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Salmonella Pathogenicity Island 2-Encoded Type III Secretion System Mediates Exclusion of NADPH Oxidase Assembly from the Phagosomal Membrane

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Salmonella typhimurium requires a type III secretion system encoded by pathogenicity island (SPI)-2 to survive and proliferate within macrophages. This survival implies that S. typhimurium avoids or withstands bactericidal events targeted to the microbe-containing vacuole, which include intraphagosomal production of reactive oxygen species (ROS), phagosomal acidification, and delivery of hydrolytic enzymes to the phagosome via fusion with lysosomes. Recent evidence suggests that S. typhimurium alters ROS production by murine macrophages in an SPI-2-dependent manner. To gain insights into the mechanism by which S. typhimurium inhibits intraphagosomal ROS production, we analyzed the subcellular distribution of NADPH oxidase components during infection of human monocyte-derived macrophages by wild-type (WT) or several SPI-2 mutant strains of S. typhimurium. We found that the membrane component of the NADPH oxidase, flavocytochrome b$_{558}$, was actively excluded or rapidly removed from the phagosomal membrane of WT-infected monocyte-derived macrophages, thereby preventing assembly of the NADPH oxidase complex and intraphagosomal production of superoxide anion. In contrast, the NADPH oxidase assembled on and generated ROS in phagosomes containing SPI-2 mutant S. typhimurium. Subversion of NADPH oxidase assembly by S. typhimurium was accompanied by increased bacterial replication relative to that of SPI-2 mutant strains, suggesting that the ability of WT S. typhimurium to prevent NADPH oxidase assembly at the phagosomal membrane represents an important virulence factor influencing its intracellular survival. The Journal of Immunology, 2001, 166: 5741–5748.

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4 Abbreviations used in this paper: SPI, Salmonella pathogenicity island; TTSS, type III secretion system; cyt b$_{558}$, flavocytochrome b$_{558}$; DPBS, Dulbecco’s PBS; GFP, green fluorescent protein; MDM, monocyte-derived macrophage; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; ssa, secretion system apparatus; sse, secretion system potential effector; WT, wild type; PAB, PBS containing 0.5 g/L sodium azide and 5 g/L BSA.

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cytosolic components, \( p74^{\text{phox}} \) and \( p67^{\text{phox}} \), of the NADPH oxidase during infection of monocyte-derived macrophages (MDMs) by WT or several SPI-2 mutant strains of \( S. \ typhimurium \). We found that most phagosomes containing \( S. \ typhimurium \) that express a complete SPI-2 TTSS lacked cyt \( b_{558} \), thereby thwarting successful assembly of the NADPH oxidase and avoiding \( O_2^- \)-derived antimicrobial agents. We discuss how preventing NADPH oxidase assembly may contribute to the ability of \( S. \ typhimurium \) to circumvent successfully host microbicidal pathways.

### Materials and Methods

**Materials**

Dulbecco’s PBS (DPBS), RPMI 1640, MEMs, L-glutamine, FBS, and goat serum were purchased from Life Technologies (Grand Island, NY). Lab-Tek 8 well-chamber slides were obtained from Nalge Nunc International (Naperville, IL). A mixture of three murine mAbs to cyt \( b_{558} \) was used in immunofluorescence microscopy. 7D5, a generous gift from Michio Nakamura (Institute of Tropical Medicine, Nagasaki, Japan), recognizes an extracellular conformational epitope on gp91 \( \text{phox} \) expressed only by the assembled \( \text{NADPH oxidase} \). The Abs recognizing cyt \( b_{558} \) and gp91 \( \text{phox} \) (44:1) were the generous gifts from A. Jessat, M. Quinn, and J. Burritt (Montana State University, Bozeman, MT) and recognize linear epitopes on the cytosolic domain of the respective subunits of cyt \( b_{558} \) (15). Anti-p74 \( \text{phox} \) and p67 \( \text{phox} \) polyclonal Abs were previously described (16). Texas Red-coupled anti-mouse and -rabbit secondary Abs were obtained from Molecular Probes (Eugene, OR), and Cy5-conjugated anti-mouse and anti-rabbit secondary Abs were obtained from J. Burritt (Montana State University, Bozeman, MT) and recognize linear epitopes on the cytosolic domain of the respective subunits of cyt \( b_{558} \) (15).

**Bacterial strains and growth conditions**

Chromosomal modifications were introduced in SL1344 \( S. \ typhimurium \) in genes coding for the TTSS apparatus (secretion system apparatus or ssa) or in genes coding for effector proteins (secretion system effector or sse). The \( S. \ typhimurium \) ssaT mutant used in this study was made using SsaT of strain P9B7 \( ssaT::\text{mTn5} \) obtained from David Holden (17). The \( ssaT::\text{mTn5} \) mutation was moved by P22-mediated transduction to \( S. \ typhimurium \) strain SL1344. The desired transductant was designated \( S. \ typhimurium \) JK14. The \( S. \ typhimurium \) sseD mutant was constructed using the following method. The \( sseD \) gene was amplified from the SL1344 chromosome using primers \( sseD1 \) (5'-GGACGCAATGATGTGCGG-3') and \( sseD2 \) (5'-CGTACACAACCC-3'). The 2.4 kb DNA fragment was cloned into pGEM-T Easy (Promega, Madison, WI) and a blunt-ended \( aphT \) gene encoding kanamycin resistance was ligated into the middle of the \( sseD \) gene digested with EcoRV. The \( sseD::\text{aphT} \) construct was cloned into the pBDJ129 vector, followed by selection for allelic exchange of the mutated \( sseD \) into the SL1344 chromosome (18). The \( sseD::\text{aphT} \) transductant was designated \( S. \ typhimurium \) JK22. SseD protein was not expressed from JK22, as determined by Western blot analysis. In addition, the mutation resulted in severe attenuation of mouse virulence (19). JK22 was complemented by a plasmid carrying the appropriate gene. 7D5, a generous gift from Michio Nakamura (Institute of Tropical Medicine, Nagasaki, Japan), recognizes an extracellular conformational epitope on gp91 \( \text{phox} \) expressed only by the assembled \( \text{NADPH oxidase} \). JK22 was complemented by a plasmid carrying the appropriate gene.

**Intraphagosomal superoxide generation**

MDMs were plated on chamber slides and peritoneal macrophages were plated on glass coverslips to achieve ~50% confluence. \( S. \ typhimurium \) SL1344, \( sseD, \ ssaT \) strains, or zymosan particles were washed, dispersed in HEPES-RPMI 1640 containing 1 ng/ml nitroblue tetrazolium (NBT), and then added to macrophages to achieve a ratio of 3 zymosan/cell or 25 bacteria/cell. Phagocytosis was synchronized using centrifugation (600 × g, 3 min, 16°C) followed by a 60-min incubation at 37°C. Samples were washed three times with DPBS, counterstained with Wright-Giemsa (SureStain; Fisher, Pittsburgh, PA), and then sealed using Permount (Fisher). Light microscopy was performed using a Zeiss Axiosplan2 microscope (Carl Zeiss, Thornwood, NY) and samples were photographed using ASA 400 color slide film. Phagosomes were monitored for the presence of blue-black formazan precipitate formed by the reduction of NBT by superoxide anion. At least 300 phagosomes were scored per experiment in triplicate samples.

**Synchronized phagocytosis**

GFP-expressing \( S. \ typhimurium \) SL1344, \( sseD, \ ssaT \) strains were suspended in complete medium (RPMI 1640 with 10 mM HEPES and 10% heat-inactivated FBS) at a concentration of 2.5 × 10^6 bacteria/ml. Phagocytosis was synchronized by centrifugation of bacteria (10^6 bacteria/well) onto the macrophage monolayer (2 min, 600 × g, 12°C), followed by incubation at 37°C in 5% CO_2 for the desired amount of time. Cells were then stained and/or processed for immunofluorescence microscopy (see below).

**Assessment of bacterial growth**

Replication of \( S. \ typhimurium \) inside macrophages was evaluated by immunofluorescence and confocal microscopy. This approach was recently described by Hensel and collaborators in the back cover of a recent issue of Molecular Microbiology (Vol. 36, issue 5, 2000). Following centrifugation of \( S. \ typhimurium \) onto the macrophage monolayer, chamber slides were incubated at 37°C in 5% CO_2 for 30 min. Gentamicin was then added at a final concentration of 6 µg/ml and incubation was extended for 90 min. We verified that this treatment effectively killed noningested bacteria by quantifying CFUs after exposing bacteria to gentamicin (5–100 µg/ml) for 10–60 min (data not shown). Furthermore, 85–95% of cell-associated WT or mutant \( S. \ typhimurium \) was intracellular at 5 min of phagocytosis at 37°C, as judged by confocal microscopy.

During the 90-min treatment with gentamicin, cells were washed once in RPMI 1640 and processed for microscopy (2-h time point) or further incubated at 37°C in 5% CO_2 in gentamicin-free complete medium for 4 or 8 h (6- or 10-h time points, respectively) to minimize antibiotic-mediated effects on intracellular \( S. \ typhimurium \). Cells were then stained with Wright-Giemsa and examined using a Zeiss Axiosplan2 photomicroscope or processed for immunofluorescence microscopy. Bacteria were quantified from 100 infected macrophages for each strain in each of three to six independent experiments. The numbers obtained were compared using paired Student’s t test with significance set at \( p < 0.05 \).

**Immunofluorescence microscopy and image processing**

\( S. \ typhimurium \)-infected MDMs were fixed in 10% neutral-buffered Formalin for 15 min at 25°C and then permeabilized in 50% methanol/50% acetic acid for 5 min at 4°C. Fixed and permeabilized cells were rinsed in DPBS supplemented with 0.5 mM sodium azide and 5 mM BSA (PAB), and then blocked in PAB containing 10% heat-inactivated normal goat serum (blocking buffer) for 1 h at 25°C or overnight at 4°C. Following incubation with primary Abs (anti-p74 \( \text{phox} \) or p67 \( \text{phox} \) rabbit Ab and/or anti-cyt \( b_{558} \) mouse Ab, diluted in blocking buffer) for 1 h at 25°C, cells were washed five times in PAB, and incubated with appropriate secondary Abs (anti-mouse Cy5 and/or anti-rabbit Texas Red, diluted in blocking buffer) or rhodamine-phyllolidin for an additional hour. After five washes in PAB, the slide was detached from the chamber and mounted using the Molecular Probes SlowFade Light Antifade kit (Eugene, OR). Cells were viewed using a Zeiss LSM 510 confocal microscope. Phagosomes were monitored for the presence of cyt \( b_{558} \) by either p74 \( \text{phox} \) or p67 \( \text{phox} \) Abs. At least 50 phagosomes were scored per sample from three separate experiments. The numbers of positive phagosomes obtained at each time point were compared using paired Student’s t test with significance set at \( p < 0.05 \).

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Analysis of infected MDMs lysates by immunoblotting

Noninfected and WT or sseD S. typhimurium-infected MDMs (2-h time point) were lysed by a 30-min incubation in lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 10 mM EDTA, 100 μg/ml leupeptin, 100 μg/ml pepstatin, and 1 mM PMSE), followed by solubilization in SDS sample buffer. Samples were resolved by 9% SDS-PAGE and then transferred to nitrocellulose. Membranes were blocked with 3% BSA in PBS containing 0.05% sodium azide and 0.1% Nonidet P-40 and probed with Abs against gp91phox (54.1) or p22phox (44.1). HRP-conjugated goat anti-mouse (Bio-Rad, Hercules, CA) Abs were used to detect immunoreactive proteins by ECL. The total protein content of the cellular lysate was visualized by staining with Ponceau S (Boehringer Mannheim; Ingelheim, Germany).

Results

Growth of S. typhimurium sseD mutant within macrophages is impaired

Because the SPI-2-encoded TTSS is required for survival or growth of S. typhimurium inside phagocytes (10–13), we assessed the ability of strains of S. typhimurium with mutations in genes encoding components of the TTSS to replicate inside MDMs. We compared the number of WT and sseD S. typhimurium that associated with MDMs after 2, 6, or 10 h. Although similar numbers of WT and sseD S. typhimurium were seen within MDMs at early time points (Fig. 1, upper panels), after 6–10 h MDMs infected with S. typhimurium WT contained clusters of bacteria in close proximity. These clusters of bacteria likely reflected intracellular replication, since no clumping of bacteria was observed at earlier time points (Fig. 1, upper panels). In both cases, intracellular bacteria were identified within spacious phagosomes (Fig. 1, lower panels), as previously described (21). Confocal microscopic examination of serial sections throughout the whole cell demonstrated that the clusters of bacteria were intracellular (Fig. 2). Comparable numbers of bacteria were cell associated at the 2-h time point in WT and sseD-infected MDMs (Fig. 3A), indicating that both strains were phagocytosed with equal efficiency. In contrast, by 10 h, sseD showed a 50% reduction of cell-associated bacteria relative to the WT (Fig. 3B). We noted significant heterogeneity with either WT or mutant S. typhimurium with respect to the number of bacteria invading a given cell. However, taken as a population, the number of intracellular bacteria was consistently reduced in sseD-infected MDMs relative to WT-infected MDMs (Fig. 3), suggesting that the sseD strain had a decreased ability to replicate within macrophages when compared with the WT strain.

Intraphagosomal superoxide anion production is impaired by WT S. typhimurium but not by ssaT or sseD S. typhimurium

The ultrastructural data suggest that intraphagosomal accumulation of reactive oxygen species (ROS) is reduced in phagosomes containing WT S. typhimurium relative to phagosomes containing the SPI-2 mutant sseB (13). We found that both murine peritoneal macrophages and human MDMs readily ingested WT S. typhimurium and the isogenic SPI-2 mutants (sseD and ssaT). All organisms were found in spacious phagosomes; however, these structures tended to be smaller in MDMs than in murine macrophages (Fig. 4). Using an intraphagosomal NBT assay to detect production of superoxide, we found that formazan precipitates, occurring as a result of a localized reduction of NBT by superoxide...
anion, were generated inside only 13–25% of phagosomes containing WT *S. typhimurium* (Fig. 4). In contrast, 75–85% of phagosomes containing the SPI-2 mutants sseD and ssaT, or control phagosomes containing zymosan particles, were NBT positive (Fig. 4 and data not shown). Because similar data were obtained using human and murine macrophages and because all organisms induced formation of spacious phagosomes, the absence of detectable superoxide in the majority of phagosomes containing WT *S. typhimurium* cannot be explained by differences in phagosome morphology or macrophage species. These results suggest that WT *S. typhimurium* interfered with superoxide generation in macrophage phagosomes in an SPI2-dependent manner and that the functions of the SseD and SsaT proteins were required for this process.

**Phagosomes containing WT *S. typhimurium** lack cyt b<sub>558</sub> and p47<sub>phox</sub>

The failure to detect phagosomal superoxide anion could reflect inhibition of NADPH oxidase assembly or activity and/or rapid quenching of ROS by WT *S. typhimurium*. To distinguish among these possible mechanisms, we used immunofluorescence and confocal microscopy to determine the distribution of the NADPH oxidase subunits in macrophages infected with WT, ssaT, or sseD strains (Figs. 5–7). Our previous studies demonstrated that the membrane component of the NADPH oxidase, cyt b<sub>558</sub>, localizes mainly in the plasma membrane and discrete intracellular compartments of resting neutrophils and is enriched at the phagosomal membrane during ingestion of zymosan or live *Neisseria meningitidis* (22). However, we found that cyt b<sub>558</sub> was absent from, or minimally present in, the phagosomal membrane of MDMs infected with WT *S. typhimurium* (Figs. 5A and 6A). In contrast, ssaT and ssaT S. typhimurium-containing phagosomes were frequently enriched for cyt b<sub>558</sub> (Figs. 5B, 6B, and 7A). Seventy to 80% of the phagosomes containing the SPI-2 mutants were enriched for cyt b<sub>558</sub> after 1 min of phagocytosis (Fig. 7A). At identical time points, only 40% of phagosomes in MDMs containing WT *S. typhimurium* were positive for cyt b<sub>558</sub> (Fig. 7A). By 20 min, only 25% of WT *S. typhimurium*-containing phagosomes were positive for cyt b<sub>558</sub> vs 80% for ssaT and sseD strains (Fig. 7A). In addition, the cytosolic oxidase proteins p47<sub>phox</sub> and p67<sub>phox</sub> colocalized with cyt b<sub>558</sub> at the phagosomal membrane of ssaT and sseD *S. typhimurium*-containing phagosomes (Fig. 7 and data not shown), demonstrating assembly of a functional NADPH oxidase complex. In contrast, the cyt b<sub>558</sub>-negative WT *S. typhimurium* phagosomes were not enriched for p47<sub>phox</sub> (data not shown), confirming the requirement for the cyt b<sub>558</sub> as a docking site for stable association of the cytosolic oxidase components with the membrane (16). Immunoblots of lysates of human MDMs infected with WT or ssaD *S. typhimurium* were probed to determine whether differential proteolysis of cyt b<sub>558</sub> might account for its absence from phagosomes containing WT bacteria. There was neither a difference in the amounts of full-length gp91<sub>phox</sub> or p22<sub>phox</sub> (data not shown) nor the presence of proteolytic fragments in MDMs infected with either strain of *S. typhimurium* (Fig. 8).

Taken together, these results indicate that the absence of intraphagosomal superoxide anion generation during phagocytosis of WT *S. typhimurium* reflected failure of the NADPH oxidase to assemble at the phagosomal membrane due to the absence of cyt...
b_{558} from the nascent phagosome. Furthermore, the cellular reorganization resulting in the absence of cyt b_{558} from the phagosome was dependent on expression of the SPI-2-encoded TTSS and specifically required the SseD and SsaT proteins.

**Discussion**

Survival in macrophages is essential for the virulence of Salmonella and reflects the ability of the bacteria to avoid or resist the innate host.
microbicidal mechanisms of phagocytes. The NADPH oxidase generates an array of microbicidal agents from molecular oxygen and thus is of central importance in the innate host defense of phagocytes. Earlier studies have shown that the neutrophil oxidative burst is diminished following phagocytosis of virulent Salmonella typhi compared with avirulent strains (23, 24). Recent studies have shown that S. typhimurium strains deficient in components of the SPI-2-encoded TTSS are greatly attenuated for virulence in WT mice but not in mice that lack functional NADPH oxidase activity (13). Furthermore, studies of intracellular hydrogen peroxide generation in Salmonella-infected murine macrophages have shown that a WT strain of S. typhimurium is associated with reduced ROS detection at the phagosomal

FIGURE 6. Serial sections (Z-series) of WT- or sseD-infected MDMs. Synchronized phagocytosis of WT (A) or sseD (B) GFP-S. typhimurium was performed and samples were stained as described in the legend to Fig. 4. Data shown are serial sections through bacteria-containing MDMs stained for cyt b558.

FIGURE 7. Quantification of oxidase recruitment to WT, sseD, and ssaT S. typhimurium-containing phagosomes. Thirty phagosomes were scored for the presence of cyt b558 (A) or p47phox (B) at each time point (mean ± SEM, n = 3 separate experiments).
The precise mechanisms by which the NADPH oxidase normally assembles at the plasma membrane of phagocytosing macrophages have not been fully elucidated. In contrast to polymorphonuclear leukocytes, in which cyt b558 is present not only at the plasma membrane but also in the membrane of specific granules, macrophages express the vast majority of cyt b558 at the plasma membrane (34). Our finding that WT- and SPI-2 mutant-containing phagosomes were differently enriched for cyt b558 and p47\textsuperscript{phox} suggests that S. typhimurium interfered with the signaling events that mediate organization and assembly of the NADPH oxidase. We hypothesize that cyt b558 enrichment at the phagosomal membrane of macrophages occurs via early clustering or lateral diffusion of the transmembrane cyt b558 toward the site of initial engagement of the bacteria at the surface of the phagocyte. The effectors of SPI-2 TTSS may disrupt the proximal signaling that normally targets cyt b558 to specific regions of the membrane engaged in phagosome formation. Although our data do not directly test this hypothesis, there is substantial evidence demonstrating dramatic reorganization of the phagocyte plasma membrane during both phagocytosis and chemotaxis (35–37). Remodeling of the surface topography need not be limited to these agonist-dependent events and may well be critical for precise spatial control of ROS generation. Alternatively, components of the TTSS may actively exclude cyt b558 from the phagosomal membrane early in host cell invasion, rapidly degrade, and/or modify cyt b558, the net result being the loss of a functional NADPH oxidase complex at the phagosomal membrane. Further characterization of the strategies successfully used by pathogens to foil cellular antimicrobial defenses will provide important insights into mechanisms underlying normal phagocyte responses.

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