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Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of *Salmonella* phagosomes

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By remodeling the phagosomal membrane, the type III secretion system encoded within the *Salmonella* pathogenicity island-2 (SPI2) helps *Salmonella* thrive within professional phagocytes. We report here that nitric oxide (NO) generated by IFNγ-activated macrophages abrogates the intracellular survival advantage associated with a functional SPI2 type III secretion system. NO congeners inhibit overall expression of SPI2 effectors encoded both inside and outside the SPI2 gene cluster, reflecting a reduced transcript level of the sensor kinase SsrA that governs overall SPI2 transcription. Down-regulation of SPI2 expression in IFNγ-treated macrophages does not seem to be the result of global NO cytotoxicity, because transcription of the housekeeping *rpoD* sigma factor remains unchanged, whereas the expression of the *hmpA*-encoded, NO-metabolizing flavohemoprotein is stimulated. Because of the reduced SPI2 expression, *Salmonella*-containing vacuoles interact more efficiently with compartments of the late endosomal/lysosomal system in NO-producing, IFNγ-treated macrophages. These findings demonstrate that inhibition of intracellular SPI2 transcription by NO promotes the interaction of *Salmonella* phagosomes with the degradative compartments required for enhanced antimicrobial activity. Transcriptional repression of a type III secretion system that blocks phagolysosome biogenesis represents a novel mechanism by which NO mediates resistance of IFNγ-activated phagocytes to an intracellular pathogen.

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Abbreviations used: FRT, Flp recombinant target; iNOS, inducible nitric oxide synthase; MOI, multiplicity of infection; NADPH, nicotinamide-adenine dinucleotide phosphate; NMMA, N^G-monomethyl L-arginine; NO, nitric oxide; RNS, reactive nitrogen species; SPI2, *Salmonella* pathogenicity island 2.

Intracellular pathogens have developed strategies to cope with a myriad of oxygen-dependent and -independent components of the antimicrobial arsenal of professional phagocytes. For instance, nonfusogenic intracellular pathogens such as *Mycobacterium* and *Salmonella* arrest the biogenesis of phagolysosomes (1–6). *S enterica* encompasses a group of related gram-negative bacteria capable of causing a variety of clinical syndromes that range from asymptomatic colonization or self-limiting diarrhea to severe fibrinopurulent necrotizing enteritis and life-threatening systemic disease. The potential of this enteric pathogen to cause disseminated disease is intimately associated with its ability to replicate within macrophages (7). The type III secretion system encoded in the SPI2 chromosomal gene cluster recently has been found to be critical to the survival of *Salmonella* within professional phagocytes (8–11). By altering the trafficking of lysosomes, SPI2 effector proteins allow *Salmonella* to reside within modified phagosomes that avoid the terminal stages of the degradative pathway (12, 13). A functional SPI2 secretion system also minimizes contact of *Salmonella* phagosomes with vesicles harboring nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase or inducible nitric oxide synthase (iNOS) enzymatic complexes (11, 14–16). Therefore, the SPI2 type III secretion system lessens exposure of *Salmonella* to a large fraction of the antimicrobial arsenal of professional phagocytes, including an array of lysosomal hydrolytic enzymes and a battery of reactive oxygen and nitrogen species.

The iNOS enzymatic complex catalyzes the oxidation of the guanidino group of L-arginine for the generation of L-citrulline and the diatomic radical NO (17–19). NO and its congeners react with a variety of metal prosthetic groups, organic and inorganic radicals, lipids, and DNA molecules (20). It is likely that this rich biochemistry mediates the broad-spectrum

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antimicrobial activity of reactive nitrogen species (RNS) against many viruses, bacteria, and fungi, as well as against protozoan and metazoan parasites. However, specific molecular mechanisms underlying NO-mediated host defense are not well understood. The gram-negative enteropathogenic bacteria *Salmonella* also is susceptible to the antimicrobial activity of RNS. Multiple independent lines of investigation assessing the cytotoxicity of chemically generated RNS or monitoring the effects of pharmacological or genetic inhibition of iNOS have demonstrated the importance of NO in host resistance to this enteropathogenic bacterium (21–26). It is becoming apparent that RNS generated by macrophages are bacteriostatic for *Salmonella* (27). This NO-mediated antimicrobial activity is opposed by *Salmonella*'s adaptive anti-nitrosative response that repairs NO-mediated lesions and detoxifies a variety of NO congeners (20, 22, 28–31). Recent studies have shown that the *Salmonella* SPI2 type III secretion system adds to the anti-nitrosative defenses of *Salmonella* by avoiding contact with iNOS-containing vesicles (16). Herein, we report the effects of IFNγ-stimulated NO synthesis on SPI2 function.

IFNγ is essential for the development of protective immunity against numerous infectious diseases. An unequivocal role for IFNγ in host defense against *Mycobacterium* and *Salmonella* has been demonstrated definitively by the increased incidence of these intracellular pathogens in individuals carrying defects in the IFNγ signaling pathway (32–35). IFNγ probably exerts diverse functions in resistance to these intracellular bacteria. Activation of macrophages by IFNγ is critical for host defense against *Salmonella* infection (1, 35). Studies from our laboratory have indicated that IFNγ synergizes with lipopolysaccharide on the surface of *Salmonella* to enhance the transcription of iNOS (36). The resultant high NO synthesis is associated with profound and long-lasting anti-*Salmonella* activity by IFNγ-activated macrophages (27, 37). However, the mechanisms by which IFNγ-activated NO synthesis mediates the anti-*Salmonella* activity of macrophages remain largely unknown. We show herein that the high NO output generated by IFNγ-treated macrophages inhibits SPI2 transcription. Inhibition of SPI2 function facilitates the maturation of the *Salmonella* phagosome along the degradative pathway, contributing to the enhanced anti-*Salmonella* activity exhibited by NO-producing, IFNγ-treated phagocytic cells. The NO-mediated inhibition of a type III secretion system represents a novel mechanism by which IFNγ overcomes the arrest in phagosomal maturation imposed by a nonfusogenic intracellular pathogen.

RESULTS

NO abrogates the SPI2-dependent intracellular survival of *Salmonella*

The SPI2 type III secretion system prevents maturation of the *Salmonella* phagosome along the degradative pathway. Recent studies have demonstrated that, in addition to thwarting trafficking of lysosomes and vesicles harboring the NADPH oxidase (11, 12, 15), SPI2 decreases contact of *Salmonella* with iNOS-containing vacuoles (16). To study further the relation of SPI2 with iNOS, intracellular survival of *Salmonella* was studied in IFNγ-treated macrophages capable of sustaining high NO output. As anticipated (8–11), after 18 h of contact with primary macrophages, WT *Salmonella* harboring a functional SPI2 were recovered in higher numbers than its isogenic *ΔspiC* mutant strain AV0201 (Fig. 1 A, left). A WT *spiC* allele expressed from the low-copy plasmid pWKS29 successfully complemented the growth defect associated with the *ΔspiC* mutation. Treatment of macrophages with IFNγ resulted in a hundred-fold reduction in the number of *Salmonella* isolated after 18 h of culture and remarkably abrogated the intracellular growth advantage associated with a functional SPI2. IFNγ treatment also eliminated the SPI2-dependent survival advantage of WT *Salmonella* strain 12023 (Fig. 1 A, right). Macrophages from iNOS-deficient mice were used to assess whether NO is involved in abrogating the survival advantage associated with a functional SPI2 secretory system. In contrast with WT macrophages, enhanced SPI2-mediated *Salmonella* survival was seen in IFNγ-treated, iNOS-deficient

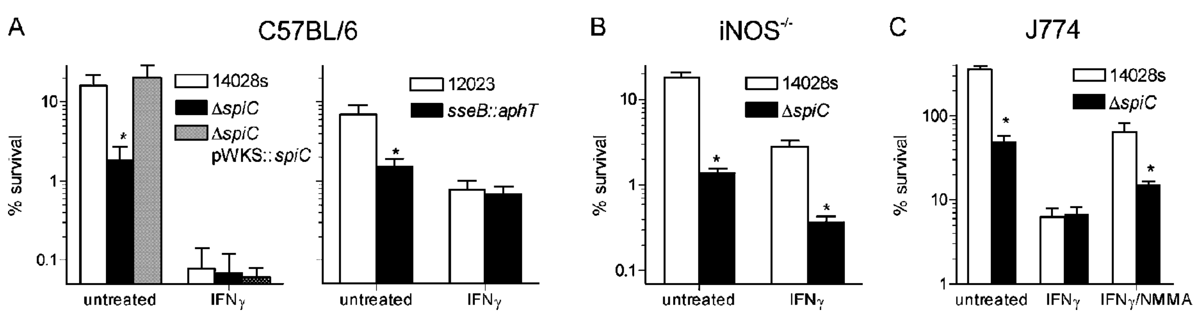

Figure 1. Abrogation of SPI2-mediated *Salmonella* survival by NO-producing, IFNγ-treated macrophages. The antimicrobial activity of untreated and IFNγ-treated periodate-elicited macrophages isolated from C57BL/6 (A) and *iNOS*$^{-/-}$ mice (B) and J774 macrophage-like cells (C) was recorded 18 h after challenge with WT *Salmonella* Typhimurium strain 14028s or 12023 or their isogenic Δ*spiC* or *sseB::aphT* mutant strains. The Δ*spiC* mutation was complemented with a WT copy expressed from the pWKS29 low copy plasmid (A). Selected groups of IFNγ-treated J774 cells were incubated with 250 μM of the NOS inhibitor NMMA at the time of *Salmonella* infection. These data are expressed as mean percent survival ± SEM of 4–18 independent observations from two to six separate experiments. *, $P < 0.05$ compared with WT controls.

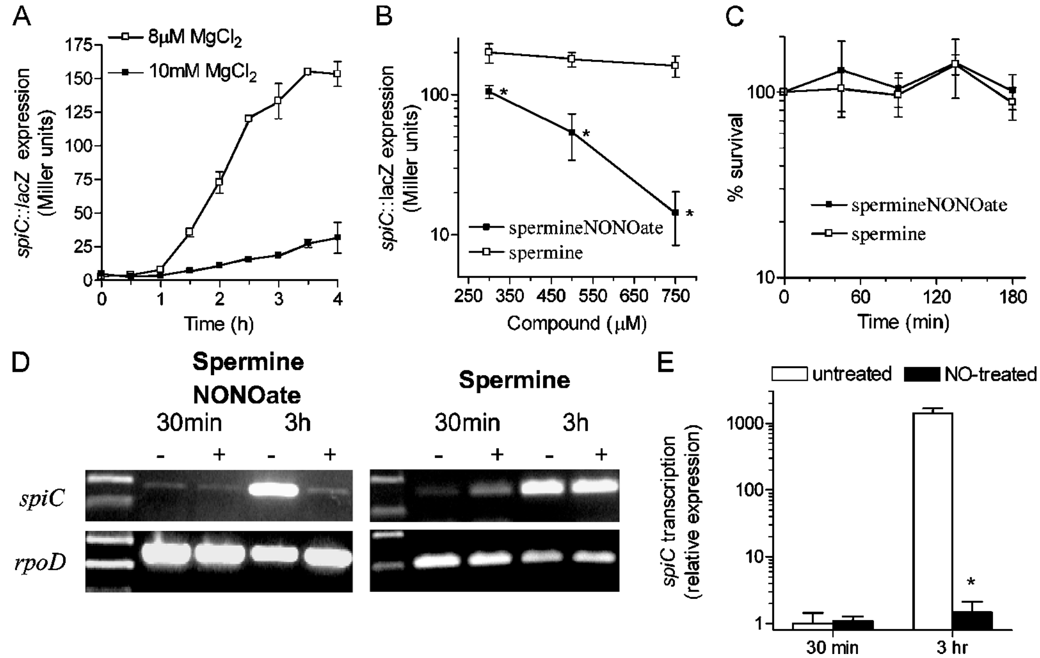

Figure 2. NO inhibits *Salmonella* SPI2 transcription. Expression of SPI2 was quantified by measuring β-galactosidase activity of strain AV0212 expressing a *spiC::lacZ* transcriptional fusion. The bacteria were grown in 8 μM or 10 mM $MgCl_2$ N salts medium (A) or in 8 μM $MgCl_2$ N salts medium in the presence of the NO donor spermine NONOate (B). Survival of strain AV0212 exposed for 3 h to 750 μM spermine NONOate was compared with untreated controls (C). Transcription of *spiC* and *rpoD* genes was assessed by RT-PCR (D) and real-time RT-PCR amplification (E) of RNA samples isolated from WT *Salmonella* cultured in 8 μM $MgCl_2$ N salts medium in the presence (+) or absence (−) of 750 μM spermine NONOate. Spermine was used as a control. Abundance of *spiC* transcripts is expressed relative to internal *rpoD* levels. The data in A–C and E represent the means ± SEM of at least three independent experiments. *, $P < 0.05$ compared with spermine-treated or untreated controls.

macrophages, suggesting that NO is critical for abrogating the survival advantage associated with SPI2 (Fig. 1 B). IFNγ also activated anti-*Salmonella* defenses that are independent of NO synthesis, as suggested by the increased toxicity exhibited by iNOS-deficient macrophages after IFNγ treatment (Fig. 1 B). Similar to the profiles of intracellular *Salmonella* growth seen in primary macrophages, the enhanced survival of SPI2-expressing *Salmonella* was lost upon the activation of J774 cells with IFNγ (Fig. 1 C). This phenotype depended upon NO synthesis, because the SPI2-dependent growth advantage was restored by adding the NOS inhibitor N^G-monomethyl-L-arginine (NMMA; Fig. 1 C). Together, these data are consistent with the hypothesis that NO produced by IFNγ-treated macrophages inhibits the intracellular survival advantage associated with the SPI2 type III secretion system.

NO inhibits the expression of *spiC* in vitro

To assess whether the NO-mediated inhibition of the intracellular growth associated with SPI2 occurs at the transcriptional level, *Salmonella* SPI2 gene transcription was assessed in vitro by measuring the activity of a *spiC::lacZ* transcriptional fusion. As anticipated (38), culture of *Salmonella* Typhimurium strain AV0212 in 8 μM $MgCl_2$ N salts medium, pH 6.9, induced the expression of *spiC::lacZ* (Fig. 2 A). To determine whether NO inhibits *spiC* expression, the NO donor spermine NONOate was added 1 h after *spiC::lacZ*-expressing *Salmonella* had been subcultured in a low magnesium N salts medium. Spermine NONOate suppressed *spiC::lacZ* transcription in a concentration-dependent manner (Fig. 2 B), and 750 μM of spermine NONOate was found to reduce *spiC* transcription to levels expressed by controls grown in noninducing high Mg^{2+} N salts medium. These inhibitory effects of spermine NONOate do not seem to be associated with cytotoxicity, because *Salmonella* viability was unaffected by the concentrations of NO donor used in these experiments (Fig. 2 C).

To rule out inadvertent side effects of the chromosomal *spiC::lacZ* transcriptional fusion, *spiC* transcription was assessed independently by RT PCR. In accord with the β-galactosidase activity, PCR amplification revealed the induction of *spiC* transcription after 3 h of growth in a low magnesium N salts medium. The expression of *spiC* was inhibited by 750 μM spermine NONOate (Fig. 2 D, left). In contrast, the expression of the housekeeping gene *rpoD* was similar after 30 min and 3 h of culture and was not affected appreciably by the addition of spermine NONOate (Fig. 2 D, left). The effects of spermine NONOate on *spiC* gene transcription seem to be dependent on the generation of NO, because comparable amounts of the parent compound, spermine, did not inhibit *spiC* expression (Fig. 2 D, right). A quantitative real-time PCR procedure, in which the number of *spiC* transcripts was normalized to internal *rpoD* levels, revealed a nearly 1,000-fold inhibition of *spiC* expression by spermine NONOate (Fig. 2 E).

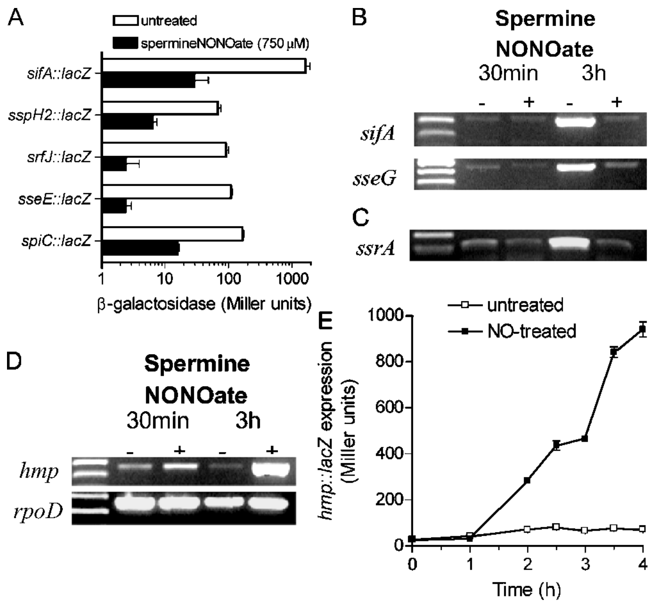

Figure 3. NO inhibits transcription of SPI2 effectors encoded in multiple loci of the *Salmonella* chromosome. (A) The effect of NO on transcription of several SPI2 *lacZ* transcriptional fusions was analyzed by measuring β-galactosidase as described in Fig. 2. These data represent the means ± SEM of two to five independent observations from two separate experiments. Transcription of the *sifA* and *sseG* SPI2 effectors (B) and the *ssrA* sensor kinase (C) was assessed by RT-PCR of RNA samples isolated from WT *Salmonella* grown in SPI2-inducing conditions in the presence or absence of the NO generator spermine NONOate as outlined in Fig. 2. (D) RT-PCR of *hmp* and *rpoD* transcripts isolated from *Salmonella* cultures grown in 8 μM $MgCl_2$ N salts medium in the presence or absence of 750 μM spermine NONOate. (E) The effects of the NO on *hmp* transcription were independently studied by measuring the activity of the *hmp::lacZ* transcriptional fusion expressed by *Salmonella* strain AV0305. The bacteria were grown in 8 μM $MgCl_2$ N salts medium in the presence or absence of 750 μM spermine NONOate. These data represent the means ± SD of three independent observations.

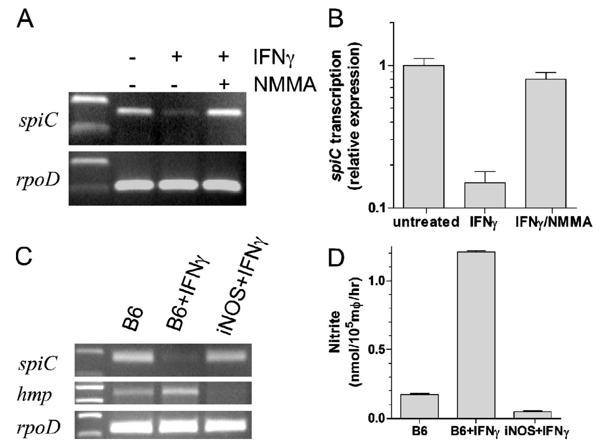

Figure 4. Inhibition of SPI2 transcription by NO-producing, IFNγ-treated macrophages. Transcription of *spiC* and *rpoD* was assessed by RT-PCR (A, C) and real-time RT-PCR (B) of samples isolated from J774 (A, B) or primary macrophages isolated from C57BL/6 (B6) or iNOS-immunodeficient (iNOS) mice (C). Total RNA was isolated from untreated or IFNγ-treated macrophages 18 h after *Salmonella* challenge. 250 μM NMMA (A and B) was added to selected J774 macrophages after *Salmonella* challenge. The rates of nitrite synthesis sustained by primary macrophages isolated from C57BL/6 or $iNOS^{-/-}$ mice were recorded after 12 h of *Salmonella* infection (D). Data in panels B and D represent the mean ± SEM of three independent experiments.

Global inhibitory effects of NO on the SPI2 regulon

The SPI2 secretion system translocates substrates encoded within the SPI2 pathogenicity island and effectors transcribed outside this gene cluster (39–41). To examine whether NO has global effects on SPI2 expression, additional *lacZ* transcriptional fusions were constructed for *sseE*, which is an effector protein encoded within SPI2, and for *sifA*, *srfJ*, and *sspH2*, which are encoded outside the island. Similar to *spiC*, the NO donor spermine NONOate inhibited transcription of *sifA*, *sspH2*, *srfJ*, and *sseE* (Fig. 3 A). The transcription of *sifA* and *sseG* also was inhibited by spermine NONOate as determined by RT-PCR analysis of RNA extracts isolated from *Salmonella* grown for 30 min or for 3 h in SPI2-inducing low Mg^{2+} N salts medium (Fig. 3 B). Together, these results suggest that NO exerts a global inhibition of transcription of SPI2 regulated genes.

The two-component SsrAB regulatory system encoded within the SPI2 gene cluster is a global regulator of SPI2 function. We therefore examined by RT-PCR whether transcription of the sensor kinase encoded by *ssrA* was affected by exposure to NO. Growth of *Salmonella* for 3 h in SPI2-inducing low Mg^{2+} N salts medium stimulated *ssrA* expression (Fig. 3 C). The transcription of *ssrA* was inhibited in response to spermine NONOate, suggesting that nitrogen oxides block SPI2 expression by directly or indirectly acting upon the master two-component SsrAB regulatory system.

Induction of *hmp* by NO

The expression of the NO-detoxifying *Salmonella* flavohemoglobin Hmp is positively induced by the transcriptional regulator Fur in response to nitrosative stress (28). The expression of *hmp* was monitored to examine whether NO-mediated inhibition of SPI2 transcription is caused by nonspecific cytotoxicity of this diatomic radical or its congeners. RT-PCR showed that the expression of *hmp* was up-regulated after 3 h of culture in the presence of the NO donor spermine NONOate (Fig. 3 D). As described earlier, the expression of the housekeeping gene *rpoD* was unaffected by exposure of *Salmonella* to this NO donor. An *hmp::lacZ* transcriptional fusion revealed a nearly 10-fold up-regulation of *hmp* transcription upon exposure to NO (Fig. 3 E).

The expression of *spiC* is down-regulated in NO-producing, IFNγ-treated macrophages

We next tested whether NO produced by IFNγ-treated macrophages decreases intracellular SPI2 transcription. PCR analysis revealed that s*piC* expression was inhibited selectively in J774 cells treated with IFNγ. Addition of 250 μM of the NOS inhibitor NMMA restored *spiC* expression to control levels, suggesting that the RNS produced by IFNγ-treated macrophages abrogate SPI2 expression (Fig. 4 A). Real-time RT-PCR showed an ~10-fold decrease in the number of *spiC* transcripts in IFNγ-treated macrophages compared with untreated controls (Fig. 4 B). Addition of the NOS inhibitor NMMA to IFNγ-treated, *Salmonella*-challenged J774 cells inhibited nitrite synthesis (unpublished data) and restored *spiC* expression. NO produced by IFNγ-treated primary macrophages also inhibited *spiC* transcription (Fig. 4 C). In contrast to this SPI2 gene, the expression of *hmp* was increased in response to NO produced by IFNγ-treated macrophages. As anticipated (27), IFNγ elevated the rate of NO synthesized by primary macrophages (Fig. 4 D). Together, these data suggest that the high rates of NO synthesized in response to IFNγ down-regulate SPI2 transcription, thus abrogating the survival advantage associated with a functional SPI2.

Functional iNOS is sufficient for the IFNγ-dependent inhibition of SPI2 transcription

The effects of IFNγ-stimulated NO synthesis on SPI2 expression were studied in more detail. IFNγ-treated macrophages exerted similar rates of killing against WT or *spiC*-deficient *Salmonella* in all the time points examined (Fig. 5 A). The increased numbers of WT *Salmonella* recovered from IFNγ-treated, iNOS-deficient macrophages were already evident after 6 h of infection. As shown in Fig. 1, the lack of iNOS had little effect on the intracellular survival of the *spiC* mutant strain, suggesting that the inhibition of the SPI2-dependent survival is a key target of NO-mediated cytotoxicity. In support of this hypothesis, intracellular *spiC* transcript levels were consistently higher at all time points examined in control macrophages lacking iNOS (Fig. 5 B). To study whether the early inhibition of *spiC* expression by NO-producing, IFNγ-activated macrophages involves metabolites generated during the respiratory burst, *spiC* expression was examined in gp91*phox*-deficient macrophages. The transcriptional analysis shown in Fig. 5 C indicates that the NO-mediated inhibition of *spiC* transcription sustained by IFNγ-treated macrophages can be achieved in the absence of a functional NADPH oxidase.

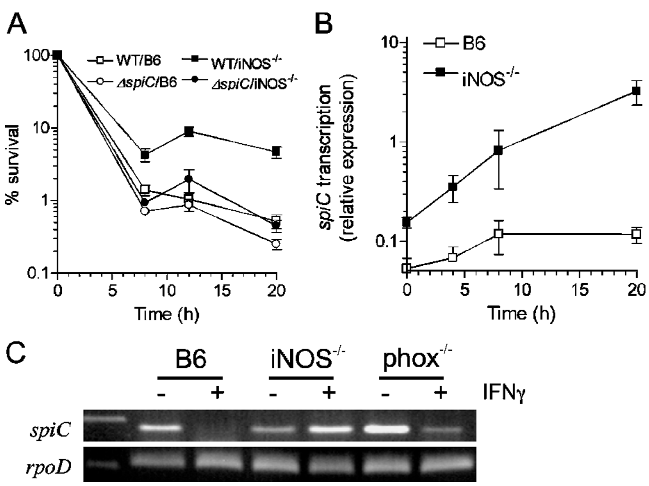

Figure 5. The NO-dependent inhibition of SPI2 expression in IFNγ-activated macrophages does not require a respiratory burst. (A) The survival of WT or Δ*spiC*::FRT *Salmonella* was determined in IFNγ-activated macrophages at several time points after infection as described in Fig. 1. (B) Temporal *spiC* transcription was tested in WT *Salmonella* grown in WT (B6) or iNOS$^{-/-}$ macrophages treated with 200 U/ml IFNγ. The abundance of *spiC* transcripts was estimated by real-time RT-PCR as described in Fig. 2. (C) *spiC* and *rpoD* expression was estimated by RT-PCR of samples isolated from WT (B6), iNOS$^{-/-}$, or gp91*phox*$^{-/-}$ peritoneal macrophages 16 h after challenge with *Salmonella* strain 14028s. Selected groups of macrophages were treated with IFNγ (+).

NO promotes the maturation of the *Salmonella* phagosome along the degradative pathway

Salmonella is a nonfusogenic intracellular pathogen (2–4, 42, 43). Through the actions of the SPI2 type III secretion system, *Salmonella* prevents the maturation of its phagosome and avoids contact with NADPH oxidase- and iNOS-containing vesicles and lysosomes (11, 12, 15, 16). Because SPI2 expression is inhibited by biologically generated NO (Figs. 4 and 5), we hypothesized that the IFNγ-induced high NO output endows macrophages with the capacity to process *Salmonella* along the degradative pathway. This hypothesis was tested by monitoring the intracellular localization of Texas red-dextran–labeled late endosomes/lysosomes and GFP-expressing *Salmonella* (Fig. 6). A functional SPI2 system allowed ~20% of contact between late endosomes/lysosomes and *Salmonella* (Fig. 6, A and H). In some instances, WT *Salmonella* were separated from late endosomes and lysosomes by the host cell nucleus. In contrast, the absence of a functional SPI2 allowed ~70% of the *Salmonella* phagosomes to acquire the late endosome/lysosome marker (Fig. 6, C and E). Treatment of J774 cells with IFNγ resulted in the maturation of phagosomes containing WT *Salmonella* along the degradative pathway, resulting in ~70% colocalization of *Salmonella* with late endosomes/lysosomes (Fig. 6, F and G). This process seems to depend on the actions of NO congeners, because 30% of *Salmonella*-containing vacuoles in IFNγ-activated J774 cells that were treated with the NOS inhibitor NMMA acquired the late endosome/lysosome marker (Fig. 6 H).

The effects of IFNγ on the maturation of the *Salmonella* phagosome were studied independently by transmission electron microscopy in primary macrophages isolated from WT C57BL/6 mice or from immunodeficient controls lacking iNOS or gp91*phox*. The maturation of *Salmonella* phagosomes was visualized by their colocalization with lysosomes that had been marked with colloidal gold-labeled BSA. Consistent with the data presented in Fig. 6, a small fraction of the phagosomes containing WT *Salmonella* matured into phagolysosomes (Fig. 7, A and G). IFNγ treatment greatly enhanced the interaction of *Salmonella* with lysosomes (Fig. 7, B, C, and G). In most cases, *Salmonella* was found in various states of digestion in the phagolysosomes of

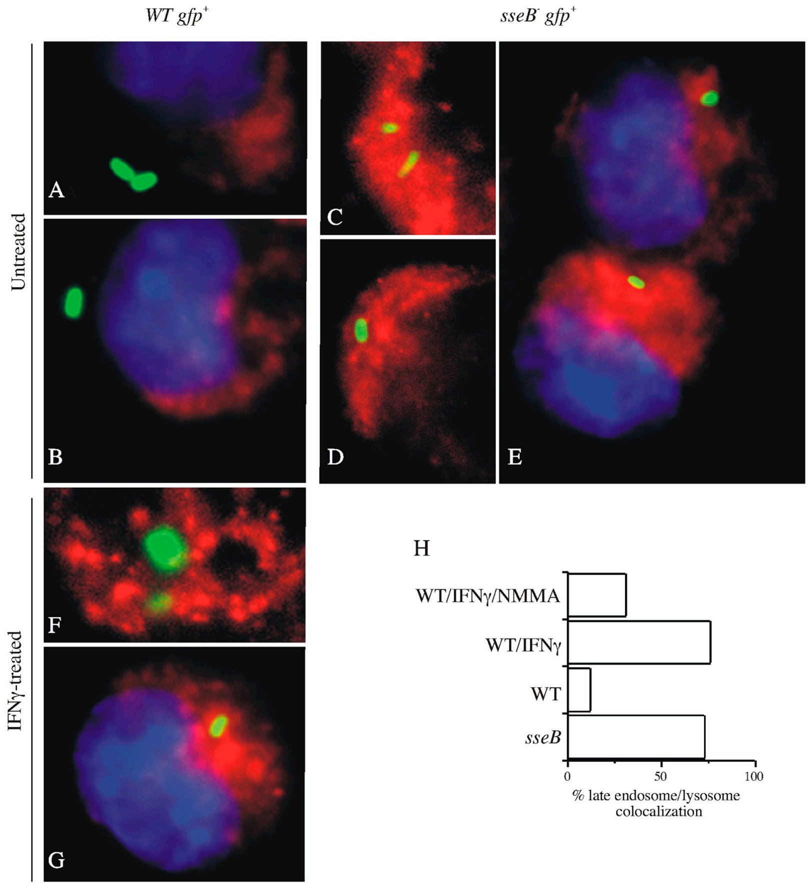

Figure 6. NO stimulates progression of the *Salmonella* phagosome along the degradative pathway. The intracellular localization of Texas red-dextran–labeled late endosomes/lysosomes (red), GFP-expressing *Salmonella* (green), and DAPI-labeled host cell nucleus (blue) was visualized by fluorescence microscopy. J774 cells were infected with either WT *Salmonella* (A and B) or its isogenic *sseB* mutant control (C–E). Selected groups of J774 cells were treated with IFNγ 20 h before infection with WT *Salmonella* (F and H). Samples were prepared for immunofluorescence microscopy 20 h after *Salmonella* infection and after 1 h of pulsing with Texas red-dextran. The percentage of *Salmonella* colocalizing with late endosomes/lysosomes is shown in H. These data represent an analysis of 330 independent observations from five separate experiments.

IFNγ-treated macrophages. However, in some instances, intact *Salmonella* were found in spacious phagolysosomes containing multiple electrodense DNA conglomerates (Fig. 7 C). The absence of iNOS resulted in a marked reduction in the proportion of *Salmonella* phagosomes that colocalized with lysosomes (Fig. 7, D and G). Remarkably, in the absence of a functional NADPH oxidase, IFNγ-activated macrophages were unable to deliver lysosomes to the *Salmonella* phagosome (Fig. 7, D and E), despite their ability to reduce SPI2 expression (Fig. 5). In fact, only 3% of the *Salmonella* vacuoles became phagolysosomes in IFNγ-treated macrophages lacking the gp91*phox* subunit of the NADPH oxidase (Fig. 7 G). Similarly, IFNγ-treated gp91phox$^{-/-}$ macrophages were unable to mature phagosomes containing *spiC*-mutant *Salmonella* along the degradative pathway (unpublished data). As anticipated by these remarkable findings, the SPI2 type III secretion system seems to be dispensable for the survival of *Salmonella* in IFNγ-treated, gp91phox$^{-/-}$ macrophages (Fig. 7 H).

DISCUSSION

The data presented herein demonstrate that NO congeners synthesized by IFNγ-activated macrophages exert a profound inhibitory effect on the transcription of the SPI2 type III secretion system. Inhibition of SPI2 transcription promotes the maturation of the *Salmonella* phagosome along the degradative pathway, facilitating a close interaction of the *Salmonella*-containing vacuole with late endosomes and lysosomes. The NO-mediated inhibition of SPI2 transcription is manifested functionally by the enhanced anti-*Salmonella* activity of IFNγ-treated macrophages. Because *Salmonella* strains that lack a functional SPI2 secretion system survive poorly in macrophages and exhibit a profound inability to cause systemic disease (8–11, 44), inhibition of SPI2 by NO may represent a mechanism by which IFNγ contributes critically to resistance to naturally acquired or experimentally induced *Salmonella* infections.

Transcriptional analysis of *Salmonella* exposed to NO generated chemically or synthesized enzymatically by acti-

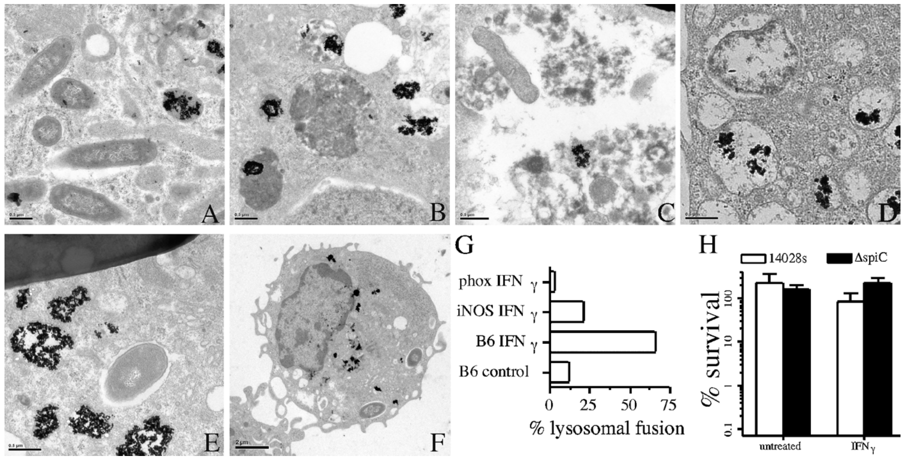

Figure 7. NO-dependent progression of *Salmonella* phagosomes along the degradative pathway in IFNγ-treated macrophages. Interaction of *Salmonella* phagosomes with lysosomes was evaluated by transmission electron microscopy 16 h after infection. Lysosomal localization was visualized as electrodense colloidal gold (25 nm)–labeled BSA particles in primary macrophages isolated from C57BL/6 (A–C), iNOS$^{-/-}$ (D), or gp91*phox*$^{-/-}$ (E and F) mice. Selected groups of macrophages (B–F) were treated with 200 U/ml IFNγ 16 h before challenge. Data in G represent the analysis of 253 phagosomes. Bars, 0.5 μm (A–E); 2 μm (F). (H) The survival of WT or *spiC*-deficient *Salmonella* was studied in untreated or IFNγ-treated gp91*phox*$^{-/-}$ macrophages 16 h after infection.

vated macrophages has revealed that nitrogen oxides inhibit overall transcription of SPI2 genes, including the effectors *sspH2*, *srfJ*, and *sifA* that are encoded outside the SPI2 gene cluster. The global inhibition of SPI2 transcription reflects the negative effects of NO on the expression of *ssrA*, which encodes a sensor kinase within the *ssrAB* operon located in the SPI2 pathogenicity island (9, 45). The two-component regulatory system SsrAB controls the expression of SPI2 genes encoding chaperons, subunits of the type III secretion apparatus and translocon, and a plethora of effectors (38, 40, 41, 45, 46). Expression of SPI2 is induced shortly after *Salmonella* enters into macrophages and is dependent upon the presence of a functional SsrAB two-component regulatory system (9, 10). Accordingly, lack of *ssrA* abrogates intracellular SPI2 expression and diminishes the resistance of *Salmonella* to the antimicrobial defenses of macrophages (9). Therefore, direct or indirect inhibition of *ssrA* expression by NO seems to be responsible for the general down-regulation of SPI2 expression and also may contribute to the enhanced anti-*Salmonella* activity of IFNγ-treated macrophages. Cysteines present in the response regulators SsrB and OmpR, both of which directly control *ssrA* transcription (47, 48), are potential targets for NO-mediated SPI2 inhibition.

Our data indicate that the inhibition of SPI2 function by NO is critical for the anti-*Salmonella* activity of IFNγ-treated macrophages. In addition, the recovery of higher numbers of SPI2-deficient *Salmonella* from activated macrophages that cannot sustain NO production (Fig. 1) suggests that nitrogen oxides also inhibit targets unrelated to SPI2 function. Many proteins, including a variety of dehydratases, organic radicals, and enzymes involved in DNA synthesis and replication, may represent such targets (20). However, our data indicate that these targets are of lesser importance than the effects of NO on SPI2. This conclusion is supported by NO's mediating a threefold reduction in the numbers of SPI2-deficient *Salmonella* recovered from IFNγ-treated macrophages, as compared with a 30-fold decrease for SPI2-sufficient controls.

The presence of a functional SPI2 does not restore complete survival of *Salmonella* in IFNγ-treated macrophages lacking iNOS. This observation is consistent with the view that IFNγ also induces anti-*Salmonella* defenses that are independent of NO synthesis. In analogy to the intracellular pathogen *Mycobacterium* (6), it is possible that IFNγ promotes degradative maturation of the *Salmonella* phagosome through the up-regulation of small guanosine triphosphatases. In support of this view, the percentage of *Salmonella* phagosomes that associate with late endosomes/lysosomes is higher upon IFNγ treatment, even in the absence of NO synthesis (Figs. 6 and 7). In addition to promoting NO-dependent and -independent phagosomal maturation, IFNγ may increase the anti-*Salmonella* activity of macrophages through its positive effects on NADPH oxidase transcription (49).

The SPI2 secretion system has been shown to prevent trafficking of vesicles containing the iNOS hemoprotein to the *Salmonella* phagosome (16). The avoidance of iNOS-containing vesicles may represent an important protection mechanism against the low NO fluxes sustained during the innate host response. Nevertheless, the SPI2 type III secretion system does not seem to prevent these relatively low levels of NO from reaching *Salmonella* completely. A possible explanation is that, even though NO possesses an unpaired electron, this diatomic radical diffuses freely through lipid membranes (50). The lipid solubility of NO may explain the stimulation of *hmp* transcription in macrophages exhibiting low rates of NO synthesis (Fig. 4). Therefore, the inhibition of SPI2 transcription by NO seems to depend on the rate of synthesis of this diatomic radical. It is likely that, at the low rates of NO that are generated by unstimulated macrophages, the NO dioxygenase Hmp (51), together with other anti-nitrosative defenses such as glutathione, homocysteine, and glutathione reductase (20, 52) allows *Salmonella* SPI2 transcription. However, at higher rates of NO synthesis, such as those sustained by IFNγ-activated macrophages (Fig. 4 D; reference 27), SPI2 transcription is inhibited, despite the presence of increased *hmp* expression (Fig. 4 C). The down-regulation of SPI2 transcription seen in IFNγ-treated macrophages could be interpreted as a key action of NO-related antimicrobial activity. Down-regulation of SPI2 in turn may allow *Salmonella* to expend its energy on an adaptive response to the nitrosative stress characteristic of the late phases of salmonellosis (25, 27).

The NO-mediated inhibition of *spiC* expression can occur in the absence of a functional NADPH oxidase. These findings suggest that $ONOO^-$ generated by the combined actions of the NADPH oxidase and iNOS hemoproteins is not responsible for the SPI2 inhibition seen in IFNγ-activated macrophages. However, these data do not rule out the possibility that $ONOO^-$ produced endogenously by the reaction of NO generated by the phagocyte and superoxide released by the bacteria mediates inhibition of SPI2 transcription. Of note, the inhibition of SPI2 expression in gp91*phox*-deficient macrophages is not followed by an increase anti-*Salmonella* activity. These unexpected findings might be explained by our observation that NADPH oxidase–deficient macrophages are unable to fuse lysosomes with *Salmonella* phagosomes. This novel role for the NADPH oxidase in the terminal stages of the degradative pathway seems to be different from the effect of the respiratory burst on the release of neutral proteases from the negatively charged proteoglycan matrix of acidic granules (53).

In summary, the present study has revealed a new mechanism by which IFNγ promotes progression of a nonfusogenic intracellular pathogen along the degradative pathway. RNS produced in response to IFNγ inhibit the transcription of the SPI2 type III secretion system that is essential for remodeling the *Salmonella* phagosomal membrane. Inhibition of SPI2 by NO may predispose *Salmonella* to a vast array of effectors from the armamentarium of activated macrophages. For instance, NO facilitates the interaction of *Salmonella* with late endosomes/lysosomes, and thus it contributes to the enhanced anti-*Salmonella* activity exhibited by IFNγ-treated macrophages.

MATERIAL AND METHODS

Bacterial strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028s was used throughout this study as WT and as the background for the construction of mutations of several SPI2 genes and the *hmp*-encoded flavohemoprotein (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050246/DC1). The mutations were engineered following the one-step, λ red–mediated gene replacement method originally described by Datsenko and Wanner (54). Briefly, primers encoding 60 nucleotides homologous to the target gene followed by 20 nucleotides homologous to the pKD13 plasmid were used for the PCR amplification of the Flp recombinant target (FRT)-flanked kanamycin resistance cassette encoded within the pKD13 plasmid. The resulting PCR product was *DpnI*-digested and electroporated into *Salmonella* Typhimurium strain TT22236 carrying the pTP2223 plasmid that expresses the λ red recombinase under P*tac* control (55). Mutations were moved into *Salmonella* Typhimurium strain 14028s by bacteriophage P22–mediated transduction, and pseudolysogens were eliminated by streaking on Evans blue uranine agar plates. The kanamycin-cassette was excised by recombining the flanking FRT sites, which serve as site-specific substrates for the Flp recombinase encoded within the pCP20 plasmid (56). The 'scars' in the *sifA, srfJ, sspH2, sseE, spiC*, and *sseG* SPI2 genes and *hmp* containing a single FRT site were confirmed by PCR, and the resultant mutants were used for the construction of chromosomal *lacZ* transcriptional fusions following the method described by Ellenmeier et al. (57). Accordingly, the pCE36 plasmid, which carries a promoterless *lacZ* gene downstream from a unique FRT site, was integrated in the SPI2 or *hmp* FRT 'scars' after transformation with the temperature-sensitive pCP20 plasmid expressing the Flp recombinase.

Macrophages. C57BL/6 and congenic $iNOS^{-/-}$ (58) or gp91phox$^{-/-}$ (59) mice were bred in our animal facility according to Institutional Animal Care and Use Committee guidelines. Peritoneal macrophages were harvested from mice 4 d after intraperitoneal inoculation of 1 mg/ml sodium periodate as described (22). The peritoneal exudate cells were resuspended in RPMI 1640 medium (Cellgro; Mediatech, Inc.) supplemented with 10% heat-inactivated FBS (BioWhittaker Inc), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich), and 100 U · ml^{-1}/100 mg · ml^{-1} of penicillin/ Lakes streptomycin (Cellgro). The peritoneal exudate cells were seeded in 24-well plates (Falcon) for *spiC* transcriptional studies or 96-well plates for macrophage-killing assays. The macrophages were selected by adherence after 48 h of culture at 37°C in a 5% CO_2 incubator. J774 murine macrophage-like cells (clone ATCC TIB-67) grown in RPMI 1640 medium supplemented as described earlier were used as an additional source of mononuclear phagocytes. Selected groups of macrophages were treated with 200 U/ml of IFNγ (Life Technologies) during the last 20 h of culture before *Salmonella* infection.

Macrophage killing assays. Macrophages were challenged at a multiplicity of infection (MOI) of 2 with WT *Salmonella* strains 14028s or 12023 and their *spiC* or *sseB* isogenic controls. The bacteria were opsonized with 10% normal mouse serum for 20 min before infection. To ensure that the phenotypes associated with the Δ*spiC*::FRT mutation are not caused by polar effects on downstream genes of the SPI2 operon, strain AV0201 was transformed with the low copy number pWKS29 vector expressing a WT *spiC* allele under the control of its native promoter. Extracellular bacteria were removed from the monolayers 25 min after challenge by washing with prewarmed RPMI 1640 medium containing 6 μg/ml of gentamicin (Sigma-Aldrich) (22). The *Salmonella*-infected macrophages were lysed at indicated time points after challenge, and the surviving bacteria were enumerated on Luria-Bertani agar plates. The results are expressed as percentage survival.

Effects of NO on in vitro SPI-2 expression. SPI2 expression was induced in vitro by culturing *Salmonella* in low osmolarity N salts medium as described (38). Briefly, *Salmonella* Typhimurium strains harboring *lacZ* transcriptional fusions were grown overnight in high Mg^{2+} N salts medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% casamino acids supplemented with 10 mM $MgCl_2$ and 100 mM Tris-HCl], pH 7.6. The bacteria were subcultured in high Mg^{2+} N salts medium and grown at 37°C in a shaker incubator until they reached an OD_{600} of 0.5. SPI2 expression was induced by switching the bacteria to 8 μM $MgCl_2$ N salts medium, pH 6.9. The expression of SPI2 *lacZ* transcriptional fusions was quantified spectrophotometrically as β-galactosidase enzymatic activity using the substrate o-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich; reference 60). β-galactosidase activity is expressed in Miller units according to the equation: $1{,}000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T_{(min)} \times V_{(ml)} \times OD_{600})$.

The NO donor spermine NONOate (Cayman Chemical) was used to determine the effects of RNS on SPI2 transcription. Spermine NONOate dissolved in 10 mM Tris-HCl, pH 7.4, was quantified spectrophotometrically using $\varepsilon_{252} = 8{,}500\ M^{-1}\ cm^{-1}$. The NO donor was added to the cultures after *Salmonella* had been grown for 1 h in 8 μM $MgCl_2$ N salts medium, a time at which the bacterial cells had reached late log phase.

Synthesis of SPI2 cDNA from bacterial cultures and *Salmonella*-infected macrophages. Total RNA was isolated from bacterial cultures grown in 8 μM $MgCl_2$ N salts medium or from *Salmonella*-infected macrophages. The samples were resuspended in TRIzol reagent (Invitrogen) containing silicon beads and processed in a bead-beater (Biospec Products, Inc.). The RNA, extracted with chloroform, was precipitated in a 1:1 mixture of isopropyl alcohol: 0.8 M sodium citrate solution, washed in ethanol, and dried in a speed vacuum. The samples were resuspended in RNAase-free H_2O and were digested with RNase-free DNase (Promega). The RNA was purified further using an RNeasy kit following the protocol recommended by the manufacturer (QIAGEN). Complementary cDNA was synthesized at 42°C for 30 min using MMLV reverse transcriptase (Promega), RNasin (Promega), dNTPs, and reverse primers for the genes of interest (Table S2, available at http://www.jem.org/cgi/content/full/jem.20050246/DC1). The cDNA was used as template for standard PCR and real-time PCR.

Quantification of SPI2 transcripts by PCR and real-time PCR. PCR reactions were performed using *Salmonella* cDNA, dNTPs, Taq DNA polymerase (Continental Lab Products), and forward and reverse primers (Table S2). PCR products were visualized under UV light after electrophoresis. The real-time PCR reactions contained cDNA, Takara OmniMi HS (Takara Bio Inc.), forward and reverse primers and fluorescent-labeled DNA probes for the *spiC* gene or the housekeeping sigma factor *rpoD* (Table S2). Real-time PCR reactions consisted of a cycle at 94°C for 45 s followed by 45 cycles at 94°C for 5 s and at 59°C for 30 s The resulting fluorescence was recorded using the SmartCycler II thermocycler (Cepheid).

Analysis of phagosomal maturation by fluorescence microscopy. J774 cells cultured on Permanox slides (Nalge Nunc International) were infected at a MOI of 2 with GFP-expressing *Salmonella* strains FF0001 or FF0002 that are proficient or deficient for the *sseB* component of the SPI2 translocon, respectively. After 20 h of infection, the cells were pulsed for 15 min with 0.1 mg/ml of 10,000 molecular weight Texas red–labeled dextran (Molecular Probes), a fluid phase marker that accumulates in late endosomes/lysosomes (61, 62). The cells were fixed in paraformaldehyde (Electron Microscopy Sciences) after 1 h of chasing and were mounted with a coverslip in Vectashield containing the DNA stain DAPI. The specimens were analyzed in a fluorescence microscope for the cellular distribution of *Salmonella* (green), late endosomes/lysosomes (red), and host cell nucleus (blue). To study the effects of macrophage activation on the maturation of the *Salmonella* phagosome, selected groups of macrophages were treated with 200 U/ml of IFNγ 20 h before *Salmonella* infection. The role of NO on the progression of *Salmonella*-containing vacuoles to phagolysosomes was studied by adding 250 μM of the NOS inhibitor N^G-monomethyl-L-arginine (Sigma-Aldrich) at the time of the infection.

Analysis of phagosome/lysosome fusion by electron microscopy. Progression of the *Salmonella* phagosome along the degradative pathway was studied independently by transmission electron microscopy. Lysosomal compartments of periodate-elicited macrophages isolated from C57BL/6 mice or congenic iNOS- or gp91*phox*-deficient controls were pulse/chased with BSA-gold (25-nm) tracer ($OD_{520} = 0.5$; Electron Microscopy Sciences). Extracellular BSA-gold particles were washed with PBS after 4 h of pulsing, and the macrophages were incubated for an additional 3 h before infection. The phagocytes were then challenged with WT *Salmonella* at a MOI of 2 as described earlier. The infected cells were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4, after 16 h of challenge, and the specimens were postfixed in 1% osmium tetraoxide, treated with uranyl acetate, dehydrated in ascending ethanol series, and infiltrated with Embed 812 (Electron Microscopy Sciences). Ultrathin sections were examined in a FEI Technai 62 electron microscope operated at 80 kV.

Statistical analysis. Data are expressed as mean ± SEM. The data were analyzed using a paired Student's *t* test.

Online supplemental material. Table S1 lists the bacterial strains and plasmids used in this study. Table S2 shows the nucleotide sequences for the PCR primers used in mutational and complementation analysis.Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050246/DC1.

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