Constitutive Acid Sphingomyelinase Enhances Early and Late Macrophage Killing of Salmonella enterica Serovar Typhimurium

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We have identified acid sphingomyelinase (ASM) as an important player in the early and late anti-Salmonella activity of macrophages. A functional ASM participated in the killing activity of macrophages against wild-type Salmonella enterica serovar Typhimurium. The role of ASM in early macrophage killing of Salmonella appears to be linked to an active NADPH phagocyte oxidase enzymatic complex, since the flavoprotein inhibitor diphenyleneiodonium not only blocked a productive respiratory burst but also abrogated the survival advantage of Salmonella in macrophages lacking ASM. Lack of ASM activity also increased the intracellular survival of an isogenic ΔspiC::FRT Salmonella strain deficient in a translocator and effector of the Salmonella pathogenicity island 2 (SPI2) type III secretion system, suggesting that the antimicrobial activity associated with ASM is manifested regardless of the SPI2 status of the bacteria. Constitutively expressed ASM is responsible for the role that this lipid-metabolizing hydrolase plays in the innate host defense of macrophages against Salmonella. Accordingly, the ASM activity and intracellular concentration and composition of ceramide, gangliosides, and neutral sphingolipids did not increase upon Salmonella infection. Salmonella triggered, nonetheless, a significant increase in the secreted fraction of ASM. Collectively, these findings have elucidated a novel role for constitutive ASM in the anti-Salmonella activity of murine macrophages.

Infection with Salmonella enterica serovar Typhimurium is a major global health problem. In the United States alone, it is estimated that over a million people contract nontyphoidal salmonellosis every year (46). Most clinical presentations of nontyphoidal Salmonella infections are manifested as gastroenteritis that resolves spontaneously without antibiotic treatment. In immunocompromised individuals, nontyphoidal Salmonella infections can become life threatening. The incidence of Salmonella enterica infections has recently been associated with host defense against systemic salmonellosis (28, 44). Nonetheless, phagocytes constitute a critical component in resistance to Salmonella. Investigations using immunodeficient mice have shown that macrophages exploit early Toll-like receptor 4 signaling and NRAMP1-dependent mechanisms in resistance to systemic salmonellosis (28, 43). Within the first few hours following phagocytosis, ROS generated by the NADPH phagocyte oxidase enzymatic complex kill many of the internalized Salmonella bacteria, while RNS generated by the inducible nitric oxide synthase (iNOS) expressed in response to gamma interferon control the replication of this intracellular pathogen later in the infection (23, 24, 41).

Lipid-mediated signaling initiated by neutral or acid sphingomyelinases (ASMs) is increasingly recognized as a critical component in diverse cellular processes, including innate immunity. In particular, ASM, which hydrolyzes sphingomyelin to ceramide in lipid rafts and endocytic vacuoles (19), has recently been associated with host defense against Listeria monocytogenes and Pseudomonas aeruginosa (13, 36). The increased susceptibility of ASM-deficient mice to Listeria correlates with the reduced listericidal activity of their macrophages. Likewise, mice lacking ASM are hypersusceptible to Salmonella serovar Typhimurium (36); however, the anti-Salmonella capacity of macrophages lacking ASM is currently unknown. Herein, macrophages from ASM-deficient mice were used to determine whether the lipid-metabolizing activity of ASM plays a role...
during the early and late anti-
Salmonella defenses of macrophages.

MATERIALS AND METHODS

Mice. Pairs of heterozygous ASM−/− mice bred in an sv129 × BL/6 background (17) were kept in our animal facility according to Institutional Animal Care and Use Committee guidelines. DNA isolated from mouse tails was used as a template for PCR amplification in a reaction mixture containing DNA poly-
merase (CLP, San Diego, CA), deoxynucleoside triphosphates, a common for-
ward primer (5′-AGCAGGTCTTCCCTCTTAC-3′) and 5′-GGAATTCGCCGATTATTGTCG-3′, respectively. PCR was performed for 30 cycles of 93°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by a final extension for 5 min at 72°C. The mutant (−500-bp) and wild-type (−300-bp) alleles were visualized following agarose gel electrophoresis and ethidium bromide staining. Homozygous mice bearing a wild-type or ASM-decient allele were used for experimentation.

Macrophages. Peritoneal macrophages were harvested from ASM−/− mice or their wild-type ASM+/+ littersmates 4 days after intraperitoneal inoculation of 1 ml/mg sodium periodate (Sigma). Peritoneal exudate cells were resuspended in RPMI 1640 containing 2 mM l-glutamine, 1 mM sodium pyruvate (all from Sigma), and 10% heat-inactivated fetal bovine serum (Cambrex) (RPMI + medium). Cells were plated at densities of 3 × 10⁶ cells/well onto 8-well Permanox chamber slides (Nalge Nunc International), 10⁵ cells/well in 96-well Microtiter-2 plates (Thermo Labsystems), 10⁶ cells/well in 24-well plates, and 2 × 10⁵ cells/well in 6-well tissue culture plates (Falcon) for killing assays, superoxide measurement, ASM activity analysis, and sphingolipid analysis, respectively. The macrophages were cultured for 16 h at 37°C in the presence of 100 U/mI 100 mg/ml of penicillin/streptomycin (Cellgro) prior to infection with Salmonella.

Phagocytosis. Salmonella bacteria grown overnight in LB medium were incu-
ated with 1 mg/ml fluorescein isothiocyanate (FITC; Sigma) in Dulbecco’s phosphate-buffered saline (PBS; Sigma) for 30 min at 37°C. The cultures were washed three times with Dulbecco’s PBS to eliminate free FITC, and the bacteria were opsonized with normal mouse serum prior to the macrophage challenge described above. Peritoneal macrophages from ASM−/− and ASM+ + mice were plated at a density of 3 × 10⁵ cells on glass coverslips placed in six-well tissue culture plates were challenged with FITC-labeled Salmonella bacteria at an MOI of 20 for 30 min at 37°C in a 5% CO₂ incubator. After most cell-free Salmonella bacteria were washed out with prewarmed PBS, 0.2% trypan blue prepared in 0.2% Triton X-100 and 0.1 mM NaCl, Cellular lysates and supernatants were incubated for 60 min at 37°C with 100 pmol of 7-nitro-2,1,3-benzoxadiazol-4-yI (NBD)-spingomyelin (Invitrogen, Carlsbad, CA) prepared as a 20 mM stock solution in 50 mM sodium acetate, pH 5.0, containing 2% Triton X-100. Enzymatic production of NBD-ceramide was stopped by extracting the lipids from the cellular lysates or supernatants in chloroform-methanol-acetic acid (190:9:1). NBD-ceramide was visualized in a Molecular Imaging FX (Bio-Rad) using a 488-nm excitation wavelength and a 530-nm emission wavelength. The amount of ceramide was quantified by regres-
sion analysis using a standard curve prepared with NBD-ceramide which was prepared by incubating 10 ml of 7-nitro-2,1,3-benzoxadiazol-4-yI (NBD)-spingomyelin (Invitrogen, Carlsbad, CA) prepared as a 20 mM stock solution in 50 mM sodium acetate, pH 5.0, containing 2% Triton X-100 and 0.1 mM NaCl. Cellular lysates and supernatants were incubated for 60 min at 37°C with 100 pmol of 7-nitro-2,1,3-benzoxadiazol-4-yI (NBD)-spingomyelin (Invitrogen, Carlsbad, CA) prepared as a 20 mM stock solution in 50 mM sodium acetate, pH 5.0, containing 2% Triton X-100. Enzymatic production of NBD-ceramide was stopped by extracting the lipids from the cellular lysates or supernatants in chloroform-methanol-PBS (1:10). Following centrifugation at the organella was dissolved in chloroform-methanol-acetic acid (190:9:1) and the lipids were dried under N₂ and resuspended in methanol. After the addition of 300 mM chloroform-methanol-water-pyridine (60:30:6:1) at 37°C while shaking the samples at 300 rpm overnight. Dena-
tured proteins were removed by passing the samples through cotton washing. The lipids were dried in a RotaVAP (Büchi, Switzerland) and resuspended in methanol, and contaminating phospholipids were removed by alkaline metha-

Superoxide measurement. Macrophages were infected with Salmonella bacte-
ria at an MOI of 10. The monolayers were washed after 15 min, and the medium was replaced with RPMI + medium containing 6 µg/ml gentamicin and 12.5 µM of the superoxide probe lucigenin (41). The flavoprotein inhibitor DPI was added to selected groups of macrophages at a final concentration of 10 µM. Chemili-

Nitrile measurement. The concentration of nitrile (NO₂⁻) produced by Sal-
monella-infected macrophages 20 h postchallenge was estimated spectropho-
tometrically at 550 nm in a VersaMax microplate reader ( Molecular Devices) after culture supernatants were mixed with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethylenediamide hydrochloride in 2.5% phosphoric acid). The NO₂⁻ concentration was calculated by regression analysis using a NaNO₂ standard curve.
as expected (9, 39, 44), both the early and late antihypersusceptibility to the antimicrobial effects of ASM, macrophages harbored an ASM activity of 360 fmol ceramide/10^5 cells/h (Fig. 1A and B). ASM-proficient and -deficient macrophages were used to measure the contribution of ASM to the anti-Salmonella activity of macrophages. The survival of wild-type Salmonella was increased twofold \((P < 0.05)\) in ASM-deficient macrophages at 2 h postinfection (Fig. 2A). By 20 h, the bacterial burden was about fivefold greater \((P < 0.05)\) in ASM-deficient macrophages than in ASM^+/+^ congenic controls. Together, these data indicate that ASM contributes to both the early and late anti-Salmonella activity of macrophages. The SPI2 type III secretion system enhances the intracellular survival of Salmonella within macrophages by reducing contact with lysosomes and NADPH oxidase- and iNOS-containing vesicles (3, 35, 38). To determine if SPI2 antagonizes the antimicrobial activity associated with ASM.

The survival advantage of Salmonella in ASM^−/−^ macrophages cannot be explained by altered bacterial internalization (14), because wild-type and ΔspiC::FRT Salmonella bacteria were not antagonize the antimicrobial activity associated with ASM. The survival advantage of Salmonella in ASM^−/−^ macrophages cannot be explained by altered bacterial internalization (14), because wild-type and ΔspiC::FRT Salmonella bacteria were

FIG. 1. ASM enzymatic activity of murine macrophages. (A) Peritoneal macrophages isolated from ASM^+/+^ and ASM^−/−^ mice were assessed for ASM activity by incubating cellular lysates with NBD-sphingomyelin (NBD-SM) for 60 min at 37°C. Enzymatic production of NBD-ceramide (NBD-cer) was visualized following high-performance TLC of lipid extracts. NBD-ceramide (1 pmol) was loaded as a control (lane 1). (B) The amount of ceramide produced by intracellular ASM was quantified by regression analysis using an NBD-ceramide standard curve. The results are expressed as fmol ceramide/10^5 macrophages (mean ± standard deviation) \((n = 3)\). \*, \(P < 0.05\), compared to ASM^+/+^ control by unpaired, two-way \(t\) test.

FIG. 2. Intracellular survival of Salmonella is enhanced in macrophages lacking ASM. The antimicrobial activities of macrophages from ASM^+/+^ and ASM^−/−^ mice against wild-type (wt) (A) and spiC-deficient (B) Salmonella bacteria were monitored over time. Percent survival is expressed as the mean ± standard deviation \((n = 6\) to 10). Logarithmically transformed data were analyzed by two-way ANOVA. \*, \(P < 0.05\) for comparison of ASM^+/+^ and ASM^−/−^ antimicrobial activities as determined by a Bonferroni posttest. (C) Internalization of bacteria by ASM^+/+^ and ASM^−/−^ macrophages was estimated by recording the number of bacteria capable of forming a colony following 35 min of infection. Data are expressed as mean numbers of CFU ± SEM \((n = 16)\). (D) Phagocytic indices were calculated for ASM^+/+^ and ASM^−/−^ macrophages by enumerating the number of intracellular FITC-labeled Salmonella bacteria per macrophage by fluorescence microscopy. A total of 50 to 60 macrophages were counted from six separate slides per group. The numbers of bacteria per macrophage are represented in a box-and-whisker plot as median, intraquartile, and total ranges.
The generation of RNS by proficient or -deficient macrophages was measured as lucigenin-depending chemiluminescence after Salmonella-infected macrophages from ASM-positive and -deficient macrophages is independent of ASM. (A) The generation of RNS by Salmonella-infected macrophages from ASM-positive and -deficient macrophages was determined by measuring the accumulation of nitrite in supernatants at the indicated times. Data are expressed as μM nitrite/10^6 macrophages (m±SEM) (mean ± SEM). (B) The oxidative burst of ASM-proficient or -deficient macrophages was measured as lucigenin-dependent chemiluminescence after Salmonella challenge. Selected samples were treated with 10 μM DPI at the time of the infection. Results are expressed as mean relative luminescence units (RLU) ± SEM. The data are from three to six independent observations.

recovered in similar numbers after 35 min of infection, regardless of the ASM status of the phagocytes (Fig. 2C). The phagocytic capacity of ASM-positive and ASM-negative macrophages was estimated by enumerating the number of intracellular FITC-labeled Salmonella bacteria following 30 min of bacterial challenge, and the intracellular bacterial load was quantified by scoring the number of bacteria per macrophage. These data revealed no significant differences (P > 0.7) in the abilities of ASM-positive and ASM-negative macrophages to phagocytize Salmonella bacteria (Fig. 2D).

ASM-mediated macrophage anti-Salmonella activity is dependent upon production of ROS. Because ASM optimizes the early and late stages of anti-Salmonella activity, we tested whether ASM activity may influence the production of ROS and RNS. Macrophages lacking ASM generated the same amounts of NO_2^- as wild-type controls (Fig. 3A). ASM-proficient and -deficient macrophages generated similar amounts of ROS 1 h after Salmonella infection (Fig. 3B). The addition of the flavoprotein inhibitor DPI completely abolished the respiratory burst (Fig. 3B). Inhibitors of the oxidative burst and synthesis of NO were used to determine whether the ASM-dependent killing is related to NADPH oxidase or iNOS activity. DPI abolished the ASM-dependent killing (Fig. 4A). The effects of DPI seemed to be related to inhibition of NADPH oxidase activity, since the NOS inhibitor MMLA, which reduced the NO_2^- produced by Salmonella-infected macrophages to levels lower than 1 μM, had no effect (P > 0.65) on ASM-dependent killing of Salmonella bacteria (Fig. 4B). The inhibitory effects of DPI cannot be explained by the inhibition of ASM activity either, since untreated and DPI-treated macrophages had similar ASM activities (Fig. 4C).

Salmonella infection reduces ASM activity. The kinetics of ASM activity were recorded upon Salmonella challenge. ASM activity peaked 30 min after infection (Fig. 5A), although this increase was not significantly different (P > 0.5) from that of uninjected controls. Overall ASM activity decreased after a few hours of infection and was independent of the SPI2 status of the bacteria (Fig. 5A). Since the elicitation process could potentially mask the effects that Salmonella infection may have upon the sphingomyelin pathway, the ASM activities of peritoneal and resident macrophages were compared. The ASM activities of Salmonella-infected and uninjected controls in both populations of phagocytes were similar (Fig. 5B). Together, these data suggest that constitutive expression of ASM optimizes the anti-Salmonella activity of macrophages.

The contents of high-order sphingolipids generated from ceramide in ASM-positive and ASM-negative macrophages were compared. The sphingolipid profile of ASM-positive macrophages (Fig. 5C, lane 1) included ceramides, gangliosides, and sphingomyelin. The patterns of gangliosides, sphingomyelin, and neutral sphingolipids were unchanged in ASM-positive macrophages after 30 min of Salmonella infection. Compared to the results with ASM-negative macrophages, a large amount of lipid comigrating with sphingomyelin accumulated in ASM-negative macrophages. Sphingolipids were also extracted from 5 × 10^7 J774 macrophage-like cells that were infected with either wild-type or spiC-deficient Salmonella. Consistent with the results obtained for wild-type bacteria in primary macrophages, the sphingolipid profiles obtained from J774 cells at 0, 4, and 20 h postinfection were similar to those from ASM-positive macrophages.
Salmonella infection decreases constitutive ASM activity of macrophages. (A) Intracellular ASM activity was assessed at the indicated times following challenge of peritoneate-elicted macrophages (md) with wild-type (wt) or spiC-deficient (m/spiC) Salmonella. (B) Comparison of the ASM activities of resident and peritoneate-elicted Salmonella-infected ASM""/"" macrophages (n = 4 to 8). ASM activities are expressed as mean fmol ceramide/10" macrophages/h \pm standard deviation. p.i., postinfection. (C) Total sphingolipids extracted from control (lanes C) or Salmonella-infected (lanes I) ASM""/"" or ASM""/"" macrophages were visualized following separation by high-performance TLC. Mixed gangliosides GM1 (6), GD1a (7), and GD1b (8) (lane MG); neutral glycosphingolipids cerebroside (1), lactosyl ceramide (2), ceramide trihexoside (4), and globoside (5) (lane S1); and sphingolipid mix of cerebrosides (1), sulfatides (3), and sphingomyelin (5) (lane S2) were used as standards.

FIG. 5. Salmonella infection decreases constitutive ASM activity of macrophages. (A) Intracellular ASM activity was assessed at the indicated times following challenge of peritoneate-elicted macrophages (md) with wild-type (wt) or spiC-deficient (m/spiC) Salmonella. (B) Comparison of the ASM activities of resident and peritoneate-elicted Salmonella-infected ASM""/"" macrophages (n = 4 to 8). ASM activities are expressed as mean fmol ceramide/10" macrophages/h \pm standard deviation. p.i., postinfection. (C) Total sphingolipids extracted from control (lanes C) or Salmonella-infected (lanes I) ASM""/"" or ASM""/"" macrophages were visualized following separation by high-performance TLC. Mixed gangliosides GM1 (6), GD1a (7), and GD1b (8) (lane MG); neutral glycosphingolipids cerebroside (1), lactosyl ceramide (2), ceramide trihexoside (4), and globoside (5) (lane S1); and sphingolipid mix of cerebrosides (1), sulfatides (3), and sphingomyelin (5) (lane S2) were used as standards.

Secreted ASM accumulated to significantly higher levels in infected cellular supernatants over 4 h than in uninfected controls (Fig. 6B). The increase in ASM activity from infected macrophages represents about 1/10 of the ASM activity lost from endosomal fractions during the first 4 h following Salmonella challenge. The increase in secreted ASM activity was, however, transitory, since it dropped below baseline levels at later times of the infection (Fig. 6B). The increase in secretory ASM following Salmonella infection cannot be explained by a previously observed subversion of the exocytic pathway by SPI2 effectors (20), because wild-type and spiC-deficient Salmonella bacteria induced similar secretions of ASM. Nor are the increased secretory ASM activities of macrophages specific to Salmonella infection, as E. coli induced similar patterns of ASM secretion (Fig. 6B).

DISCUSSION

We present the first characterization of lysosomal and secreted ASM activities following the infection of macrophages with Salmonella. Most macrophage ASM was found to reside intracellularly, likely reflecting enzymatic activity of the lysosomal fractions. Remarkably, lysosomal ASM activity was reduced by almost 100 fmol/10" macrophages/h a few hours after infection, coinciding with a surge of 10 fmol/10" macrophages/h ASM activity in the secreted fraction. These dynamic changes in the cellular distribution of ASM activity in Salmonella-infected macrophages were neither associated with global changes in sphingolipid profiles nor required for bacterial uptake (Fig. 2C), as has been shown for other pathogens (12, 14). Rather, ASM was associated with the stimulation of the early innate host defenses of macrophages against Salmonella. Together, these findings have uncovered a role for constitutive ASM in the anti-Salmonella activity of macrophages.

The contribution of ROS to defense against Salmonella is manifested by the increased incidence of salmonellosis in individuals harboring a cadre of defective variants in the cytosolic or membrane components of the NADPH oxidase complex (21). The respiratory burst dominates the early innate defenses of
macrophages against Salmonella (23, 41). Two independent lines of experimentation suggest that the involvement of ASM in the anti-Salmonella activity of macrophages is linked to the NADPH oxidase function. First, ASM-mediated killing was evident as early as 2 h after infection, during a period in which the lack of the membrane gp91phox subunit of the NADPH phagocyte oxidase completely abrogates intracellular killing of Salmonella (41). Second, the NADPH oxidase inhibitor DPI not only increased the number of Salmonella bacteria recovered from macrophages but also abolished the differences in killing between ASM⁶⁷ and ASM⁻/- phagocytes. The inhibitory effects associated with DPI appear to be specific to the NADPH oxidase enzymatic complex since specific inhibition of iNOS did not abrogate the ASM-dependent phenotype. Collectively, these findings indicate that ASM optimizes early macrophage killing of Salmonella by acting synergistically with a functional NADPH oxidase.

The synergism of ASM and the respiratory burst appears to be independent of stimulation of NADPH oxidase activity by mediators of the sphingomyelin pathway. In accord with previous data examining ROS production by Listeria-infected phagocytes (36), ASM-deficient macrophages sustained a normal respiratory burst in response to Salmonella. Stimulation of K⁺ fluxes into phagosomes that promote the activation of proteases from the proteoglycan matrix in the lysosomal lumen (33) may provide an intriguing scenario for the association between ASM and ROS-mediated killing. In view of this, ASM has been shown to activate the lysosomal aspartyl-protease cathepsin D (15). Alternatively, ASM may target ROS production to the Salmonella phagosome. It should be noted that an important fraction of the NADPH oxidase-mediated anti-Salmonella activity of macrophages, likely representing conventional ROS-mediated cytotoxicity, occurs in the absence of the sphingomyelin pathway.

The SPI2 type III secretion system has been associated with inhibition of both lysosomal fusion and trafficking of NADPH oxidase- or iNOS-containing vesicles (3, 35, 38). More recently, a functional SPI2 type III secretion system has been associated with the segregation of Salmonella phagosomes along the exocytic pathway and the acquisition of ceramide (20). It is tempting to imagine that the extracellular flux of ASM seen in Salmonella-infected macrophages is associated with the SPI2-mediated segregation of Salmonella bacteria along the exocytic pathway. However, several lines of evidence indicate that this scenario is incorrect. On the one hand, ASM contributed equally to the killing of wild-type and spiC-deficient Salmonella bacteria. On the other hand, the overall makeup of sphingolipids and the amount of secreted ASM in Salmonella-infected macrophages were unaffected by the SPI2 status of the bacteria. More-likely possibilities for the enhanced secreted ASM activity seen shortly after Salmonella infection include the export of ASM to the outer leaflet of the host cell membrane triggered during the initial contact of host cells and pathogen. Fusion of lysosomal vesicles with the plasma membrane during phagosomal biogenesis (6, 30) may deliver secreted ASM to the nascent phagosome. The fact that E. coli also triggered an early surge in the extracellular secretion of ASM activity further supports the idea that at least part of the cellular redistribution of ASM is activated during phagocytosis. Extracellular secretion of ASM in response to two enterobacterial species suggests that the role of ASM uncovered here is not specific to Salmonella but may be required for the bactericidal activity of murine macrophages against a variety of pathogenic microorganisms.

In summary, our data have elucidated a hitherto unknown role for ASM in the anti-Salmonella arsenal of professional phagocytes. Future experimentation will be required to determine the origin of the ASM enzymatic activity secreted extracellularly in response to Salmonella and to elucidate the association of ASM with the anti-Salmonella activity of the NADPH phagocyte oxidase.

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