Antimicrobial Actions of the NADPH Phagocyte Oxidase and Inducible Nitric Oxide Synthase in Experimental Salmonellosis. II. Effects on Microbial Proliferation and Host Survival In Vivo

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Abstract
The roles of the NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS) in host resistance to virulent Salmonella typhimurium were investigated in gp91phox−/−, iNOS−/−, and congenic wild-type mice. Although both gp91phox−/− and iNOS−/− mice demonstrated increased susceptibility to infection with S. typhimurium compared with wild-type mice, the kinetics of bacterial replication were dramatically different in the gp91phox−/− and iNOS−/− mouse strains. Greater bacterial numbers were present in the spleens and livers of gp91phox−/− mice compared with C57BL/6 controls as early as day 1 of infection, and all of the gp91phox−/− mice succumbed to infection within 5 d. In contrast, an increased bacterial burden was detected within reticuloendothelial organs of iNOS−/− mice only beyond the first week of infection. Influx of inflammatory CD11b+ cells, granuloma formation, and serum interferon γ levels were unimpaired in iNOS−/− mice, but the iNOS-deficient granulomas were unable to limit bacterial replication. The NADPH phagocyte oxidase and iNOS are both required for host resistance to wild-type Salmonella, but appear to operate principally at different stages of infection.

Key words: Salmonella • virulence • innate immunity • oxidative • nitrosative

Introduction
Salmonella infections afflicting animals and humans pose a serious medical and veterinary problem worldwide. Despite considerable progress in understanding the genetic determinants of Salmonella virulence, rational vaccine design is still hampered by an inadequate understanding of the essential elements of host resistance to Salmonella. Much of the current knowledge of mechanisms of natural resistance and acquired immunity in salmonellosis has been generated in the mouse typhoid model (1–3), in which polymorphonuclear and mononuclear phagocytes play a critical role by limiting initial bacterial replication (4–10). In immunocompetent mice, bacterial growth in the reticuloendothelial system is suppressed by the end of the first week of infection, resulting in a plateau phase (11). This requires an influx of bone marrow–derived mononuclear cells and coincides with the onset of hepatosplenomegaly and granuloma formation, but does not require functional CD4+ or CD8+ T cells (11). Soluble factors including TNF-α, IFN-γ, IL-12, and IL-18 are essential for the suppression of bacterial growth during sublethal Salmonella infections (9, 12–22), and are specifically required for granuloma formation (9, 12, 17) and macrophage activation (14, 20). Secondary infection in immunized mice additionally involves Salmonella-immune CD4+ and CD8+ T cells and Salmonella-specific antibodies (3, 17).
The mechanisms by which phagocytes inhibit or kill virulent salmonellae are incompletely characterized, but the NADPH phagocyte oxidase has been strongly implicated (23, 24). The NADPH phagocyte oxidase catalyzes the production of superoxide (O$_2^-$) that can be metabolized to a variety of toxic reactive oxygen species (ROS). Patients deficient in the NADPH phagocyte oxidase are susceptible to recurrent microbial infections, including salmonellosis (25), in a clinical condition known as chronic granulomatous disease (CGD; for a review, see reference 26). The gp91phox gene encoding an essential component of the NADPH phagocyte oxidase has been targeted in mice, resulting in the elimination of oxidative burst activity in neutrophils and macrophages and an increased susceptibility to microorganisms including Salmonella typhimurium (24), A. fumigatus, Staphylococcus aureus (26), and Mycobacterium tuberculosis (27).

The role of the inducible nitric oxide (NO) synthase (iNOS) in the ability of macrophages to inhibit or kill Salmonella has been somewhat less clear (28, 29). Dimeric iNOS catalyzes the conversion of l-arginine to l-citrulline with the production of NO, which can be further metabolized to a variety of congeners (reactive nitrogen species [RNS]) with antimicrobial activity (30). Activated macrophages display increased expression of iNOS and elevated production of NO derivatives (for a review, see reference 30). The role of NO derivatives in host resistance to microbes has been documented in vitro and in vivo in a large number of infection models (for a review, see reference 31).

Generation of O$_2^-$ and NO derivatives in response to Salmonella infection has been documented both in vivo (20, 32) and in vitro (24, 33–35). The activity of iNOS is positively regulated by several cytokines (IL-12, IFN-γ, TNF-α) known to be essential for host resistance to Salmonella (12, 20, 30). Evidence obtained using metabolic inhibitors and immunodeficient knockout mice suggests that oxygen radicals mediate resistance to Salmonella in mice and are required for maximal bacterial killing by macrophages (24, 29). Furthermore, data obtained using NO synthase (iNOS) inhibitors L-NMMMA or aminoguanidine support a role for NO derivatives in host resistance to Salmonella (32, 33, 36). However, some evidence from in vitro models has suggested that the respiratory burst oxidase, but not iNOS, is essential to kill virulent Salmonella, whereas both ROS and RNS are involved in the killing of some mutant Salmonella strains (24, 28, 29). Moreover, observations using the NOS-inhibitor aminoguanidine have suggested that iNOS may regulate infiltration of inflammatory cells in the tissues, rather than exert direct antimicrobial activity against Salmonella (36).

Many of the published studies examining phox-dependent and iNOS-dependent host defenses in salmonellosis have used attenuated bacterial strains and innately susceptible mice, making it somewhat difficult to ascertain the role of these defenses in infections with virulent Salmonella (29, 32, 33, 36). The goal of this study was to clarify the roles of the NADPH phagocyte oxidase and iNOS in limiting bacterial replication during experimental infection with virulent S. typhimurium, using gp91phox$^{-/-}$ mice in a Salmonella-susceptible C57BL/6 (N ramp1) genetic background, and iNOS$^{-/-}$ mice in both C57BL/6- (N ramp1) and Salmonella-resistant 129Sv (N ramp1) genetic backgrounds. Sublethal inocula of wild-type Salmonella were administered to facilitate the detection of phox-dependent and iNOS-dependent antimicrobial actions.

### Materials and Methods

A. Mice were purchased from Harlan Olac Ltd. Mice homozygous for a targeted mutation in the gp91 subunit of the NADPH oxidase (gp91phox$^{-/-}$) on a C57BL/6 genetic background (26) were bred at the University of Colorado School of Medicine Center for Laboratory Animal Care. Mice homozygous for a targeted mutation in the iNOS gene (iNOS$^{-/-}$ [37, 38]) on a C57BL/6 or 129Sv background were bred at either BoK Universal, Ltd. or the Imperial College animal unit.

**Bacteria.** S. typhimurium M525P, a variant of S. typhimurium M525 (18), is a wild-type strain of intermediate virulence. S. typhimurium C5 is a highly virulent strain (6). For intravenous inoculation, bacteria were grown at 37°C as stationary overnight cultures in Luria-Bertani (LB) broth (Difco). Aliquots were snap frozen and stored in liquid nitrogen. The inoculum was diluted in PBS and injected in a lateral tail vein. The number of viable bacteria in each inoculum was checked by dilution and pour plating onto LB agar plates.

**Bacterial Enumeration in Organ Homogenates.** Mice were killed by cervical dislocation. Spleens and livers were aseptically removed and homogenized in a Colworth Stomacher in 10 ml of cold distilled water (11). Viable counts were determined using pour plates of LB agar.

**LD$_{50}$ Determinations.** Groups of mice were injected intravenously with 10-fold decreasing doses of S. typhimurium M525P or S. typhimurium C5. Parallel groups of age-matched uninfected mice were observed in parallel during each experiment. Mortality was scored over a 30-d period. LD$_{50}$ values were calculated according to the method of Reed and Muench (39).

**IFN-γ ELISA.** Mice were bled from a lateral tail vein. Sera were collected and stored at −70°C. IFN-γ was measured by capture ELISA using antibody pairs and cytokine standards purchased from BD PharMingen.

For IFN-γ determinations, 96-multiwell ELISA plates (MaxiSorp Immuno Plate; Nunc) were coated overnight at 4°C with 50 μl/well of a rat anti-mouse IFN-γ IgG1 monoclonal capture antibody (clone R 4-6A2) in 0.1 M NaHCO$_3$ buffer, pH 9.5 at 2 μg/ml. After blocking with PBS supplemented with 10% FCS at 37°C for 1 h, twofold serum dilutions were loaded in 50 μl in triplicate, and the plates were incubated at 37°C for 2 h. Serial twofold dilutions of rIFN-γ ranging from 20 ng/ml to 40 pg/ml were included as standards. Biotinylated rat anti-mouse IFN-γ IgG1 mAb (clone XM G1.2; 100 μl/well) at 1 μg/ml in PBS supplemented with 10% FCS was added for 1 h at 37°C, after which 100 μl/well of peroxidase-labeled streptavidin at 2.5 μg/ml (Sigma-Aldrich) in PBS supplemented with 10% FCS was added for 45 min at room temperature. O-Phenylenediamine
in parallel experiments. Log10 LD50 values 1 mo after infection with Salmonella C57BL/6 or 129Sv control mice were infected with highly virulent S. typhimurium C5 strain, respectively. Congenic C57BL/6 wild-type control mice were infected intravenously with a 10-fold increasing dose ranging from 10^5 to 10^6 CFU of moderately virulent S. typhimurium M525P or the highly virulent S. typhimurium C5 strain, respectively. Congenic wild-type C57BL/6 or 129 Sv control mice were infected in parallel experiments. Log10 LD50 values 1 mo after infection with each strain were found to be 3.35 ± 0.06 in C57BL/6 and 4.29 ± 0.19 in 129Sv mice, compared with 5.42 ± 0.22 and 5.28 ± 0.07 in the respective control groups. Thus, infection with O5 S^-/- mice show increased susceptibility to Salmonella, but can still survive infection with low bacterial inoculum over a 30-d observation period. This contrasts with the gp91phox^-/- mice, for whom even a very modest inoculum was rapidly lethal.

On the basis of the LD50 determinations, seven C57BL/6 wild-type mice and seven congenic wild-type control mice were infected intravenously with ~10^6 CFU of S. typhimurium M525P (Fig. 1 B). Similarly, seven 129Sv mice and nine congenic 129Sv mice were infected with ~10^6 CFU of the highly virulent S. typhimurium C5 strain. Results are expressed as number of survivors and compared to the different experimental groups. Differences between experimental groups were considered significant for P values <0.05.

Results

Mortality of S. typhimurium Infection in Wild-type, gp91 phox^-/-, and congenic wild-type mice infected with S. typhimurium. (A) Eight gp91phox^-/- mice and eight congenic C57BL/6 controls (Nram1^+, Salmonella susceptible) were infected intravenously with ~600 CFU of S. typhimurium M525P, a strain of intermediate virulence. (B) Seven IN O5 S^-/- mice and seven congenic C57BL/6 controls (Nram1^+) were infected intravenously with ~10^6 CFU of S. typhimurium M525P. (C) Nine IN O5 S^-/- mice and nine congenic 129Sv mice were infected with ~10^6 CFU of the highly virulent S. typhimurium C5 strain. Results are expressed as number of survivors at times after infection.
wild-type mice were infected with $6.5 \times 10^3$ CFU of the highly virulent wild-type strain. Spleen and liver bacterial counts were similar in C57BL/6- and iNOS$^{-/-}$-deficient mice during the first 5 d of infection, despite the administration of higher inocula than those administered to the gp91phox$^{-/-}$ mice. However, by days 8 and 12, the counts in the livers of iNOS$^{-/-}$ mice were significantly higher than those of C57BL/6 control mice; by day 21, the bacterial counts in both spleens and livers of C57BL/6 iNOS$^{-/-}$ mice were considerably higher in comparison to controls. The course of infection and differences in bacterial counts between mutant and wild-type mice were similar when a lower inoculum ($6.5 \times 10^2$ CFU) was administered, except for lower absolute values and an absence of deaths observed throughout the 30-d experiment (data not shown). Fig. 2, E and F shows the course of the infection with S. typhimurium C5 in 129Sv and congenic iNOS$^{-/-}$ mice. Bacterial counts in iNOS$^{-/-}$ mutant and wild-type mice were similar until day 8 of the infection. Thereafter, both spleen and liver counts were significantly higher in the iNOS$^{-/-}$ mice. Repeat experiments gave similar results (data not shown).

Histopathology of S. typhimurium Infection in Wild-type, gp91phox$^{-/-}$, and iNOS$^{-/-}$-Derivative mice. Spleen and liver histology was monitored at specific times in the experiments described in Fig. 2. The spleens of infected gp91phox$^{-/-}$ mice were increased in size more rapidly ($P < 0.01$) than those of congenic controls. Histopathological examination of the spleen after infection with S. typhimurium M525P, a wild-type strain of intermediate virulence, did not show increased susceptibility to infection that leads to death of the animals during the second and third week of infection. No deaths were observed in parallel groups of five uninfected iNOS$^{-/-}$ or wild-type mice of either strain.

In parallel experiments, iNOS$^{-/-}$ and congenic C57BL/6 wild-type mice were infected with $6.5 \times 10^4$ CFU of S. typhimurium M525P, whereas iNOS$^{-/-}$ and congenic 129Sv wild-type controls were infected with $8.5 \times 10^4$ CFU of S. typhimurium C5. Fig. 2, C and D shows that spleen and liver bacterial counts were similar in C57BL/6- and iNOS$^{-/-}$-derivative mice during the first 5 d of infection, despite the administration of higher inocula than those administered to the gp91phox$^{-/-}$ mice. However, by days 8 and 12, the counts in the livers of iNOS$^{-/-}$ mice were significantly higher than those of C57BL/6 control mice; by day 21, the bacterial counts in both spleens and livers of C57BL/6 iNOS$^{-/-}$ mice were considerably higher in comparison to controls. The course of infection and differences in bacterial counts between mutant and wild-type mice were similar when a lower inoculum ($6.5 \times 10^2$ CFU) was administered, except for lower absolute values and an absence of deaths observed throughout the 30-d experiment (data not shown). Fig. 2, E and F shows the course of the infection with S. typhimurium C5 in 129Sv and congenic iNOS$^{-/-}$ mice. Bacterial counts in iNOS$^{-/-}$ mutant and wild-type mice were similar until day 8 of the infection. Thereafter, both spleen and liver counts were significantly higher in the iNOS$^{-/-}$ mice. Repeat experiments gave similar results (data not shown).

**Figure 2.** Bacterial counts in liver and spleen of gp91phox$^{-/-}$, iNOS$^{-/-}$, and congenic wild-type mice infected with S. typhimurium. (A and B) gp91phox$^{-/-}$ mice and congenic C57BL/6 controls (N ramp1) were infected intravenously with $\sim$600 CFU of S. typhimurium M525P, a wild-type strain of intermediate virulence. All Salmonella-infected gp91phox$^{-/-}$ mice were dead by day four. (C and D) iNOS$^{-/-}$ mice and congenic C57BL/6 controls (N ramp1) were infected with $6.5 \times 10^4$ CFU of S. typhimurium M525P. (E and F) iNOS$^{-/-}$ mice and congenic 129Sv controls (N ramp1) were infected with $8.5 \times 10^4$ CFU of the highly virulent wild-type S. typhimurium C5 strain. Spleen and liver counts were determined at time points thereafter. The results are expressed as Log$_{10}$ viable count (mean ± SD) obtained from groups of four mice per data point.
C57BL/6 wild-type control mice (Fig. 3 A) after infection with 600 CFU S. typhimurium M 525P, in parallel with organism burden. As shown in Fig. 3 B, a similar increase in spleen weight was observed during the first 8 d of the infection in C57BL/6 IN O S⁻⁻⁻ mice and C57BL/6 mice infected with 6.5 x 10⁴ CFU of S. typhimurium M 525P. The mean spleen weight was slightly higher in C57BL/6 mice than in C57BL/6 IN O S⁻⁻⁻ on day 12 (P = 0.02), but no significant differences in spleen weight between IN O S⁻⁻⁻ mutant and wild-type mice were observed by day 21 of the infection. A similar experiment performed with a lower challenge dose (6.5 x 10³ CFU per mouse) showed similar spleen weights in IN O S⁻⁻⁻ mutant and C57BL/6 wild-type mice at all time points (data not shown). Fig. 3 C shows that significant (P = 0.02) differences in spleen weight between IN O S⁻⁻⁻ mice and congenic 129Sv wild-type mice infected with 8.5 x 10⁴ CFU of S. typhimurium C5 appeared by day 8 after infection and persisted until at least day 14.

Microscopic examination of livers of C57BL/6 and congenic gp91phox⁻⁻⁻ and IN O S⁻⁻⁻ mice revealed the early formation of microabscesses rich in polymorphonuclear neutrophils by day 3 of infection (Fig. 4, A–C), with more extensive lesions noted in the gp91phox⁻⁻⁻ animals. On days 8, 11, and 14, the focal lesions in the spleens and livers of the infected C57BL/6 and IN O S⁻⁻⁻ mice consisted mainly of mononuclear cells and were surrounded by normal tissue. This evolution of the inflammatory response (10, 18) occurred similarly in both IN O S⁻⁻⁻ mice (Fig. 4, D and F) and C57BL/6 mice (Fig. 4, E and G), showing no iNOS dependence on the formation of mononuclear cell-rich granulomas. In several IN O S⁻⁻⁻ mice it was visually evident that the average size of focal granulomas was larger than in congenic wild-type control mice. On day 21 of infection, granulomatous lesions present in the livers and spleens of IN O S⁻⁻⁻ mice in the C57BL/6 background showed a variable degree of central necrosis (Fig. 4, H–K); macroscopically visible abscesses were present in the livers and occasionally in the spleens. The histological picture in organs of wild-type 129Sv and congenic IN O S⁻⁻⁻ mice infected with S. typhimurium C5 was similar to that described in the C57BL/6 strain background, except for the absence of central necrosis in the hepatic and splenic lesions.

The focal lesions in several liver sections were counted at low-power magnification. As shown in Fig. 5, similar numbers of inflammatory foci were present in the livers of IN O S⁻⁻⁻ and congenic C57BL/6 mice on day 3 (predominantly neutrophils) and day 8 (predominantly mononuclear cells) of infection, but the numbers of mononuclear cell-rich lesions were significantly higher in C57BL/6 IN O S⁻⁻⁻ mice by days 12 and 21. Similar numbers of inflammatory foci were found within the livers of IN O S⁻⁻⁻ and congenic 129Sv mice on day 3 of infection, but thereafter the number of lesions was significantly higher in the livers of the IN O S⁻⁻⁻ mice. Thus, both IN O S⁻⁻⁻ mice and congenic wild-type mice develop splenomegaly and form granulomas in the spleen and liver during experimental salmonellosis.

Flow Cytometry of Splenocyte Populations in Salmonella-infected Wild-type and IN O S⁻⁻⁻ Mice. Studies in mice treated with iNOS inhibitors have suggested that iNOS might be required for the recruitment of inflammatory cells to the spleen (36). The percentages of CD11b⁺ cells and I-A b⁺ CD11b⁺ cells were determined on days 3, 8, and 15 or 16 in the spleens of Salmonella-infected wild-type and IN O S⁻⁻⁻ mice in experiments parallel to those depicted in Fig. 2. CD11b⁺ cells and I-A b⁺ CD11b⁺ cells were recruited to infected spleens of IN O S⁻⁻⁻ mice and wild-type mice to a similar degree and, at some time points, higher percentages of inflammatory cells were present in the spleens of IN O S⁻⁻⁻ mice compared with wild-type mice (Table I). The percentages of CD4⁺ T cells, CD8⁺ T cells, CD25⁺ cells, and B220⁺ cells in the spleens of wild-type and

Figure 3. Spleen weights of gp91phox⁻⁻⁻, IN O S⁻⁻⁻, and congenic wild-type mice infected with S. typhimurium. Mice were infected as described in the legend to Fig. 2. Spleen weights were measured from (A) infected gp91phox⁻⁻⁻ and congenic C57BL/6 mice, (B) IN O S⁻⁻⁻ and congenic C57BL/6 mice, and (C) IN O S⁻⁻⁻ mice and congenic 129Sv mice at designated time points. Results are expressed as mean spleen weight ± SD for four mice per data point.
iNOS−/− mice were also comparable (not shown). The results shown are representative of three individual experiments that yielded similar results. Thus, the greater bacterial burden observed in iNOS−/− mice (Fig. 2, C–F) cannot be attributed to an impairment in the infiltration of inflammatory cells.

Serum IFN-γ Levels in Salmonella-infected Wild-type and iNOS−/− Mice. Serum IFN-γ was measured by ELISA in the sera of six Salmonella-infected wild-type and six congenic iNOS−/− mice at days 3, 7, 11, 16, and 21 of infection. No statistically significant differences (P > 0.05) in serum IFN-γ levels between iNOS−/− and C57BL/6 wild-
type mice after infection with S. typhimurium M52SP were detected on days 3, 7, and 11. Serum IFN-γ levels at later time points were statistically higher (P < 0.05) in iNOS−/− mice (243 ± 109 pg/ml on day 16; 430 ± 44 pg/ml on day 21) than in C57BL/6 wild-type controls (82 ± 62 pg/ml on day 16; 75 ± 9 pg/ml on day 21). Similarly, no differences in serum IFN-γ levels between iNOS−/− and congenic 129Sv wild-type mice infected with S. typhimurium C5 were detected on days 3, 7, and 11, but IFN-γ levels on days 16 and 21 of infection were statistically higher (P < 0.05) in the iNOS−/− mice (500 ± 99 pg/ml on day 16; 710 ± 144 pg/ml on day 21) than in the 129Sv wild-type controls (156 ± 130 pg/ml on day 16; 200 ± 49 pg/ml on day 21). Thus, iNOS deficiency does not impair circulating levels of IFN-γ during Salmonella infection. Higher levels of IFN-γ at later time points are probably attributable to increased organism burden, but may also reflect regulatory effects of NO on cytokine production.
Detailed study of the course of Salmonella infection in gp91phox−/− or iNOS−/− mice has revealed major differences in the temporal contribution of the NADPH phagocyte oxidase and iNOS to host defense in salmonellosis. Enhanced bacterial proliferation was observed in gp91 phox−/− mice as early as day 1 after infection. In contrast, iNOS−/− mice effectively controlled bacterial growth in the tissues during the first week of infection, but a lethal increase in bacterial burden appeared later in the course of infection.

Early killing of bacteria by resident phagocytes and acute inflammatory cells during the first 24 h of infection appears to be heavily dependent on the presence of an intact NADPH phagocyte oxidase. These in vivo observations correlate well with in vitro studies, suggesting a crucial role for the oxidative burst in the rapid killing of ingested Salmonella by macrophages (28, 29, 35). The accelerated replication of Salmonella in gp91phox−/− mice that already possess an innately susceptible N ramp1(169D) allele (40) reveals a degree of independence between N ramp1 and oxidative burst-dependent host defenses, and shows that N ramp1−/− mice still retain potent antimicrobial mechanisms during the early phases of Salmonella infection. Indeed, N ramp1 (lyt) is believed to limit bacterial growth rather than enhance bacterial killing in vivo (41), whereas NADPH-mediated oxidative mechanisms have been shown to mediate killing of Salmonella by mononuclear cells (28, 29, 35).

iNOS expression in the tissues can be detected very early in the course of a Salmonella infection (within 24 h) (32, 34; our unpublished observations), raising the possibility of synergistic antimicrobial interactions between ROS and RNS, as have been shown for Escherichia coli and Salmonella typhimurium in vitro (35, 42) and for sodC-deficient S. typhimurium in vivo (24). However, such interactions are unlikely to be essential for the early killing of wild-type S. typhimurium in this experimental model, because iNOS was dispensable for the control of bacterial replication during the initial days of infection.

The early bactericidal role of the NADPH phagocyte oxidase complex (44), and perhaps even its direct inhibition by nitrogen oxides (45), might contribute to the evolution of a variety of oxidant species, the subsequent predominant production of NO· over time would be predicted to lead to a progressive conversion to nitrosative chemistry (35, 43). The tenuous stability of the activated NADPH phagocyte oxidase complex (44), and perhaps even its direct inhibition by nitrogen oxides (45), might contribute to the evolution of the host response from ROS to RNS dependence. This temporal progression of host defense may also be accounted for by the greater reliance of acute inflammatory neutrophils and resident mononuclear cells on oxidative killing mechanisms, succeeded by an influx of NO·-producing activated macrophages. Although oxidative bacterial killing by neutrophils is highly effective (46), organisms persisting within mononuclear cells (10) may require slower iNOS-dependent mechanisms for their eventual clearance.

**Table I. Flow Cytometry**

| Days after infection | CD11b+ | I-A b+CD11b+ |
|---------------------|--------|--------------|
| Day 3               |        |              |
| C57BL/6             | 12.45  | 5.0          |
| C57BL/6 iNOS−/−     | 6.4    | 5.5          |
| 129                 | 8.82   | 5.3          |
| 129 iNOS−/−         | 12.8   | 8.2          |
| Day 8               |        |              |
| C57BL/6             | 12.11  | 7.69         |
| C57BL/6 iNOS−/−     | 15.1   | 7.3          |
| 129Sv               | 14.4   | 11.3         |
| 129Sv iNOS−/−       | 25.1   | 22.7         |
| Days 15–16          |        |              |
| C57BL/6             | 12.8   | 6.83         |
| C57BL/6 iNOS−/−     | 21.3   | 9.05         |
| 129Sv               | 14.0   | 8.6          |
| 129Sv iNOS−/−       | 23.0   | 12.7         |

Percentages of CD11b+ and CD11b−I-A b+ double positive cells in the spleens of iNOS−/− and congenic C57BL/6 mice infected with S. typhimurium M525P, or iNOS−/− and congenic 129Sv mice infected with S. typhimurium C5. Mice were infected as described in the legend to Fig. 2. Results are expressed as percentages of total cells. The data are representative of three individual experiments that yielded similar results.
In murine typhoid, iNOS is positively modulated by IFN-γ and TNF-α (12, 20); therefore, it could be hypothesized that IFN-γ and TNF-α mediate the suppression of bacterial growth in the tissues of infected mice via NO-dependent mechanisms. However, there are important distinctions among the courses of infection in cytokine-deficient, gp91phox−/−, and iNOS−/− mice. In contrast to iNOS−/− mice, whole body X-irradiated mice, IFN-γ−/− mice, IFN-γR−/− mice, TNFR55−/− mice, and mice treated with anti–IFN-γ or anti–TNF-α neutralizing antibodies fail to suppress the early growth of Salmonella in the reticuloendothelial system (11–13, 16; our unpublished observations), and succumb within 7–8 d after challenge with wild-type Salmonella under experimental conditions similar to those used in this study (our unpublished observations). These observations indicate that IFN-γ and TNF-α do not exclusively enhance innate immunity via NO synthesis. Later stages of Salmonella infection are controlled by T cell–mediated immunity, and mouse strains lacking T cells such as nude mice, CD-α/β−/− mice, recombination activating gene (rag)1−/− mice, or sld mice fail to clear Salmonella and succumb to infection despite initial suppression of early bacterial replication (13, 47; our unpublished observations). At this time, we cannot exclude that some degree of impairment in T cell responses to Salmonella might occur in iNOS−/− gene-targeted mutant mice, but recent evidence from mouse models of Trypanosoma brucei and Mycobacterium avium infection shows that iNOS-deficient mice can mount effective T cell responses despite the absence of high-output NO production (48, 49).

Early histopathological examination of Salmonella-infected mice revealed microabscesses containing neutrophils in all strains, but lesions were most extensive with surrounding necrosis in gp91phox−/− animals. iNOS−/− and wild-type mice progressed to develop macrophage-rich granulomas by day 8, but all gp91phox−/− had succumbed to uncontrollable infection by this time point. We observed the development of central necrosis in some granulomas during later stages of infection in N ramp1 C57BL/6 iNOS−/− mice but not in wild-type mice or N ramp1 129Sv iNOS−/− mice, possibly reflecting increased bacterial proliferation leading to the death of the phagocytic and parenchymal cells in the more susceptible mouse strain. Dysregulation of cytokine production in the absence of RNS may also be contributing to the development of necrosis (50, 51).

Results obtained after chemical iNOS inhibition during Salmonella infection have suggested that impaired RNS production can lead to a reduction of macrophage tissue infiltration and granuloma formation (32, 36). Nitrogen oxides are known to play a role in the positive regulation of macrophage inhibitory protein (MIP)-1x, a powerful chemoattractant for macrophages (51), and have additional important immunoregulatory properties (52). However, in this study we found the morphology of granulomatous lesions to be very similar in the tissues of iNOS−/− and congenic wild-type mice infected with S. typhimurium. In fact, focal lesions and numbers of CD11b+ cells were even more numerous in iNOS−/− mice during late stages of infection compared with wild-type mice. Increased granuloma formation, which has also been noted in iNOS−/− mice infected with M. avium (53), may reflect inhibitory effects of NO on leukocyte recruitment (54). We have also shown that the influx of inflammatory cells in the spleen as measured by flow cytometry, the development of splenomegaly, the elevation of serum IFN-γ levels, and the activation of inflammatory cells as measured by l-arginine expression (12, 55) are not impaired in iNOS−/− mice. Elevation of serum IFN-γ levels has also been described in iNOS−/− mice infected with T. brucei or L. donovani major (38, 56). The discrepant findings in iNOS−/− mice and animals administered iNOS inhibitors may reflect NOS-independent actions of inhibitors such as aminoguanidine (57), or residual or compensatory mechanisms in the mice (58). Our results indicate that NO production plays an important antimicrobial effector role during salmonellosis. This conclusion is further supported by observations in murine listeriosis, in which NO inhibition exacerbates infection and bacterial burden without apparent effects on hepato-splenic macrophage inflam-mation and granuloma formation (59).

In conclusion, the course of experimental salmonellosis in congenic mice lacking a functional NADPH phagocyte oxidase or iNOS has revealed critical temporal differences in the roles of these host immune effector mechanisms. The early induction of the NADPH phagocyte oxidase results in rapid bacterial killing, but this phase is rapidly succeeded by a prolonged plateau phase with eventual bacterial clearance dependent upon the induction of NO production. This coordinated response may maximize antimicrobial actions of the innate immune system while limiting collateral tissue injury from exposure to reactive oxidants.

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