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Inhibition of neutral sphingomyelinase protects mice against systemic tuberculosis

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1. ABSTRACT

Tuberculosis is one of the most serious infectious diseases worldwide. The initial pulmonal localization of the pathogens often develops into systemic infection with high lethality. We investigated the role of the mammalian neutral sphingomyelinase (Nsm)/ceramide system in systemic infection of mice and murine macrophages with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). Our results demonstrate that BCG infection of RAW cells, a macrophage cell line, results in rapid activation of Nsm but not of acid sphingomyelinase (Asm). Activation of Nsm is associated with a massive release of superoxide. Genetic knock-down of Nsm in RAW cells prevented superoxide production upon BCG infection. Superoxide suppressed autophagy in BCG-infected macrophages *in vitro* and *in vivo*: Knock-down of Nsm or inhibition of superoxide restored autophagy in macrophages and increased killing of intracellular bacteria upon BCG infection. Most importantly, autophagy was also massively increased

in Nsm-heterozygous mice, protecting these mice from systemic BCG infections, granuloma development, and chronic infections of liver and spleen. These findings indicate that the Nsm/ceramide system plays a role in protecting mice against systemic tuberculosis by preventing superoxide-mediated inhibition of autophagy.

2. INTRODUCTION

Infections with *Mycobacterium tuberculosis* are among the most common severe diseases worldwide, with more than 8 million new cases and more than 2 million deaths per year (1). The initial localization of the pathogens after infection is usually the lung. A dreaded complication of lung tuberculosis is the generalization of bacteria in other organs, such as liver and spleen; this condition is often fatal. To control systemic infection, the organism forms granulomas in several tissues, such as liver and spleen, and these granulomas contain infected macrophages, epithelioid macrophages, and multinucleated giant cells (2).

The role of neutral sphingomyelinase (Nsm) in this process is presently unknown. Sphingomyelinases are enzymes characterized by their pH dependency: acid, neutral, and alkaline sphingomyelinases have been identified. Sphingomyelinases catalyze the breakdown of sphingomyelin into phosphocholine and ceramide, often in response to environmental stress, inflammatory cytokine stimuli, or mediating stress-induced events such as apoptosis or inflammation (3,4). Ceramide, produced by acid sphingomyelinase (Asm), resides in the anti-cytoplasmic or outer leaflet of the cell membrane and causes the formation of outer-membrane ceramide-enriched platforms that modulate membrane-associated events, such as the clustering of receptors (5-7). These modulations result in the amplification of signaling events. In contrast, Nsm releases ceramide in the cytoplasmic leaflet of cellular membranes.

The role of the Asm/ceramide system in the infection of several pathogens has been well described. These pathogens include *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli,* and *Mycobacterium avium* (8-14). The role of the Nsm/ceramide-system in pathogen/host interaction, however, requires definition. It was recently shown that Nsm is required for PorB-dependent invasion of *N. gonorrhoeae* into epithelial cells. This invasion involves the recruitment of phosphatidylinositol-3-kinase (PI3K) to caveolin, and this recruitment leads to further activation of PI3K downstream events that mediate the invasion of bacteria (15). The same pathogen exploits the Asm/ceramide system to invade epithelial cells via another ligand, namely Opa proteins (13). Furthermore, the uptake of measles viruses by dendritic cells requires both the Asm/ceramide system and the Nsm/ceramide system. After binding carbohydrate structures on the pathogen ligands to the dendritic cell receptor dendritic cell–specific intercellular adhesion molecule-3–grabbing non-integrin (DC-SIGN), both sphingomyelinases are transiently activated within several minutes (16).

The Asm/ceramide-system is also crucially involved in triggering the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Ceramide domains cluster subunits of NADPH oxidase, thereby releasing superoxide (17). Although superoxide is necessary for bacterial killing after infection with several pathogens (18,19), they also inhibit the stimulation of autophagy (20-22). Autophagy is an intracellular pathway and mechanism by which cells eliminate misfolded proteins, damaged organelles, and pathogens. Autophagy is important in human diseases such as cancer and neurodegeneration (23,24) and serves as a control and as a cellular defense mechanism against many intracellular pathogens, including mycobacteria, *S. typhimurium*, *L. monocytogenes*, *Shigella flexneri*, *S. aureus,* and distinct herpes viruses (25-29). Stimulation of autophagy in macrophages infected with either *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) or *M. tuberculosis* causes mycobacterial phagosomes to mature into phagolysosomes, and this maturation results in increased killing of intracellular pathogens and thereby serves as a defense mechanism for infected host cells (22). However, the role of Nsm in the release of superoxide and the regulation of autophagy is unknown.

In the study reported here we investigated the role of Nsm in the infection of murine macrophages with BCG. Our findings provide evidence that the Nsm/ceramide system plays a central role in BCG-induced production of superoxide by controlling autophagy; this control mediates the killing of intracellular pathogens. Infection of wild-type (wt) cells results in a massive release of superoxide that inhibits autophagy and thereby impairs the killing of intracellular BCG. Down-modulation of Nsm or pharmacological inhibition of superoxide restores autophagy and results in enhanced killing of bacteria. *In vivo*, unlike wt mice, mice heterozygous for Nsm ($Nsm^{+/-}$) are able to control granuloma formation and bacterial burden and, thus, are protected from BCG infection.

3. MATERIALS AND METHODS

3.1. Mice and cells

Nsm-heterozygous mice (sphingomyelin phosphodiesterase 3 heterozygote; $Smpd3^{+/-}$) and syngenic wt littermates maintained on a 129sv genetic background were purchased from the European Mouse Archive (EMMA; France). Mice were housed in the animal facility of the University of Duisburg-Essen under pathogen-free conditions according to the criteria of the Federation of Laboratory Animal Science. The

genotype was verified by polymerase chain reaction (PCR) analysis before experimentation. *In vivo* infections were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV), animal grant Gr. 1312/12. The *in vitro* experiments were performed with RAW 264.7 cells that were transfected either with short hairpin RNA (shRNA) targeting Smpd3 to suppress Nsm activity or with a control plasmid (scrambled artificial sequence). RAW cells were maintained in Dulbecco´s modified Eagle's medium (DMEM; Gibco/Invitrogen, Karlsruhe, Germany) with 10 mM HEPES (Carl Roth GmbH, Karlsruhe, Germany; pH 7.4), 2 mM L-glutamine, 100 microM non-essential amino acids, 100 units/ml penicillin, and 100 microg/ml streptomycin (all from Gibco) (MEM/HEPES), supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria). We added 750 microg/ml hygromycin (AppliChem, Darmstadt, Germany) to maintain the transfected plasmid in genome. Cells were maintained at 37°C in a 10% CO_2 atmosphere. At least two days before infection, hygromycin was removed. One day before infection, the desired numbers of cells were seeded in plates and incubated at 37°C in a 5% CO_2 atmosphere.

3.1.1. Transfection of RAW 264.7 cells

We transfected RAW 264.7 cells with a SureSilencing shRNA Plasmid targeting Smpd3 (Qiagen, Hilden, Germany) to suppress the activity of Nsm. The plasmid was amplified by transformation of competent *E. coli* DH5alpha for 30 min on ice, heat shock for 90 sec at 42°C and incubation on ice for 2 min. Bacteria were shaken in 1 mL lysogeny broth (LB) medium (Carl Roth) for 45 min at 37°C, transferred to LB medium supplemented with ampicillin, and incubated at 160 rpm at 37°C overnight. The plasmid was purified with the Qiagen Plasmid Purification Maxi Kit (Qiagen) according to the manufacturer's instructions, linearized with BsaI-HF (New England BioLabs, Ipswich, MA, USA), precipitated, washed, and transfected. Transfection was done on an ECM 830 Square Wave Electroporation System (BTX, Holliston, MA, USA) with five 450-V pulses of 1 msec each. Cells were cultured for 36 h and selected with 1 mg/ml hygromycin B (Boehringer Mannheim, Mannheim, Germany; catalog number 843555). We performed experiments on bulk cultures to prevent any outgrowth of specific clones.

3.1.2. Infection experiments

All *in vivo* and *in vitro* infections were performed with green fluorescent protein (GFP)-expressing BCG (GFP-BCG). To construct the GFP-BCG strain, BCG were transformed with the dual reporter plasmid pSMT3LxEGFP (30). For infection experiments, bacteria were shaken at 120 rpm at 37°C in Erlenmeyer flasks with 10 ml Middlebrook 7H9 Broth with Glycerol (BD Biosciences, Heidelberg, Germany), supplemented with 50 microg/ml hygromycin to maintain the GFP plasmid. Bacteria were used for infection experiments after 5 to 7 days of culture. Bacteria were collected by centrifugation at 2000 rpm for 10 min. The bacterial pellet was resuspended in HEPES/Saline buffer (H/S) consisting of 130 M NaCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 20 mM HEPES (pH 7.3), 5.4 mM KCl, and 0.8 mM $MgSO_4$ and was vortexed for 5 min. Samples were bath-sonicated for 5 min at 4°C and passed 10 times through a syringe with a needle 0.8 mm in diameter. Clumps of bacteria were removed by centrifugation for 2 min at 1000 rpm. The supernatant containing single cells of GFP-BCG was carefully collected. The bacterial number was calculated with a 100× oil lens and an inverted fluorescence microscope (DMIRE2; Leica, Heidelberg, Germany). For *in vivo* infections, bacteria were pelleted at 3200 rpm for 10 min and resuspended in 0.9% NaCl, after which 1×10^6 bacteria were injected intravenously. After the indicated time, the animals were sacrificed by cervical dislocation. Liver, spleen, and brain were removed for further processing.

For *in vitro* assays, cells were infected with GFP-BCG in MEM/10 mM HEPES at a bacteria-to-host cell ratio (multiplicity of infection, MOI) of 5:1 to 10:1. If indicated, 10 mM N-acetylcysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA) or 2.5 mM 3-Methyladenine (3-MA) (CAS 5142-23-4, Calbiochem) was added 30 min before infection. Synchronous infection conditions and enhanced interactions between bacteria and host cells were achieved by 8-min centrifugation (500 rpm) of the bacteria onto the cells. The end of the centrifugation was defined as the starting point of infection. The infection was terminated by fixation or lysis, as described below.

3.1.3. Assay for neutral sphingomyelinase activity

For determination of Nsm activity in macrophages, cells were left uninfected or were infected for the indicated time, the supernatant was removed, and cells were pelleted. The supernatant was centrifuged, and the adherent cells were lysed in a buffer consisting of 100 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 0.2% Igepal, and 10 microg/ml aprotinin/leupeptin (A/L). The cells were then scraped from plates with a rubber policeman and combined with pelleted supernatant. Samples were lysed for 10 min on ice, shock-frozen in liquid N_2, and stored at -80°C until the assay was performed.

To confirm Nsm expression levels we used murine brain tissue as a control. We removed half of each brain and froze it in liquid nitrogen. We then added 1 mL lysis buffer, consisting of 100 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1% Igepal, 10 microg/ml A/L and 2.5 mM DTT to the brain and homogenized the samples by sonication. Samples had then been diluted 1:5 in a buffer containing 100 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 10 microg/ml A/L and 2.5 mM DTT to 0.2% Igepal. Aliquots were taken for protein measurements, and 15 microliter of diluted sample extract was used for lipase

reaction in a total volume of 300 microliter of the lysis buffer. The enzyme reaction was initiated by the addition of 0.05 microCi [^{14}C]-sphingomyelin (Perkin Elmer, Waltham, MA, USA; product number NEC663010UC, specific activity: 2 GBq/mmol) per sample. Before the assay, the substrate was dried in a SpeedVac (Thermo Scientific Sarant DNA 120), dissolved in 30 microliter of buffer per sample, and sonicated in ice water for 10 min. We then added 30 microliter of sphingomyelin to each 300 microliter lysate, incubated the lysates for 60 min at 37°C with agitation, and extracted samples with 1 mL of $CHCl_3$:CH_3OH (2:1, v/v). The samples were vortexed, phases were separated by centrifugation for 5 min at 14,000 rpm, and the upper aqueous phase was carefully transferred to a scintillation vial and measured by liquid scintillation counting. Hydrolysis of [^{14}C]-sphingomyelin by Nsm results in the release of [^{14}C]-choline chloride into the aqueous phase, whereas ceramide and unreacted [^{14}C]-sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]-choline chloride serves to determine the activity of Nsm.

3.1.4. Assay for acid sphingomyelinase activity

For determination of Asm, 2×10^5 RAW cells were infected with GFP-BCG for various time periods, washed, and lysed in 300 microliter ice-cold Asm lysis buffer (1% Igepal, 250 mM sodium acetate, pH 5.0) per sample. The aliquots were diluted to 0.1% Igepal and 250 mM sodium acetate (pH 5.0) and incubated with [^{14}C]-sphingomyelin for 30 min at 37°C. The [^{14}C]-sphingomyelin was dried before use and was solubilized into micelles in 0.1% Igepal and 250 mM sodium acetate (pH 5.0) in a bath sonicator for 10 min. Cell lysates were incubated for 60 min at 37°C with 0.05 microCi of [^{14}C]-labeled sphingomyelin per sample. Lipids were then extracted by the addition of 1 mL $CHCl_3$:CH_3OH (2:1, v/v) per sample, after which they were vortexed for 15 sec and centrifuged at 14,000 rpm for 5 min. An aliquot of the aqueous phase was applied for liquid scintillation counting.

3.1.5. Ceramide and sphingomyelin measurements via mass spectrometry

Ceramides and sphingomyelin were extracted and quantified as recently described (31,32). Briefly, cells were infected for various time periods and were then subjected to lipid extraction with C17 ceramide and C16 D31 sphingomyelin as internal standards. Sample analysis was carried out by rapid-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) with a Q-TOF 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive electrospray ionization (ESI) mode. The precursor ions of ceramides (C16 ceramide (m/z 520.508), C17 ceramide (m/z 534.524), C18 ceramide (m/z 548.540), C20 ceramide (m/z 576.571), C22 ceramide (m/z 604.602), C24 ceramide (m/z 632.634), and C24:1 ceramide (m/z 630.618)) were cleaved into the fragment ion m/z 264.270. The precursor ions of sphingomyelin (C16 sphingomyelin (m/z 703.575), C16 D31 sphingomyelin (m/z 734.762), C18 sphingomyelin (m/z 731.606), C20 sphingomyelin (m/z 759.638), C22 sphingomyelin (m/z 787.669), C24 sphingomyelin (m/z 815.700), and C24:1 sphingomyelin (m/z 813.684) were cleaved into the fragment ion m/z 184.074. Quantification was performed with Mass Hunter Software (Agilent Technologies).

3.1.6. Measurement of production of superoxide

Superoxide production was measured by electron spin resonance (ESR), as previously described (33). We infected 1×10^6 cells with GFP-BCG for the indicated time, removed the medium, scraped the cells into 20 mM HEPES (pH 7.5), 1 mM EDTA, and 255 mM sucrose, and then shock-froze them in liquid nitrogen. Proteins were isolated and resuspended with modified Krebs-HEPES buffer containing deferoxamine (100 microM, Sigma) and diethyldithiocarbamate (5 microM, Sigma). A spin trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen Science Transfer & Diagnostics, Elzach, Germany; 1 mM final concentration), was then added to the mixture in the presence or absence of manganese-dependent superoxide dismutase (SOD, 200 U/mL; Sigma-Aldrich). The mixture was loaded into glass capillaries and immediately kinetically analyzed for $O_2^{\cdot}$ production for 10 min. The SOD-inhibited fraction of the signal was used to calibrate the system. The ESR settings were as follows: biofield, 3,350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; points of resolution, 4,096; receiver gain, 100; and kinetic time, 10 min. The ESR signal strength was recorded in arbitrary units, and the final results were expressed as the fold changes from control strength, as previously described (34).

3.1.7. Western blotting

RAW macrophages were left uninfected or were infected, washed in cold H/S-buffer, and lysed for 5 min on ice in 125 mM NaCl, 25 mM Tris (pH 7.4), 10 mM EDTA, 10 mM sodium pyrophosphate, and 3% NP40 supplemented with 10 microg/ml Aprotinin/Leupeptin (A/L). Nonadherent cells in the supernatant were collected and centrifuged at 1200 rpm for 5 min. Adherent cells were lysed in lysis buffer as described above. Lysates and pellets were combined and insoluble material was pelleted by centrifugation for 10 min at 14,000 rpm. 5 x sodium dodecyl sulfate (SDS) sample buffer was added, and samples were boiled for 5 min and separated by 8.5% to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were blotted on nitrocellulose membranes (Amersham Protran Premium 0.2 micrometer, product code 10600005; GE Healthcare, Freiburg, Germany) at for 2 hr at 4°C (80 V). For detection of proteins, blots were washed with

phosphate-buffered saline (PBS) and blocked for 1 h at room temperature in Starting Block Tris-buffered saline (TBS) buffer (Thermo Scientific, Grand Island, NY, USA; supplier number 37542). After two another washing steps in PBS, specific primary antibodies against Beclin-1 (BECN1 (H-300), Santa Cruz Biotechnology, Dallas, TX, USA; catalog number, sc-11427) or LC3B (Sigma Aldrich L7543, rabbit IgG) were incubated overnight at 4°C in PBS (BECN1) or PBS, 4% BSA (LC3B). After being subjected to 6 washing steps in TBS/Tween, blots were incubated for 1 h at room temperature in TBS/Tween with alkaline phosphatase (AP)-conjugated secondary antibodies (Santa Cruz Biotechnology). Samples were washed extensively and developed with CDP Star (Perkin Elmer; product number NEL616001KT).

3.1.8. Immunocytochemistry

Cells were grown on coverslips, infected, or left uninfected. They were then fixed in 1% paraformaldehyde (PFA; Sigma-Aldrich), buffered in PBS consisting of 137 mM NaCl, 2.7 mM KCl, 7 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.4 mM KH_2PO_4, and 6.5 mM Na_2HPO_4 (pH 7.2-7.4) for 15 min for staining of Beclin or 4% PFA in PBS for 25 min for visualization of LC3B, and washed in PBS. Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (pH 7.4) for 10 min at room temperature, washed once with H/S and once with H/S with 0.05% Tween-20 (Sigma), and blocked for 15 min in H/S with 0.05% Tween-20. Samples were incubated for 45 min with anti-ceramide antibodies (mouse immunoglobulin M (IgM); Glycobiotech, Borstel, Germany) in H/S supplemented with 5% FCS (Gibco/Invitrogen). Cells were washed three times in H/S with 0.05% Tween-20 and incubated for an additional 45 min with Cy3-labeled IgM anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After additional washings in PBS with 0.05% Tween-20, samples were incubated with antibodies against either Beclin-1 or LC3B in H/S supplemented with 5% FCS. Cells were then washed three times in H/S with 0.05% Tween-20 and incubated for another 45 min with Cy5-labeled anti-rabbit antibodies (all from Jackson ImmunoResearch). After a final wash with H/S, cells were mounted on glass microscope slides with Mowiol (Kuraray Specialities Europe GmbH, Frankfurt, Germany). Cells were examined with a Leica TCS DMIRE confocal microscope (Leica, Mannheim, Germany).

3.1.9. Histopathologic assessment

Mice were sacrificed, livers and spleens were removed, embedded in Tissue-Tec (Sakura Finetek USA, Torrance, CA, USA), and shock-frozen in liquid nitrogen. Sections 6 micrometer thick were cut with a cryotome (Leica CM1850 UV). For stainings, sections were thawed and fixed in ice-cold acetone for 10 min. After a washing step with PBS, tissues were either stained with a Truant TB Fluorescent Stain Kit (Fisher Scientific, Pittsburgh, PA, USA) or stained with Cy5-coupled anti–Beclin-1 (BECN1 (H-300), Santa Cruz Biotechnology, sc-11427) or LC3B (Cell Signaling Technology, Danvers, MA, USA; product number 2775) antibodies as described above. The fluorescent staining of mycobacteria (Truant; BD Difco, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) was performed according to the manufacturer's instructions. For evaluation of numbers of granulomas, bacteria in granulomas and single bacteria were counted in serial sections (50 fields per sample). Samples were analyzed by fluorescence or confocal microscopy.

3.1.10. Quantification of colony-forming units

To quantify BCG colony-forming units (CFU) in tissue, we removed the liver and spleen from infected mice, added 5 mg/ml saponin (Serva Electrophoresis GmbH, Heidelberg, Germany) in H/S, and homogenized the tissues in a loose Dounce homogenizer (Braun, Kronberg, Germany). The homogenates were incubated for 30 min at 37°C on a thermomixer at 350 rpm for the release of intracellular bacteria. Samples were centrifuged for 2 min at 1000 rpm, supernatant was diluted in PBS, and plated on Middlebrook 7H10 agar plates enriched with oleic acid, albumin, dextrose, and catalase (OADC; BD Biosciences, Heidelberg, Germany). For CFU assays, infected cells were washed once with MEM/10 mM HEPES after indicated infection time to remove non-adherent bacteria and were then lysed in 3 mg/ml saponin for 30 min at 37°C. We plated 100-microliter aliquots and counted bacterial CFU after the plates had been incubated for approximately 2 weeks in a humidified 37°C atmosphere.

3.1.11. Statistical analysis

Data are displayed as mean +/- SD as indicated. All data were tested for normal distribution with the David-Pearson-Stephens test. Statistical analysis was performed with Student's *t*-test for single comparisons and with analysis of variance (ANOVA) for multiple comparisons. Statistical significance was set at the level of *P* less than 0.05.

4. RESULTS

4.1. *Mycobacterium bovis* Bacillus Calmette Guérin infects macrophages via the neutral sphingomyelinase/ceramide system

To determine whether Nsm plays a role in the infection of macrophages with BCG, we incubated RAW cells for the indicated times with BCG and determined the activity of Nsm. These experiments showed rapid activation of Nsm upon infection (Figure 1A). Nsm activation reached a maximum as early as 5 min after the initiation of infection. Knock-down of Nsm in RAW cells reduced the absolute activity of Nsm but did not change the relative increase in enzyme activity after BCG infection (Figure 1A). This increased activity is associated with an early decrease in sphingomyelin concentrations in control-transfected and Nsm-suppressed RAW

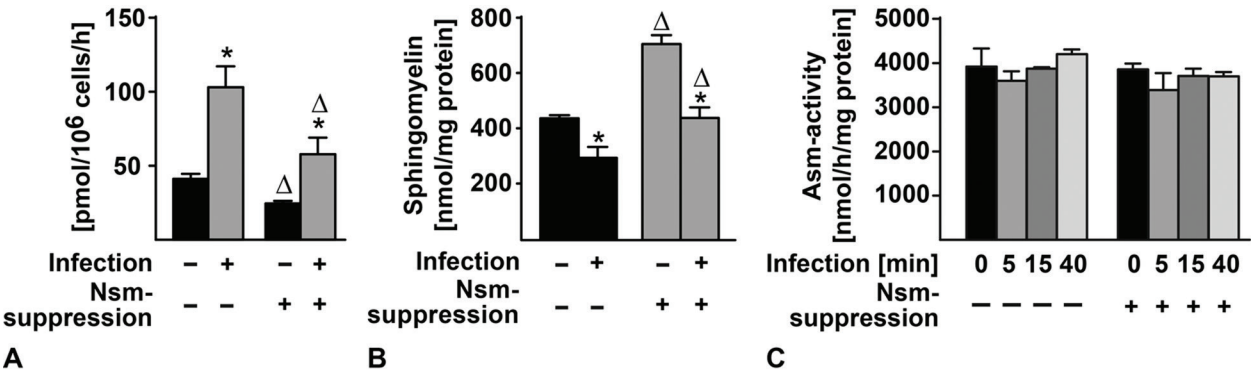

Figure 1. *Mycobacterium bovis* Bacillus Calmette Guérin infection of RAW macrophages activates neutral sphingomyelinase and triggers sphingomyelin consumption. RAW cells were transfected either with shRNA targeting neutral sphingomyelinase (Nsm) or with scrambled control shRNA. The cells were selected, and bulk cultures were infected with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) for 5 min (A,B) or the indicated time (C) at a multiplicity of infection (MOI) of 5:1. Cells were lysed, and Nsm activity (A), sphingomyelin consumption (B), and acid sphingomyelinase (Asm) activity (C) were determined. Sphingomyelinase activity was determined by enzyme assays using [^{14}C]-sphingomyelin as a substrate. Cellular sphingomyelin was measured by mass spectrometry. Shown are means +/- SD of at least three independent experiments each. Significant differences (*P* less than 0.05, as determined by ANOVA) compared to uninfected control cells are indicated with asterisks; significant differences between corresponding control-transfected or Nsm-targeted groups are indicated with deltas (Δ).

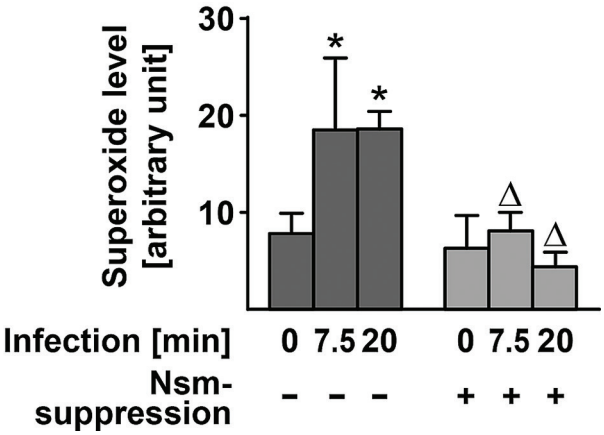

Figure 2. *Mycobacterium bovis* Bacillus Calmette Guérin infection of RAW macrophages induces a release of superoxide via neutral sphingomyelinase. Infection of RAW cells with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) induces the production of superoxide; this induction is prevented by down-regulation of neutral sphingomyelinase (Nsm) expression. Control-transfected or Nsm-suppressed RAW cells were left uninfected or were infected with BCG at a multiplicity of infection (MOI) of 5:1 for 7.5 min or 20 min. Superoxide production was measured by electron spin resonance. Shown are means +/- SD of three independent experiments each. Significant differences (*P* less than 0.05, ANOVA) compared to uninfected control cells are indicated with asterisks; significant differences between corresponding control-transfected or Nsm-targeted groups are indicated with deltas (Δ).

macrophages after infection with BCG (Figure 1B). However, the absolute levels of sphingomyelin in Nsm-suppressed cells are higher than in control-transfected cells, because of the suppressed Nsm-activity (Figure 1B). The decrease in sphingomyelin concentrations was due to Nsm activity, not to Asm activity, because Asm activity actually decreased slightly after infection of control-transfected or Nsm-deficient macrophages (Figure 1C). Taken together, these findings indicate that Nsm, but not Asm, is activated upon infection of macrophages with BCG.

4.2. Neutral sphingomyelinase activation in macrophages by *Mycobacterium bovis* Bacillus Calmette Guérin results in the production of superoxide

Superoxide has been found to play an important role in host-pathogen interactions (17,35). To determine whether BCG also induces the production of superoxide and whether this process depends on Nsm activity, we incubated RAW cells with mycobacteria for various time periods and measured the production of superoxide. The results showed that infecting control-transfected RAW cells with BCG leads to a rapid and strong increase in the production of superoxide after 7.5 or 20 min of infection. This increase in superoxide production is abrogated in RAW cells with Nsm knock-down (Figure 2).

4.3. Neutral sphingomyelinase regulates autophagy via superoxide

Superoxide exhibits multiple functions in cells infected with pathogenic bacteria. On the one hand, they serve to kill pathogens (18,19); on the other hand, excess production of superoxide also induces cell death and inhibits cellular defense systems such as autophagy, a process that has been shown to play a crucial role in the cellular defense against intracellular mycobacteria (20-22). To learn whether Nsm-dependent autophagy plays a role in BCG infection, we used Western blotting and immunofluorescence staining to determine the levels of the autophagy marker Beclin-1 and LC3B after the infection of macrophages. In the immunofluorescence experiments, we also analyzed the cellular distribution of ceramide to investigate whether autophagy proteins co-localize with ceramide

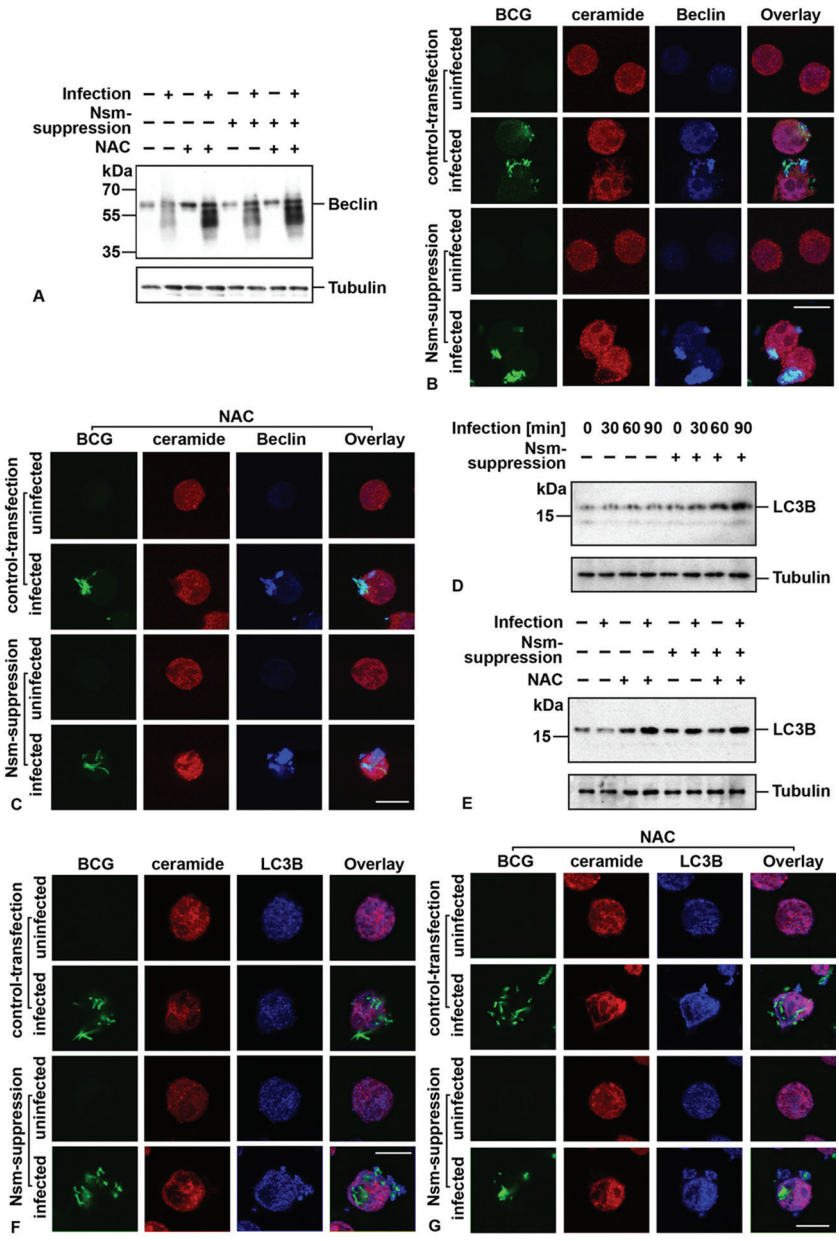

Figure 3. Neutral sphingomyelinase expression controls Beclin-1 and LC3B expression after infection with *Mycobacterium bovis* Bacillus Calmette Guérin. Suppression of neutral sphingomyelinase (Nsm) enhances *Mycobacterium bovis* Bacillus Calmette Guérin (BCG)-induced autophagy, as indicated by the finding that the expression of Beclin-1 and LC3B in RAW macrophages transfected with shRNA targeting Nsm was markedly higher than that in control-transfected RAW macrophages. Inhibition of superoxide by the application of N-acetylcysteine (NAC) increases autophagy in control-transfected cells. Control-transfected or Nsm-suppressed RAW cells were left uninfected or were infected with green fluorescent protein (GFP)-transfected BCG at a multiplicity of infection (MOI) of 5:1 for westernblots or 10:1 for immunofluorescence. Infection time was 60 min in A, B, C, F, G and 30 min or the indicated time in D, E. As indicated, cells were preincubated with NAC for 30 min before infection. Expression of Beclin-1 (A) or LC3B (D, E) was analyzed by westernblotting. Alternatively, cells were fixed with 1% paraformaldehyde for 15 min for Beclin-1 or 4% paraformaldehyde for 25 min for LC3B, permeabilized, and stained for Beclin-1 or LC3B with Cy5-coupled antibodies and for ceramide with Cy3-coupled antibodies (B, C, F, G). Scale bars represent 10 micrometer. Shown are representative results from three independent experiments each.

and, second, to confirm that bacteria are internalized into vesicles that contain ceramide. The results showed that the levels of Beclin-1 and LC3B increased after BCG infection only if the expression levels of Nsm were reduced, whereas the levels of these autophagy proteins were unchanged in control-transfected RAW cells after BCG infection (Figure 3A, B, D, E, F). Preincubation of control-transfected RAW cells with the antioxidant NAC increased the levels of Beclin-1 or LC3B after infection with BCG to the levels observed in infected macrophages with Nsm knock-down, whereas NAC did not further increase the levels of Beclin-1 or LC3B in Nsm-suppressed

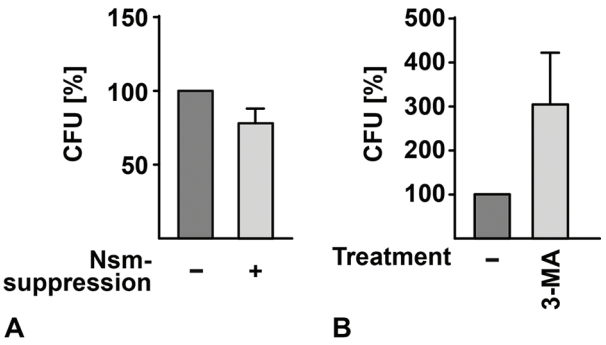

Figure 4. Neutral sphingomyelinase suppression promotes intracellular killing of *Mycobacterium bovis* Bacillus Calmette Guérin. (A) Control or Nsm-suppressed RAW macrophages were infected with BCG for 26 hrs and the number of BCG-CFU was determined after 2 weeks of culture. (B) RAW macrophages were incubated with 3-methyladenine (3-MA) 30 min before infection with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) for 26 h. Infected cells were washed and lysed. BCG were cultured for 2 weeks, at which time colonies were counted. Shown are the means +/- SD of the colony-forming units (CFU) from 4 independent experiments each.

macrophages after infection (Figure 3A, C, E, G). Taken together, these findings indicate that Nsm negatively regulates autophagy via superoxide after BCG infection.

4.4. Survival of *Mycobacterium bovis* Bacillus Calmette Guérin in macrophages depends on neutral sphingomyelinase-controlled autophagy

To investigate the impact of Nsm-suppression on bacterial survival, we compared the number of alive BCG in control with that in Nsm-suppressed cells after infection with BCG for 26 hrs. The results show that suppression of Nsm-expression reduces BCG survival in RAW-macrophages (Figure 4A).

To further determine whether Nsm-dependent autophagy leads to reduced survival of intracellular BCG, we inhibited autophagy with 3-methyladenine (3-MA) in macrophages and tested whether this inhibition impairs the ability of macrophages to kill the pathogen. The results of these studies show that BCG killing is dependent on cellular autophagy: BCG killing was greatly reduced in macrophages after inhibition of autophagy (Figure 4B). These findings indicate that BCG-induced activation of Nsm, which results in superoxide-mediated inhibition of autophagy, is used by the intracellular pathogen to protect itself from killing in macrophages.

4.5. Stimulation of autophagy reduces *Mycobacterium bovis* Bacillus Calmette Guérin overload in infected mice

To test the *in vivo* relevance of Nsm-dependent autophagy in mycobacterial infection, we systemically infected mice intravenously with 1×10^6 bacteria for 1, 3, or 6 weeks to investigate the status of acute and chronic infection. We determined the formation of granulomas and the number of single bacteria by using a fluorescence staining kit, and we determined the number of viable bacteria by CFU assays of liver and spleen homogenates. Intravenous infection of wt mice with BCG resulted in a small number of granulomas even after 1 week of infection in wt mice, whereas almost no granulomas were detectable in $Nsm^{+/-}$ mice. Between 3 and 6 weeks after intravenous infection of wt mice with BCG, we observed massive formation of granulomas in wt mice, but this formation was much less pronounced in $Nsm^{+/-}$ mice infected with BCG (Figure 5A-F). The granulomas were also smaller in $Nsm^{+/-}$ mice than in wt mice. Likewise, the number of of free single bacteria was greatly reduced in $Nsm^{+/-}$ mice compared to wt mice (Figure 5A-F).

CFU assays measuring viable bacteria in liver and spleen showed that these organs of $Nsm^{+/-}$ mice contained approximately 30% fewer bacteria than did those organs in wt mice (Figure 5G).

To assess the chronic state of the infections, we performed an extended time course and determined granuloma and bacteria in liver and spleen 12 weeks after infection. These results confirm the notion that Nsm-heterozygosity protects against BCG-infection: After 12 weeks of infection the number of granuloma and of mycobacteria in liver and spleen was much lower in $Nsm^{+/-}$ mice than in wt mice (Figure 5A,B).

To investigate autophagy in tissues after mycobacterial infection, we stained cryosections from liver and spleen with the autophagy markers Beclin-1 and LC3B. The levels of both Beclin-1 and LC3B were significantly higher in the liver and spleen of $Nsm^{+/-}$ mice than in these same organs in wt mice. The difference was most pronounced after 6 weeks of infection, which corresponds to the chronic state of BCG infection (Figure 6A-6D). These findings indicate that reduction of Nsm expression and function protects mice from systemic tuberculosis by stimulating autophagy.

5. DISCUSSION

The results of this study demonstrate that genetic inhibition of the Nsm/ceramide system protects mice against systemic tuberculosis. Pathogenic mycobacteria such as *M. tuberculosis* and *M. avium* reach the lung via aerosols and are internalized by alveolar macrophages. Within macrophages the pathogenic mycobacteria reside within phagosomes and are at least partially able to prevent the fusion of phagosomes with late endosomes and lysosomes. As a consequence, they survive and replicate in macrophages (36, 37). To control the infection, the organism forms granulomas that contain infected macrophages, epitheloid macrophages, and multinucleated giant cells in the lung, but if the bacteria generalize, granulomas are also formed in other organs,

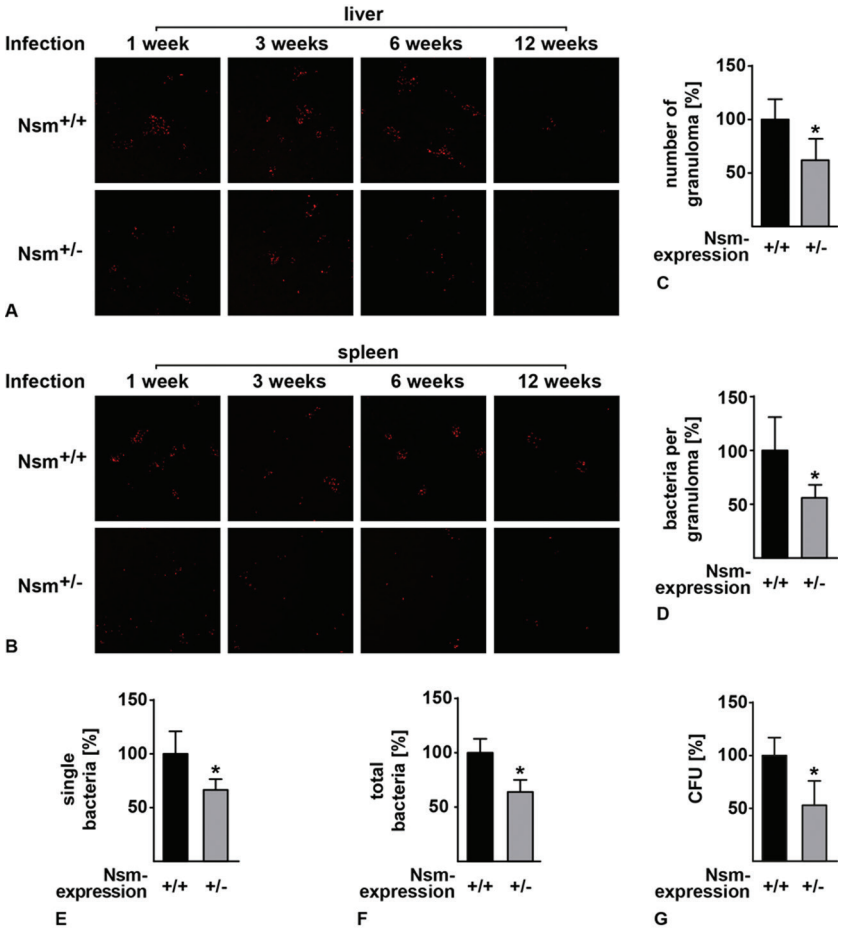

Figure 5. Neutral sphingomyelinase heterozygosity promotes killing of *Mycobacterium bovis* Bacillus Calmette Guérin *in vivo*. Wild-type ($Nsm^{+/+}$) or neutral sphingomyelinase-heterozygous ($Nsm^{+/-}$) mice were infected with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) for 1 to 12 weeks. The livers (A) and spleens (B) were removed, fixed, and stained with Truant for visualization of the mycobacteria. Nsm heterozygosity results in improved elimination of BCG after *in vivo* infection. (C-G) Quantitative analysis of 10 serial sections from livers and spleens demonstrates that Nsm heterozygosity reduces the number of granulomas (C), the number of bacteria per granuloma (D), and the number of single (E) and total (F) bacteria. Likewise, culture of tissues homogenates shows lower numbers of bacteria in the livers and spleens of Nsm-heterozygous mice than in the tissues of wt mice (G). Shown are the means +/- SD of at least 6 independent experiments each. Significant differences (*P* less than 0.05, ANOVA) between wt and Nsm-heterozygous mice are indicated with asterisks.

such as liver and spleen (2). Our findings indicate that genetic inhibition of Nsm prevents bacterial persistence in tissues and reduces the number of single bacteria. This protective effect of Nsm heterozygosity is particularly evident during the chronic stage of infection. In accordance with improved killing and impaired intracellular survival of the pathogen, the findings of *in vitro* experiments showed that macrophages with Nsm knock-out also contain less bacteria than do wt macrophages.

Our findings show that Nsm plays a crucial role in the infection of macrophages with BCG, both *in vitro* and *in vivo* (Figure 7). Nsm mediates the release of superoxide that seem to kill mainly extracellular BCG and thereby contribute to the host defense. On the other hand, Nsm-controlled superoxide also inhibits autophagy. Autophagy is a defense mechanism inhibiting the survival of BCG and *M. tuberculosis* bacteria in infected macrophages (22). The best-characterized form of autophagy is the so-called macroautophagy: After an autophagosome forms from an isolated membrane protrusion, it fuses with a lysosome to form an autophagolysosome, in which enzymatic degradation of the autophagosomal content takes place. Ceramide generated at the outer mitochondrial membrane has been shown to be involved in regulating mitophagy (i.e., autophagosomes containing mitochondria) by binding to LC3B (38, 39). In addition, Anes *et al.* (2003) demonstrated that sphingomyelin and ceramide, among other lipids, trigger actin nucleation on phagosomes and thereby promote phagolysosomal fusion, resulting in mycobacterial killing (40). Thus, our data indicate a dual role of ceramide in regulation of superoxide release and autophagy after BCG infection: While activity of the Nsm and the formation of ceramide from sphingomyelin results in a massive release of superoxide that initially kills the

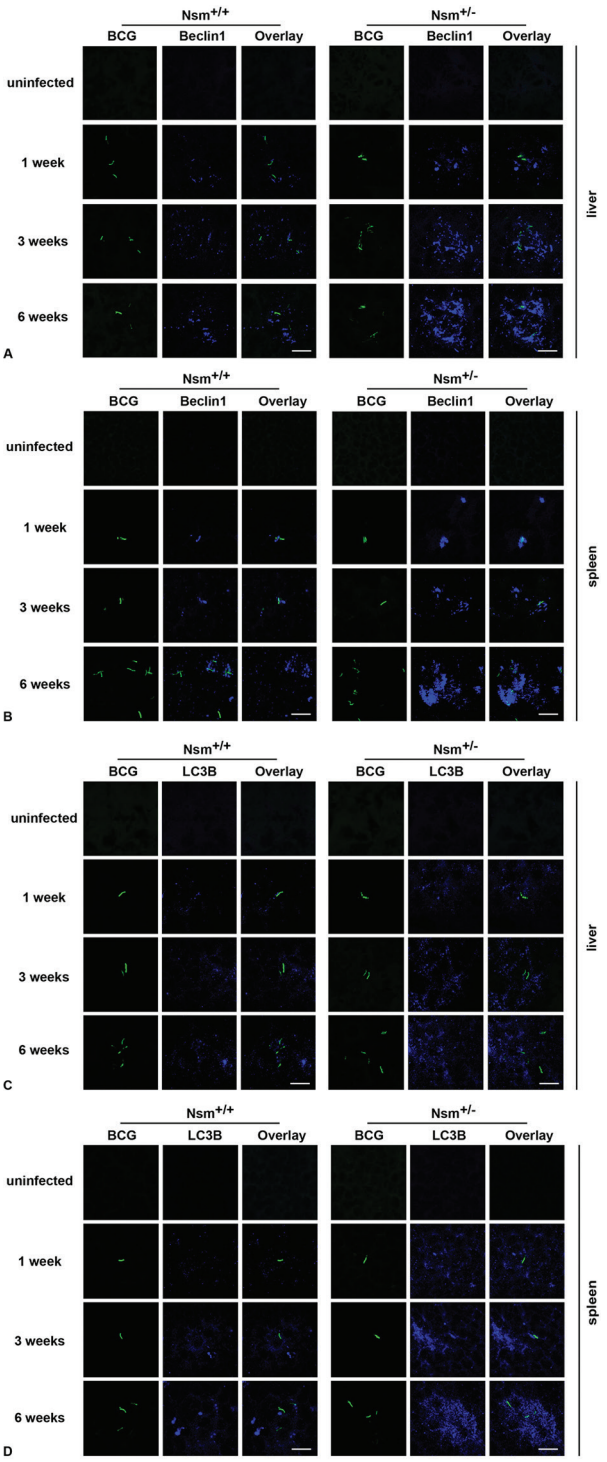

Figure 6. Heterozygosity for neutral sphingomyelinase promotes autophagy *in vivo*. Heterozygosity of neutral sphingomyelinase (Nsm) markedly promotes autophagy in murine liver and spleen cells infected with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG). Wild-type mice ($Nsm^{+/+}$) and Nsm-heterozygous mice ($Nsm^{+/-}$) were infected with BCG for 1, 3 or 6 weeks or left uninfected. The livers (A, C) and spleens (B, D) were removed, sectioned, fixed, and stained with Cy5-coupled anti-Beclin-1 (A, B) or anti-LC3B (C, D) antibodies. Green fluorescent protein (GFP)-positive BCG are visualized in green. Scale bars represent 10 micrometer. Shown are representative results from 3 independent experiments each.

pathogen, intracellular pathogens protect themselves from killing by exploiting this release of superoxide to reduce autophagy. Thus, the generation of ceramide is part of the attack of the pathogen against the host. This is confirmed by our *in vitro* findings showing that inhibition of autophagy prevents killing in macrophages with Nsm suppression, whereas activation of autophagy after BCG infection by treatment of wt macrophages with antioxidants enables these cells to eliminate the pathogen. These findings also confirm the previously reported crucial role of autophagy in the host response against BCG (22,27,41,42). Most importantly, infection of $Nsm^{+/-}$ mice indicates the crucial role of autophagy *in vivo*: The autophagy markers Beclin-1 and LC3B are much more up-regulated in livers and spleens of $Nsm^{+/-}$ mice after acute or chronic infection with BCG than in these organs in wt mice, and this up-regulation is associated with the number of bacteria in granulomas.

Our findings identify a completely novel pathway upstream of autophagy, i.e. the activation of Nsm by BCG and the Nsm-controlled release of superoxide that directly or indirectly inhibit autophagy upon infection with BCG. It is important to note that ceramide produced by neutral sphingomyelinase does not seem to regulate autophagy directly, but indirectly via the release of superoxide. In addition to ceramide, an increase in sphingomyelin levels has also been shown to be important for autophagy (41); however, we observed a decrease in sphingomyelin levels after BCG infection, suggesting that sphingomyelin does not regulate autophagy after BCG infection.

At present it is unknown how BCG activates Nsm, which has been previously shown to be regulated by redox systems (43). The present findings suggest that Nsm is upstream of superoxide, because genetic down-regulation of Nsm prevents the release of superoxide.

Additional studies have shown that Nsm is also regulated by heat shock protein 60 and by the phosphatase calcineurin (44,45). Although calcineurin has been previously shown to be involved in mycobacterial infections, it is presently unknown whether calcineurin also plays a role in mycobacteria-induced activation of Nsm (46).

Nsm activity hydrolyzes sphingomyelin and generates ceramide. Depending on the pathway of ceramide generation, ceramide will be formed in membranes facing the cytosol or in anticytoplasmic membranes. Ceramide generated by Asm activity will locate in the anticytoplasmic leaflet of membranes, for instance, plasma or lysosomal membranes. Asm-released ceramide has been implicated in infections with several pathogens, such as *N. gonorrhoeae*, *S. aureus*, *P. aeruginosa*, *M. avis*, rhinovirus, and measles virus (8-13,16,47,48) and is involved in the regulation of the inflammasome in cystic fibrosis lungs (49). Ceramide generated by Asm may be also primarily involved in the

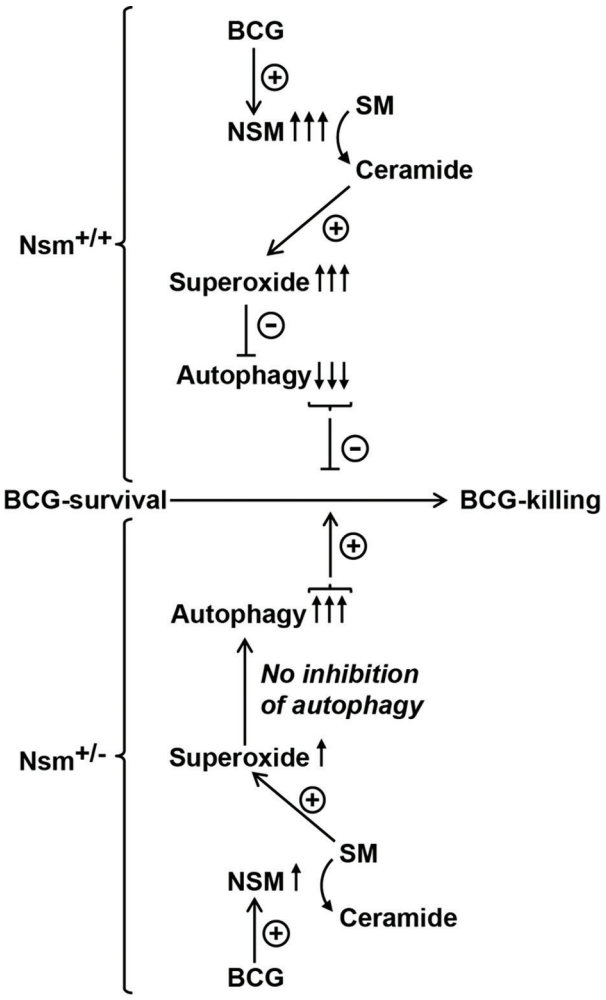

Figure 7. Diagram of the role of the neutral sphingomyelinase in BCG infection and autophagy. BCG infection activates the Nsm, which triggers a release of high amounts of superoxide. Superoxide inhibits autophagy and thereby prevents killing of intracellular pathogens. Heterozygosity of the Nsm limits ceramide release and superoxide production and, thus, allows killing of intracellular pathogens by autophagy.

fusion of lysosomes with phagosomes, as has been shown for *L. monocytogenes* (8,10). In contrast, Nsm-generated ceramide faces the cytosol and locates in the cytoplasmic leaflet of membranes, predominantly the endoplasmic reticulum and the plasma membrane. Nsm-generated ceramide has been previously shown to be involved in signaling of the TNF receptor or in the action of some chemotherapeutic drugs, but its role in bacterial infections is poorly understood (50,51). While Asm-generated ceramide forms membrane platforms that serve to cluster receptor and signaling molecules, the topology of intracellular ceramide is unknown. Ceramide-enriched membrane platforms in the outer leaflet of the plasma membrane cluster NADPH oxidase subunits, thereby activating the enzyme and triggering the generation of mainly extracellular superoxide. This superoxide serves to kill extracellular bacteria (17). In contrast, the source of Nsm-generated intracellular superoxide is unknown, but it is attractive to speculate that a modification of the mitochondrial metabolism by ceramide results in the generation of large amounts of superoxide.

Our findings suggest that Nsm plays a complex role in the defense against mycobacteria: Although the enzyme serves to produce superoxide and to kill some bacteria early after infection, this superoxide also inhibits autophagy, which is required for killing intracellular bacteria and resolving an infection. The *in vivo* findings demonstrate very clearly that inhibiting Nsm by approximately 50% almost completely prevents granuloma formation and facilitates elimination of the pathogen. At present, only a few cellular molecules are known (22,52-54) to confer resistance to BCG and, thus, may serve as targets for treating the infection. Nsm inhibitors may be particularly attractive novel target molecules for treating mycobacterial infections, because $Nsm^{+/-}$ mice have no phenotype; therefore, a temporary and partial pharmacological inhibition will be sufficient to allow elimination of mycobacteria with minimal adverse effects.

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