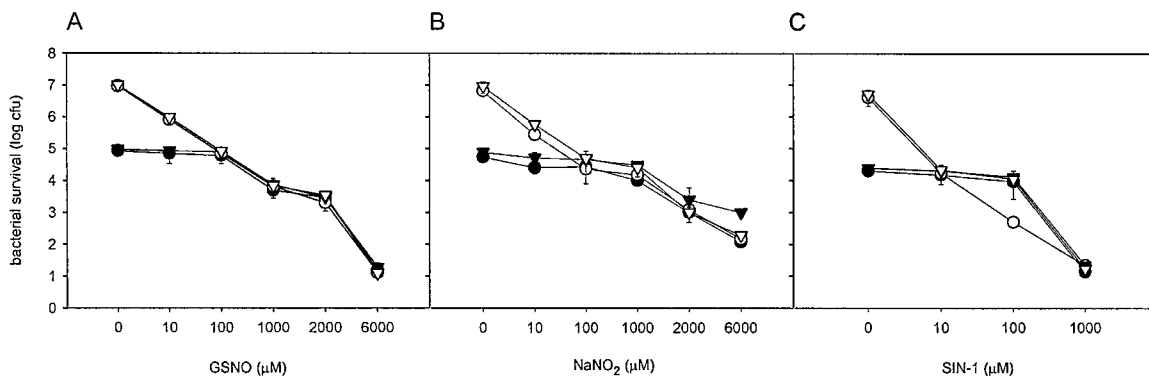


**Figure 3.** Intracellular proliferation of *Salmonella* in the presence or absence of iNOS inhibitor L-NMMA. RAW267.4 macrophages were infected with wild-type (wt) *S. typhimurium* and various mutant strains in SPI2 genes at a MOI of 10. Non-internalized bacteria were removed by washing and killed by gentamicin. L-NMMA was added at a concentration of 10  $\mu\text{M}$  if indicated. The number of intracellular bacteria was determined by plating serial dilutions of macrophage lysates on LB-agar for counting of cfu. Intracellular replication was expressed as the ratio of cfu at 2 to 16 h after infection.

creased the intracellular proliferation of the SPI2 mutants (Fig. 3). This observation indicates that iNOS contributes to the intracellular killing of *Salmonella*. However, the presence of L-NMMA also resulted in increased intracellular proliferation of the wild-type strain, which suggests that a proportion of wild-type *Salmonella* was also killed by RNI (Fig. 3).

To control if the observed effects are the result of higher susceptibility of SPI2 mutant strains to nitrosative damages, in vitro tests were performed (Fig. 4). Wild-type *S. typhimurium* and a representative SPI2 mutant strain (*ssaV*) were killed to the same extent by GSNO,  $\text{NaNO}_2$ , or SIN-1. Therefore, increased intracellular survival of SPI2 mutant strains in the presence of L-NMMA is not the result of higher sensitivity of these strains to nitrosative damage.

*Localization of iNOS in Salmonella-infected Macrophages Is Dependent on SPI2 Function.* As the previous experiments clearly demonstrated a role of RNI in controlling the intra-

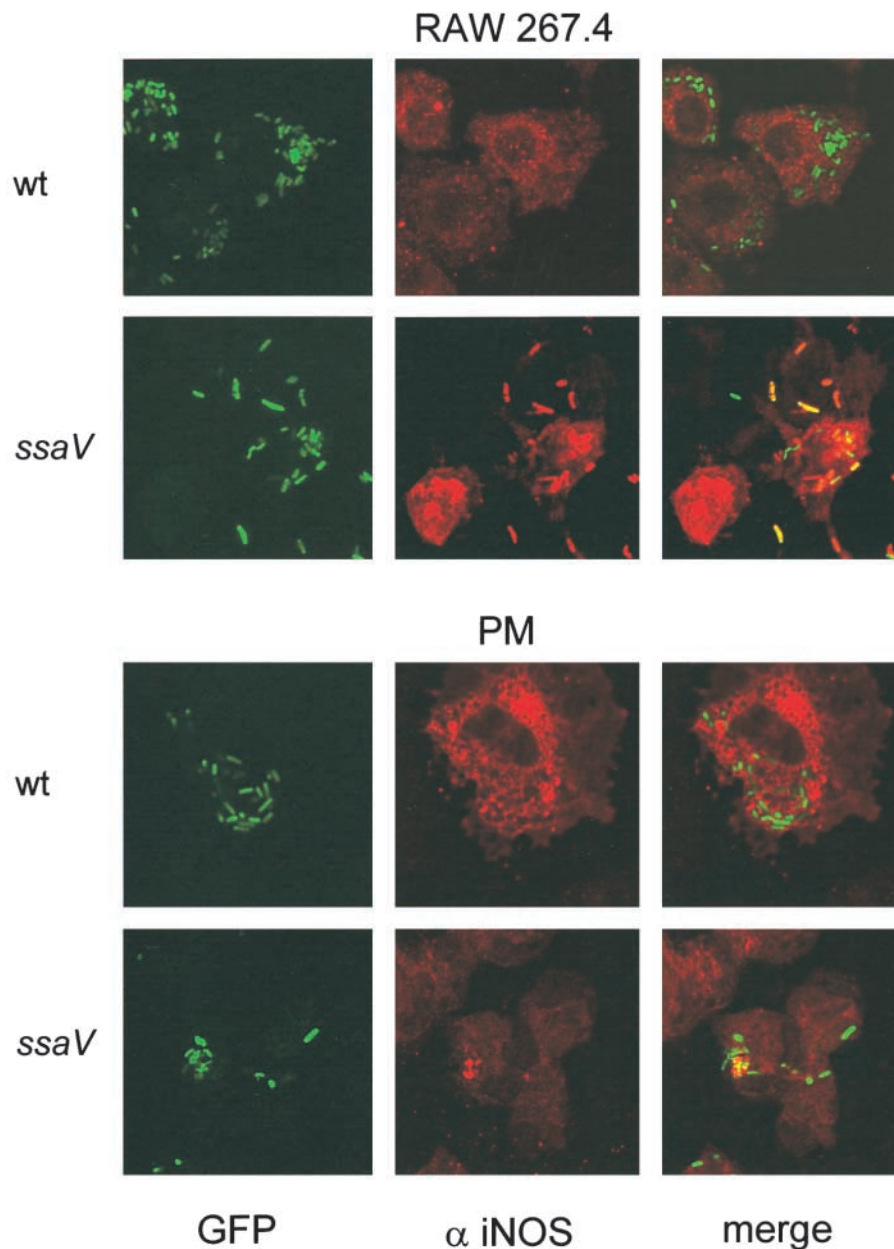


**Figure 4.** In vitro survival of *S. typhimurium* under different nitrosative stress conditions. Wild-type (circles) or SPI2 deficient (*ssaV* mutant, triangles) *S. typhimurium* were exposed to various concentrations of GSNO (A) or  $\text{NaNO}_2$  (B) in LB medium (adjusted to pH 5.0) or SIN-1 (C) in LB medium (adjusted to pH 7.0). At 3 h (open symbols) or 9 h (filled symbols) after exposure serial dilutions were plated and cfu were counted. Results are expressed as mean cfu  $\pm$  SD of two independent experiments in triplicate.

cellular proliferation of *Salmonella*, we set out to localize iNOS in *Salmonella*-infected macrophages. RAW267.4 macrophages or peritoneal macrophages were infected with wild-type *S. typhimurium* and various SPI2 mutants and analyzed by confocal laser-scanning microscopy for the localization of iNOS. In case of macrophages infected with wild-type *S. typhimurium*, iNOS was only rarely colocalized with the bacteria and a diffuse staining for iNOS was observed (Fig. 5). In contrast, in macrophages infected with a SPI2 mutant strain defective in *ssaV*, iNOS frequently colocalized with the bacteria (Fig. 5). The iNOS staining was dense around the SPI2-deficient bacteria. Comparable results were obtained with an antiserum raised against aa 1,125–1,144 (Fig. 5) and an antiserum raised against aa 1137–1144 of mouse iNOS (data not shown). Similar pat-

terns of colocalization with iNOS were obtained with various strains harboring mutations in SPI2 genes *sseB*, *ssaV*, *ssaJ*, *ssrA*, or *ssrB*. Previous work demonstrated that the virulence defect of a strain harboring a mutation in *sseB* can be complemented by a plasmid-born copy of *sseB* (15). Analysis of a *sseB* strain harboring *psseB* revealed the same pattern of iNOS localization as observed for wild type *Salmonella* (data not shown).

We next investigated whether the altered cellular localization of iNOS is a result of reduced intracellular proliferation of mutant strains in SPI2 or directly related to SPI2 function and analyzed infections with an isogenic strain proficient for SPI2 function but defective in *purD*. The *purD* mutant is deficient in purine metabolism and attenuated in virulence (31) and intracellular proliferation (data



**Figure 5.** Localization of iNOS in *Salmonella*-infected macrophages. RAW267.4 macrophages and mouse peritoneal macrophages (PM) were infected with GFP-expressing wild-type (wt) *S. typhimurium* or SPI2 mutant strain defective in *ssaV*. Non-internalized bacteria were killed by addition of gentamicin. At 12 h after infection, the cells were fixed and permeabilized for immunostaining. iNOS was detected by incubation with a polyclonal antibody against iNOS and a Cy3-labeled secondary antibody. The localization of iNOS (red) and *S. typhimurium* (green) was analyzed by fluorescence microscopy (not shown) and confocal laser-scanning microscopy. Colocalization of *S. typhimurium* and iNOS resulted in orange to red staining of the bacterial cells.

not shown) to a similar extent as the SPI2-deficient strains. Although the number of intracellular *purD* bacteria was lower than those of wild-type *Salmonella*, *purD* bacteria were not colocalized with iNOS (data not shown).

The proportion of bacterial cells colocalized with iNOS after different time intervals was quantified (Fig. 6 A). At 8 or 16 h after infection, the proportion of wild-type *S. typhimurium* colocalized with iNOS remained lower than those of SPI2 mutant strains. Similar to a recent report by Webb et al. (32) we did not observe significant colocalization to either strain at earlier time points after infection.

Our observations indicate that the altered localization of iNOS is an effect of the function of SPI2. SPI2 mutant strains are still able to survive and replicate, to a certain extent, in infected macrophages (15). This result is in line with the observation that a significant percentage of SPI2 mutants (~40%) was not associated with iNOS. Such bacteria may escape the macrophage defense and be able to replicate intracellularly. On the other hand, a low percentage (~10%) of wild-type *Salmonella* colocalized with iNOS, indicating that RNI also control the intracellular proliferation of fully virulent bacteria.

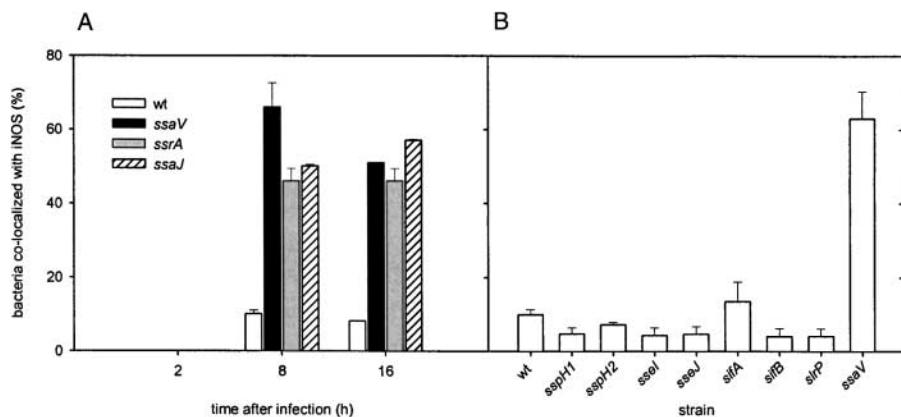
**Effect of Mutations in Genes for STEs on iNOS Delivery to Salmonella-containing Phagosomes.** A family for proteins that are translocated by the TTSS of SPI2 is encoded by genes outside of the SPI2 locus (33). The gene products, namely SlrP, SspH1, SspH2, SifA, SifB, SseI, and SseJ are referred to as STEs and have been proposed to interact with host cell functions after translocation into the infected host cell. We set out to analyze if an individual STE protein is required for the prevention of iNOS delivery to SCVs and constructed a set of strains harboring mutations in STE genes. These strains were analyzed for iNOS delivery to SCVs after infection of macrophages. In contrast to strains deficient in the TTSS of SPI2, mutants in STE genes had characteristics similar to wild-type *Salmonella*, i.e., only 5–10% of the intracellular *Salmonella* colocalized with iNOS (Fig. 6 B). Colocalization of iNOS to the *sifA* strain was observed more frequently (~15%). A role of SifA for maintenance of SCVs was observed (24) and this effector

may also, to a certain extent, interfere with iNOS delivery to SCVs.

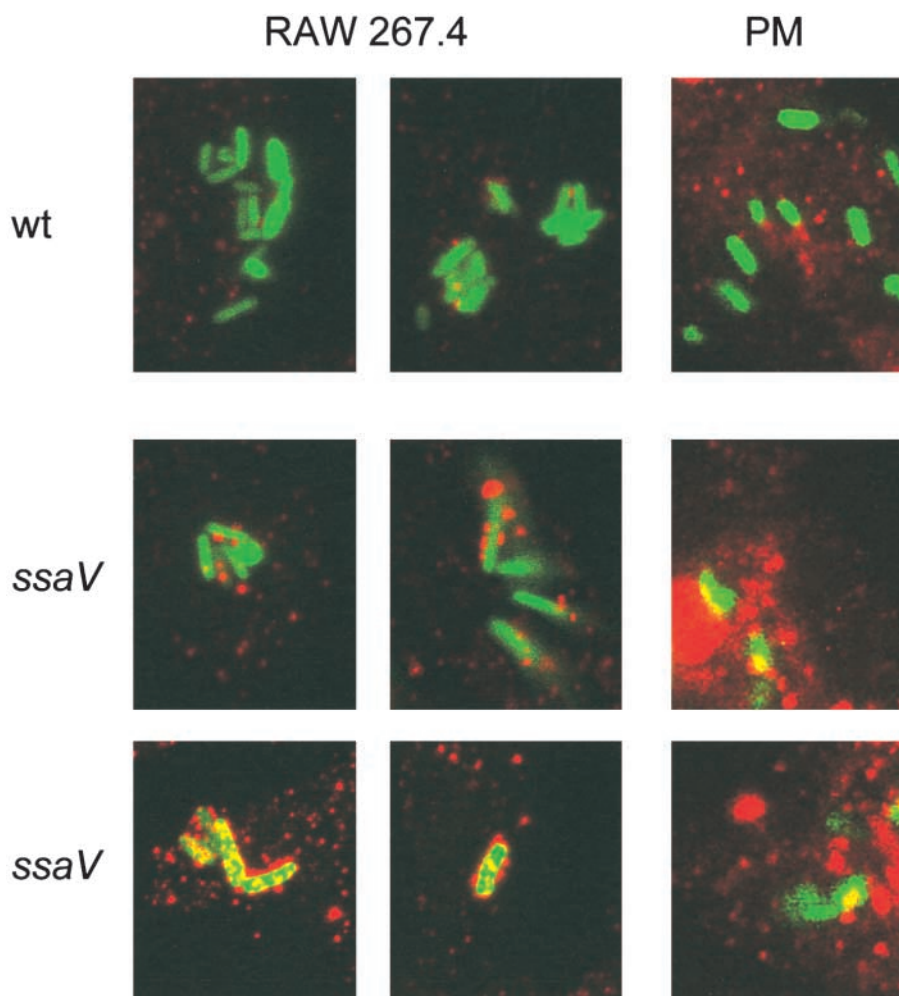
These observations indicate that a single STE is not sufficient to modify the delivery of iNOS to SCVs. The concerted action of several STE or the function of further, yet unknown effector proteins of the SPI2-encoded TTSS may be required for this phenotype.

**Peroxynitrite Formation in Salmonella-infected Macrophages Is Localized with SPI2-deficient Bacteria.** NO per se is not a potent cytotoxic molecule and most potent bactericidal effect of NO appears to be via a reaction with  $O_2^-$  (2). NO reacts with  $O_2^-$  to yield peroxynitrite ( $OONO^-$ ). Peroxynitrite generated in the cells cannot be detected directly, but sites of damage due to the reaction of peroxynitrite can be assessed by immunohistochemical staining of nitrotyrosine. In case of macrophages infected with wild-type *Salmonella*, the nitrotyrosine staining was not, or to a low extent colocalized with the bacteria (Fig. 7). Similar observations on the nitrotyrosine formation in *S. typhimurium*-infected macrophages were recently reported (22). In the case of SPI2 mutant strains, nitrotyrosine staining was localized in the vicinity of the bacteria (Fig. 7), indicating events where proteins were targeted by formation of peroxynitrite.

**Control of Intracellular Proliferation of *S. typhimurium* in Primary Peritoneal Macrophages.** After infection of primary peritoneal macrophages with *S. typhimurium*, the nitrite production was higher in primary macrophages ( $15 \pm 1.5 \mu\text{M}$  nitrite) than in the RAW267.4 cell line ( $8 \pm 0.5 \mu\text{M}$  nitrite). There was no difference observed in the nitrite production between macrophages infected with wild-type *Salmonella* or SPI2 mutant strains. Overall, the number of intracellular wild-type *Salmonella* was lower than those in RAW267.4 macrophages, but again only few events of colocalization with iNOS or nitrotyrosine were observed. On the other hand, in cells infected with SPI2 mutants, colocalization with iNOS or nitrotyrosine was frequently observed, suggesting that in primary macrophages iNOS contributes to the killing of the SPI2 mutants and not of wild-type *Salmonella*. Inhibition of iNOS by L-NMMA in-



**Figure 6.** Colocalization of iNOS with wild-type *S. typhimurium* and various strains deficient in SPI2 genes or “*Salmonella* translocated effectors.” (A) RAW267.4 macrophages were infected with wild-type *S. typhimurium* (wt) and strains deficient in the SPI2-encoded secretion system (*ssaV*, *ssaA*, *ssaJ*). At 2, 8, or 16 h after infection, the percentage of bacteria colocalized with iNOS was scored by examining randomly selected 50 microscopic fields (1,000 $\times$  magnification) and counted for total green and colocalized yellow or red bacteria. (B) RAW267.4 macrophages were infected with wild-type *S. typhimurium*, or mutant strains in SPI2 (*ssaV*) or various STE genes (*sspH1*, *sspH2*, *sseI*, *sseJ*, *sifA*, *sifB*, *slrP*). 8 h after infection, macrophages were processed for immuno-staining and colocalization was quantified as described in panel A. The data represent two independent experiments.

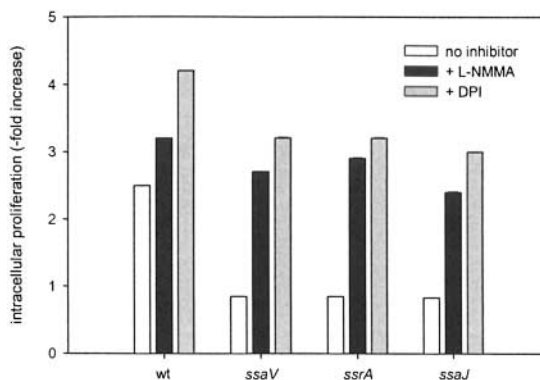


**Figure 7.** Localization of peroxynitrite in *S. typhimurium*-infected macrophages. Infection of RAW267.4 macrophages and mouse peritoneal macrophages (PM) was performed for 12 h as described in the legend to Fig. 5. The sites of peroxynitrite formation were detected by immunostaining with an anti-nitrotyrosine antibody and a Cy3-conjugated secondary antibody. Samples were analyzed by confocal laser-scanning microscopy and representative images for the localization of *S. typhimurium* expressing GFP (green) and peroxynitrite (red) are shown. Various levels of nitrotyrosine formation were observed for the SPI2 mutant strain. wt, wild-type.

creased the growth of intracellular wild-type *Salmonella* and SPI2 mutant strains. Furthermore, in infection studies with primary macrophages we used the inhibitor DPI, which inhibits both NADPH oxidase and iNOS. The addition of DPI resulted in a higher recovery of intracellular bacteria than L-NMMA (Fig. 8). The addition of phenylarsine oxide, which inhibits only NADPH oxidase but not iNOS, resulted in a lower recovery of intracellular bacteria compared with DPI (data not shown). This observation indicates additive effects of ROI and RNI in controlling intracellular proliferation of *S. typhimurium* in macrophages.

**Interaction of SPI2 and iNOS during Infection.** The role of iNOS in the control of *Salmonella* infection was analyzed by comparison of systemic salmonellosis in *iNOS*<sup>+/+</sup> and *iNOS*<sup>-/-</sup> mice. Here, we used a mixed infection model in which a mixture of equal amount of wild-type *S. typhimurium* and a SPI2 mutant strain was applied via the intraperitoneal route. At various time points after infection, the bacterial burden of each strain was determined in infected organs, and the competitive index (CI) for wild-type *S. typhimurium* versus SPI2 mutants was determined (Table III). The CI is a highly sensitive and reproducible estimate of the virulence of a mutant strain in competition with the

wild-type strain (24). In accordance with this previous study, a SPI2 mutant strain was deficient in the colonization of the C57BL/6 (*iNOS*<sup>+/+</sup>) mice. At early time points after infection, proliferation of wild-type and SPI2-defi-



**Figure 8.** Intracellular replication in primary macrophages. Peritoneal macrophages were prepared from C57BL/6 mice and infected with *S. typhimurium* wild-type (wt) or various strains harboring mutations in SPI2 genes. After infection, inhibitors L-NMMA (10  $\mu$ M) and DPI (10  $\mu$ M) were added as indicated. The intracellular proliferation was determined as described in the legend of Fig. 3.



cient *Salmonella* was similar in iNOS<sup>+/+</sup> and iNOS<sup>-/-</sup> mice. However, a dramatic difference was observed at the late phase of infection. At day 5 after infection a CI of < 0.00005 was obtained for iNOS<sup>+/+</sup> mice, indicating that the SPI2-deficient strain was out-competed by wild-type *S. typhimurium*. In contrast, in iNOS<sup>-/-</sup> mice CI of 1.2 and 0.16 in liver and spleen, respectively, were determined at day 5 after infection. These results demonstrate that iNOS<sup>+/+</sup>, but not iNOS<sup>-/-</sup> mice, are able to control the proliferation of SPI2-deficient *S. typhimurium*, indicating an interaction in vivo of SPI2 and RNI-dependent defense mechanisms of the host.

## Discussion

RNI produced by iNOS have important functions in the host defense against intracellular pathogens such as *Leishmania* spp., *Mycobacterium* spp., and *Salmonella* spp. (2, 34). Inhibition of iNOS resulted in increased bacterial burdens in organs and reduced survival of *Salmonella*-infected mice (35). An increase in the intracellular proliferation of *Salmonella* was observed in macrophages after inhibition of iNOS (22). Furthermore, it was recently demonstrated that iNOS plays an important role in controlling the growth of *Salmonella* at the later stages of infection in the model of murine salmonellosis: iNOS<sup>-/-</sup> mice succumbed to infection with a *S. typhimurium* strain of intermediate virulence several days after infection, while iNOS<sup>+/+</sup> mice survived the infection (21). Inhibition of iNOS also led to increased intracellular replication of *Salmonella* in macrophages and mutant strains that downregulate the nitrite production also showed increased proliferation in macrophages (36). These evidences suggest a role of iNOS in *Salmonella* pathogenesis.

*Salmonella* possesses several systems to detoxify RNI as well as enzymes to repair damages induced by the action of RNI (for a review, see reference 2). Recent observations also indicate that intracellular *Salmonella* utilize a variety of mechanisms to actively avoid antimicrobial activities of their host cells (17, 18). As RNI are important antimicrobial effector molecules of phagocytes, we analyzed here if *S. typhimurium* also has mechanisms to prevent synthesis or circumvent the effects of RNI. We are interested to understand the cellular microbiology of SPI2, a virulence factor

that is essential for the proliferation of *S. typhimurium* within infected host tissues and intracellular replication. No difference was observed between wild-type *Salmonella* and SPI2 mutants in terms of iNOS protein levels or nitrite production, showing that SPI2 is not involved in reduction of RNI production.

The subcellular localization of iNOS was dramatically different in macrophages infected with wild-type *Salmonella* or SPI2 mutants. We observed that iNOS was closely localized with SPI2 mutant strains, but rarely with wild-type bacteria. As SPI2 mutants show highly reduced intracellular proliferation, the colocalization of iNOS with SPI2 mutants suggests the involvement of nitrogen-derived radicals in the killing of SPI2 mutants. Inhibition of iNOS by the inhibitor L-NMMA resulted in increased proliferation of SPI2-deficient *Salmonella* in macrophages, indicating a role of RNI in limiting intracellular proliferation within macrophages.

The reaction of RNI with ROI generates peroxynitrite (ONOO<sup>-</sup>), a reactive molecule with potent antimicrobial activity against *S. typhimurium* in vitro (2). It was recently reported that peroxynitrite did not colocalize with the wild-type *Salmonella* and may not be responsible for the killing of intracellular bacteria (22). In fact, we confirm that peroxynitrite rarely colocalizes with wild-type *Salmonella*. In contrast, nitrotyrosine residues were frequently located in the vicinity of SPI2 mutants, indicating that the peroxynitrite formed can reach the target bacteria and may act as a potent molecule to kill the bacteria. This observation stresses the role of peroxynitrite in eradication of intracellular *Salmonella*. Production of ROI appears to be a crucial defense mechanism in the early phase of *Salmonella* infections as mice deficient in the phagocyte oxidase succumb shortly after infection (for a review, see reference 37). During the late phase of infection, the combined activities of ROI and RNI appear to be important for the control of proliferation of *Salmonella* in infected organs. We assume that peroxynitrite formation is a potent antimicrobial mechanism of macrophages and efficiently inactivates microorganisms that are not adapted to an intracellular lifestyle by virulence factors such as SPI2.

The observations reported here raise important new questions about the mode of delivery of iNOS to SCVs of the host cell and how intracellular *Salmonella* influence the

**Table III.** Competitive Indexes (CI) of a SPI2-deficient Mutant versus Wild-type *S. typhimurium* After Infection of iNOS<sup>+/+</sup> and iNOS<sup>-/-</sup> Mice

Time after infection (d)	CI in iNOS <sup>+/+</sup>		CI in iNOS <sup>-/-</sup>	
	Liver	Spleen	Liver	Spleen
2	0.24 ± 0.056	0.075 ± 0.035	0.52 ± 0.69	0.066 ± 0.091
3	0.0047 ± 0.0026	0.00078 ± 0.0001	0.0092 ± 0.0016	0.0028 ± 0.0005
5	<0.000005	<0.000005	1.20 ± 0.49	0.16 ± 0.04

localization of iNOS. If *Salmonella* can actively prevent the colocalization of iNOS with SCVs, under normal conditions iNOS may be transported to phagosomes in a vesicular fashion. iNOS of mouse macrophages is reported to reside not only free in the cytosol, but is also present in small vesicles (38). This has led to suggestions that the targeted delivery of such vesicles to the appropriate phagosomes possibly allows the interaction between iNOS and the pathogens, thereby exerting the antimicrobial activity. The association of iNOS with actin has been reported (32), but a recruitment of iNOS to phagosomes containing *S. typhimurium* was not observed under the specific experimental conditions of the study.

We speculate that transport of iNOS and NADPH-oxidase to phagosomes is tightly controlled to ensure that highly reactive antimicrobial radicals are preferentially produced at the appropriate subcellular location, without causing damage to host cell structures. The first model for the function of SPI2 postulates an inhibition of cellular trafficking, i.e., by inhibition of transport of iNOS-containing vesicles to SCVs. A second explanation of the phenotype described here may be the altered structure of SCVs. Recent observations indicate that intracellular *S. typhimurium* employ the SPI2 system to interfere with the organization of actin of the host cell and to accumulate actin at SCVs (39). This difference between the phagosomal composition of SCVs containing wild type *Salmonella* or SPI2 mutants may affect the access of iNOS to the bacteria. The modification of the pathogen-containing vesicle, or coating, induced by the pathogen was also observed in macrophages infected with *M. tuberculosis* (40).

A large number of STE proteins of the TTSS of SPI2 have been identified (33), and it is likely that the various effectors have different targets and functions in the host cell. However, our study did not indicate a significant role of single STE in protection of intraphagosomal *Salmonella* against nitrosative damages. STE proteins may have overlapping or partially redundant functions in protection of intracellular *Salmonella*, or additional, not yet characterized effector proteins of the SPI2 are required for this function.

Various intracellular phenotypes in macrophages have been related to the function of SPI2 of *Salmonella*: (a) Uchiya et al. reported the inhibition of vesicular trafficking as a function of SPI2 (17). (b) Vazquez-Torres et al. observed that intracellular *Salmonella* employ the SPI2 system to exclude the NADPH-oxidase from SCVs (18, 19). (c) Finally, we report here that the SPI2 system is involved in altering the localization of iNOS. Further work has to reveal whether the different cellular phenotypes are all consequences of the same function of SPI2, e.g., by a general inhibition of cellular vesicle transport. It is likely that the combined activities of NADPH-oxidase and iNOS at the SCVs are required to generate potent antimicrobial effectors.

Finally, the *Salmonella*-induced production of RNI might have deleterious effects on the host cells. If high amounts of RNI are produced but proper delivery to phagosomes is inhibited by SPI2, damage to the host cell

may occur. Such autotoxicity by incorrectly delivered antimicrobial effector molecules might also contribute to increased intracellular proliferation of *Salmonella*. It was reported that uric acid, which quenches peroxynitrite formation, improves killing of *Salmonella* by macrophages (22), suggesting that the peroxynitrite formed cannot reach the target bacteria and becomes cytotoxic to the host cell itself. This effect may enable the spread of *Salmonella* from infected macrophages and dissemination into other tissues, finally leading to a systemic infection.

Other mechanisms have been described that also contribute to the protection of bacteria against RNI and peroxynitrite. These include superoxide dismutase (41), flavohemoglobin (42), and peroxynitrite reductase (43), that are present in pathogenic bacteria and nonpathogenic relatives. Several of these mechanisms contribute directly to the virulence of *S. enterica*, while other mechanisms such as superoxide dismutase are duplicated with redundant functions (for a review, see reference 2). In contrast, the SPI2 locus represents an acquisition only present in the *S. enterica* (13) and is considered as an adaptation to survival and replication in a host organism (10). The large number of bacterial defense mechanisms evolved to counteract antimicrobial activities of RNI and peroxynitrite underline the role of these mechanisms in the innate immunity of the host.

Our observations are summarized in the following model for the interaction of *Salmonella* with macrophages: macrophages react to the contact with bacteria with expression of iNOS and increased production of RNI. *Salmonella* that invade macrophages or are taken up by phagocytosis are confronted with the potent antimicrobial activity of RNI produced by iNOS. In contrast to nonpathogenic bacteria, *Salmonella* employs the TTSS encoded by SPI2 to translocate effector proteins that prevent the colocalization of iNOS and the SCVs, so that RNI cannot react with the phagocytosed bacteria. In addition to bacterial mechanisms that interfere with production of RNI, detoxification of RNI, and repair of damage caused by RNI, the exclusion of RNI- and ROI-producing enzymes appears to be an important virulence mechanism of intracellular pathogens. To our knowledge, *S. typhimurium* is the first example of a pathogen that actively avoids the delivery of antimicrobial effectors to the pathogen-containing vesicle by means of a TTSS. It will now be of interest to analyze if other intracellular pathogens use similar strategies to evade antimicrobial activities of their host cells.

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