Acid Sphingomyelinase Amplifies Redox Signaling in *Pseudomonas aeruginosa* -Induced Macrophage Apoptosis

Yang Zhang, Xiang Li, Alexander Carpinteiro and Erich Gulbins

*J Immunol* 2008; 181:4247-4254; doi: 10.4049/jimmunol.181.6.4247
http://www.jimmunol.org/content/181/6/4247
Acid Sphingomyelinase Amplifies Redox Signaling in Pseudomonas aeruginosa-Induced Macrophage Apoptosis

Yang Zhang,2 Xiang Li,2 Alexander Carpinteiro, and Erich Gulbins3

Recent studies indicate that distinct membrane microdomains, also named lipid rafts, and ceramide play an important role in infectious biology. Ceramide forms larger ceramide-enriched membrane platforms that are required for diverse signal transduction. In this study, we demonstrate that ceramide-enriched membrane platforms are critically involved in redox signaling that regulates alveolar macrophage apoptosis upon infection with Pseudomonas aeruginosa. In freshly isolated alveolar macrophages, P. aeruginosa infection results in rapid activation of acid sphingomyelinase (Asm), release of ceramide, and formation of ceramide-enriched membrane platforms, which are required for P. aeruginosa-induced activation of NADPH oxidase and production of reactive oxygen species (ROS). Inhibition of NADPH oxidase or removal of intracellular ROS reduced P. aeruginosa-induced activation of the Asm and formation of ceramide-enriched membrane platforms, suggesting that NADPH oxidase-derived ROS regulate Asm-initiated redox signaling in a positive feedback manner. Furthermore, stimulation of JNK and induction of apoptosis upon P. aeruginosa infections are dependent on NADPH oxidase-derived ROS. These findings indicate that ceramide-enriched membrane platforms are essential for amplification of Asm-mediated redox signaling, which mediates JNK activation and thereby apoptosis of alveolar macrophages upon P. aeruginosa infection. The Journal of Immunology, 2008, 181: 4247–4254.

Pseudomonas aeruginosa causes pulmonary infections particularly in immunocompromised individuals or patients with cystic fibrosis (CF)4 (1). CF patients are highly susceptible to P. aeruginosa infections, developing chronic lung infections that result in fibrosis and destruction of lung tissue, and finally die. P. aeruginosa infections in CF patients often develop a mucoid biofilm in the respiratory tract, and the host defense system is unable to efficiently clear P. aeruginosa in this form (1). The mechanisms by which P. aeruginosa cause chronic lung infections are complex, and may at least partially be due to the suppression of host defense systems in the lung.

Alveolar macrophages serve as the first line of host defense to clear extracellular bacteria from the lung. In addition, these lung-resident macrophages are critically involved in coordinating the innate immune response during the bacterial infection (2). Alveolar macrophages are known as long-lived tissue cells with a low incidence of constitutive apoptosis (2, 3). Therefore, modulation of macrophage lifespan is an important mechanism for regulation of macrophage function and the host defense system (3). Many pathogens, including P. aeruginosa, induce both apoptotic and necrotic cell death in macrophages, which may contribute as a mechanism of immune suppression leading to exacerbation of inflammation.

In the present study is focused to define the molecular mechanisms that cause chronic lung infections, induce both apoptotic and necrotic cell death in macrophages, which may contribute as a mechanism of immune suppression leading to exacerbation of inflammation.

In fact, macrophage cell death may also provide a host response that signals for help from other immune cells (i.e., neutrophils, monocytes, and lymphocytes) and contribute to bacterial killing. Furthermore, apoptosis in macrophages and lung epithelial cells may down-modulate the immune response, because apoptotic cells are known to suppress the immune system, and, thus, prevent an overshooting immune response that may result in a cytokine storm and death (3, 8). Indeed, our previous studies have suggested that apoptosis plays a central role in the balance between host defense and invading P. aeruginosa (8, 9), although this study did not investigate the particular role of macrophage death but concentrated in epithelial cells. In vivo, mice unable to respond with host cell apoptosis are highly sensitive to pulmonary P. aeruginosa infection, release uncontrolled levels of cytokines, develop sepsis, and die (8, 10). Therefore, a controlled apoptotic response of lung cells is required for efficient clearance of invading P. aeruginosa and prevention of lung infection. At present, the role of alveolar macrophage apoptosis in the context of CF is not known. The present study is focused to define the molecular mechanisms that regulate the cellular apoptotic response of alveolar macrophages upon P. aeruginosa infection.

Ceramide generated by activity of the acid sphingomyelinase (Asm) was shown to play a key role in the infection of mammalian cells with P. aeruginosa. Ceramide reorganizes distinct membrane microdomains, named lipid rafts, that are enriched in sphingolipids and cholesterol, into larger ceramide-enriched signaling platforms. These platforms are required for diverse signal transduction, including initiation of apoptotic and stress signaling (11–16). In vivo, ceramide-enriched membrane platforms are central to the host defense against P. aeruginosa and are critical for internalization of the pathogen and induction of cell death of and controlled release of cytokines by epithelial cells (9). Failure to generate ceramide-enriched membrane platforms results in an unabated inflammatory response, massive release of IL-1, and septic death of mice (9). However, the role of ceramide and the Asm for the infection of macrophages, the mechanisms regarding how these platforms link to downstream signaling, and how their formation is regulated during the infection process are unknown.
NADPH oxidase is highly abundant in phagocytes and is the major enzyme responsible for respiratory burst of reactive oxygen species (ROS) upon bacterial infection, a key event in the defense against pathogenic bacteria. Recently, ceramide-enriched membrane platforms have been implicated in NADPH oxidase-derived ROS and endothelial dysfunction (17, 18). In endothelial cells, Asm activation and ceramide generation promote clustering of small resting lipid rafts to form larger active redox signaling platforms upon CD95 ligand and endothelial dysfunction (17, 18).

In this study, we demonstrate that *P. aeruginosa*-induced Asm activation and ceramide-enriched membrane platform formation are critical for NADPH oxidase-derived ROS production and are feed-forwardly regulated by ROS. ROS derived from this Asm-mediated feed-forward loop determines JNK activation and consequent macrophage apoptosis.

**Materials and Methods**

**Isolation of alveolar macrophages from mice**

Lung macrophages were isolated by bronchoalveolar lavage from 6- to 8-wk-old C57BL/6 wild-type (WT) mice or spingomyelin phosphodiesterase 1 knockout (Smnphd1−/−) mice, which are deficient in Asm activity. To this end, the trachea was opened and cannulated with a polyethylene tube. Then, the lung was lavaged with a total of 15 ml of ice-cold PBS in 20 aliquots (0.75 ml/ aliquot). Approximately 0.5–1 × 10^6 cells per mouse were consistently obtained. Cells were pelleted by centrifugation at 300 × g for 15 min, resuspended, and cultured for 1 h in RPMI 1640 (Life Technologies) with 1 mM HEPES (pH 7.4) in 24-well plates at density of 10^5 cells/well. Alveolar macrophages are extremely adhesive cells. Therefore, after washing off other blood cells, a pure cell culture was obtained, with more than 99% of cells being macrophages, as confirmed by FACS analysis after staining with FITC-coupled anti-CD11b Abs (BD Biosciences).

**Infection experiments**

The laboratory strain *P. aeruginosa* ATCC 27853 was plated overnight on tryptic soy agar plates at 37°C, resuspended in tryptic soy broth at an OD of 0.550 nm at 0.25, shaken at 120 rpm for 1 h at 37°C, harvested during early logarithmic growth phase, washed twice in RPMI 1640 supplemented with 1 mM HEPES (pH 7.4), and maintained in the same medium during infection. To detect apoptosis of macrophages, the cells were infected with *P. aeruginosa* at a multiplicity of infection (MOI) of 100 (1 macrophage was infected with 100 bacteria). Synchronous infection conditions and an enhanced bacterium-host cell interaction were achieved by a 2-min centrifugation (300 × g) of the bacteria onto the cells. The end of the centrifugation step was defined as the starting point of all infections.

**Detection and quantification of apoptosis**

Apoptosis was quantified by TUNEL analysis and confirmed by FITC-annexin V staining (9). To this end, cells were cultured on coverslips and infected, as described above. Cells were fixed in 2% paraformaldehyde (PFA) for 10 min, washed, and stained with FITC-coupled deoxyuridine triphosphate (dUTP) in the presence of TdT. The samples were washed in PBS, stained with alkaline phosphatase-coupled anti-FITC Abs, and developed using Fast Red tablets (Roche) as a substrate, which is converted by the alkaline phosphatase to a red dye. For FITC-annexin V and propidium iodide (PI) staining, cells were infected, as above, washed once, and incubated with FITC-annexin V (dilution in PBS 1/50; Roche) and PI (1 μg/ml; Calbiochem) on ice for 15 min. Cells were then fixed with 4% PFA in PBS for 15 min, washed once with PBS, and embedded in mounting medium on coverslips. FITC-annexin V and PI staining was observed on a Leica DMIRE2 fluorescence microscope. At least 200 cells/sample were counted.

**Asm activity assay**

Activity of the Asm was measured, as previously described (9). Briefly, 10^6 cells in a 12-well plate were infected, washed, and lysed in 200 μl of ice-cold buffer containing 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, and a mixture of protease (Roche) and phosphatase inhibitors (Sigma-Aldrich). The cells were removed from the plate, transferred into Eppendorf tubes, and immediately sonicated three times (3 × 10 s). The lysates were incubated with 0.02 μCi of [3H]spingomyelin for 30 min at 37°C. The reaction was stopped by addition of 1 ml of CHCl3:CH3OH (v/v). The substrate was dried before use and resuspended in 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, followed by 10-min bath sonication to promote the formation of micelles. Phases were separated by 5-min centrifugation at 14,000 rpm, and an aliquot of aqueous phase was applied for liquid scintillation counting. Hydrolysis of [3H]spingomyelin by spingomyelinase results in release of [3H]choline chloride into the aqueous phase, whereas ceramide and unreacted [3H]spingomyelin remain in the organic phase. Therefore, the release of [3H]choline chloride (pmol/10^6 cells/h) serves to determine the activity of the Asm.

**Ceramide measurement by 1,2-diacylglycerol (DAG) kinase assay**

Cellular ceramide was measured by DAG kinase assay, as previously described (9). Cells were infected as above and extracted in CHCl3:CH3OH:1 N HCl (100:100:1, v/v/v), and the lower phase was collected, dried, and subjected to alkaline hydrolysis of diacylglycerol in 0.1 N methanolic KOH at 37°C for 60 min. Samples were re-extracted, the lower phase was dried, and 20 μl of a detergent solution consisting of 7.5% (w/v) α-oleylgluco- pyranoside and 5 mM cardiolipin in 1 mM diethylentriaminepentaacetic acid was added to the samples. Samples were sonicated for 10 min in a bath sonicator, and 50 μl of assay buffer consisting of 0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM MgCl2, 2 mM EGTA, 2.8 mM DTT, 5 μM ATP, 10 μM of [32P]ATP, and 10 μl of diluted DAG kinase (dilution buffer: 1 ml of diethylentriaminepentaacetic acid (pH 6.6), 0.01 M imidazole/HCl) was added. The kinase reaction was performed for 30 min at room temperature, and the samples were extracted in 1 ml of CHCl3:CH3OH:1 N HCl (100:100:1, v/v/v), 170 μl of buffered saline solution (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM HEPES (pH 7.2), and 30 μl of a 100 mM EDTA solution. Samples were vortexed and centrifuged, and the lower phase was collected, dried, and dissolved in 20 μl of CHCl3:CH3OH:1 (1/1/1). Lipids were separated on silica G-60 TLC plates with CHCl3:CH3OH:CH3COOH (65:15:5, v/v/v), and the plates were dried and exposed. Ceramide spots were identified by comparison with a C16-ceramide standard and removed from the plate, and incorporation of 32P into ceramide was quantified by liquid scintillation counting. Comparison with a standard curve using C16-ceramide permitted the determination of ceramide amounts.

**Determination of ROS burst**

Cells were cultured in 96-well plates (10^4 cells/well) and incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes) for 30 min at 37°C. The nonfluorescent dye is oxidized by ROS and becomes fluorescent. Cells were infected with *P. aeruginosa* for 30 min, and the fluorescence was determined by fluorescence microplate reader (BMG LABTECH) at excitation/emission: 485/520 nm. Relative fluorescence unit (RFU) over time (ΔRFU/min) was used to represent ROS release.

**FACS analysis of surface ceramide and intracellular ROS**

To detect surface ceramide in the plasma membrane, cells were left uninfected or infected with *P. aeruginosa*, as described above, and fixed in 2% PFA in PBS for 10 min. The cells were incubated in PBS/5% FCS to block unspecific binding sites and stained with an anti-ceramide Ab (Glyobiotech; mouse IgM) for 60 min. Cells were then washed in PBS containing 1% FCS and stained for 45 min with Cy3-goat anti-mouse IgM (Jackson ImmunoResearch Laboratories). After a final PBS wash, cells were analyzed on a FACS Calibur (BD Biosciences).

To determine intracellular ROS production, cells were loaded with 10 μM H2DCFDA (Molecular Probes) for 30 min at 37°C. After incubation, cells were extensively washed with PBS and further incubated at 37°C for 10 min to allow cleavage of H2DCFDA to 2′,7′-dichlorodihydrofluorescein by intracellular esterase. After infection, the cell fluorescence was determined by FACS analysis.

**Fluorescence microscopy**

Cells were left uninfected or infected with *P. aeruginosa*, as above, and fixed in 2% PFA/PBS for 10 min. For intracellular staining, cells were permeabilized with 0.1% Triton X-100/PBS for 15 min at room temperature. Cells were washed again with PBS and incubated in 30 min in PBS supplemented with 5% FCS to block nonspecific binding sites. Cells were washed and incubated for 45 min with either mouse monoclonal anti-ceramide IgM (Glyobiotech; rabbit IgG/mouse IgM) and anti-goat IgG H+L (BD Biosciences; 0.5 μg/ml) or a rabbit polyclonal anti-phospho-c-Jun IgG (Cell Signaling Technology; 0.5 μg/ml). Cells were then washed in PBS with 0.05% Tween 20 and incubated for an additional 45 min with FITC-labeled...
goat anti-mouse IgG, Cy3 anti-rabbit IgG, or Cy5 anti-mouse IgM F(ab')2 Abs (all from Jackson ImmunoResearch Laboratories and diluted in PBS/5% FCS). After a final PBS wash, cells were mounted on glass coverslips with moviol. Control experiments were performed with irrelevant mouse or rabbit Abs and secondary Abs. Control Abs did not significantly bind to the cells. Cells were examined on a Leica TCS SP confocal microscope equipped with a ×100 oil objective, and images were analyzed using LeicaConfocal software (Leica Microsystems).

### Immunoblot analyses

Cells were lysed in SDS-sample buffer (62.5 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 0.04% bromphenol blue, and 5% 2-ME), boiled, and sonicated (3 × 10 s) on ice to break chromosomal DNA and decrease sample viscosity. Then proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in 4% BSA in TBS supplemented with 0.1% Tween 20 and incubated overnight at 4°C with anti-phospho-JNK (Cell Signaling Technology; 1/1000 dilution in 4% BSA) or anti-JNK1 (Santa Cruz Biotechnology; 1/1000 dilution in 4% BSA) Abs. Blots were labeled with alkaline phosphatase-coupled secondary Abs (Santa Cruz Biotechnology) and developed using the Tropix system.

### Results

P. aeruginosa induces caspase-dependent cell death of alveolar macrophages

Infection of mammalian cells with *P. aeruginosa* is known to induce cell death (5,8). However, the form of cell death induced by this bacterium may vary from apoptosis to necrosis, depending on the bacteria to cells ratio (MOI), infection time, and/or cell type tested (5, 19). We previously reported that *P. aeruginosa* primarily induced apoptosis in lung epithelial cells, whereas the response of alveolar macrophages to *P. aeruginosa* requires definition. To define the form of death induced by *P. aeruginosa* in alveolar macrophages, we investigated the role of caspases in *P. aeruginosa*-induced death of these cells. *P. aeruginosa* strain ATCC27853 induced cell death in 36 ± 5% of alveolar macrophages 4 h after infection.

### FIGURE 1.

*P. aeruginosa* induces caspase-dependent cell death. Alveolar macrophages were isolated by bronchoalveolar lavage of mouse (C57BL/6) lungs, cultured in RPMI 1640, and infected with *P. aeruginosa* ATCC27853 (MOI = 1:100) for 1, 2, 4, and 6 h. Cell death was determined by TUNEL in the absence or presence of the caspase inhibitor zVAD-cmk (1 μM) (A) or by FITC-annexin V and PI staining (B). Data are mean ± SD of four separate experiments. Significant differences were determined by Student’s t test. *, Infected vs noninfected controls; Δ, infected cells treated with zVAD-cmk vs infected cells without zVAD-cmk treatment (p < 0.05).

### FIGURE 2.

*P. aeruginosa*-induced cell death involves Asm activation and ceramide generation. Freshly isolated mouse alveolar macrophages were infected, lysed, and assayed for Asm activity (A) and ceramide content using DAG kinase assay (B). C, Macrophages isolated from C57BL/6 WT or Snpd1<sup>−/−</sup> (KO) mice lacking Asm activity were left uninfected (thin line) or infected (bold line) with *P. aeruginosa* for 15 min. Surface ceramide release was determined by FACS using Cy3-labeled anti-ceramide Abs. A representative of three experiments is shown. D, Macrophage apoptosis requires Asm activity. Deficiency of Asm inhibited macrophage apoptosis. Macrophages from WT or Smpd1<sup>−/−</sup> mice were infected with *P. aeruginosa* (PA) for 4 or 6 h and analyzed by TUNEL. Data are mean ± SD of four independent experiments. Significant differences between infected and noninfected controls or between infected WT or Snpd1<sup>−/−</sup> cells were determined by Student’s t test and are indicated by * or Δ, respectively (p < 0.05, Student’s t test).
infection, which was partially inhibited by a general caspase inhibitor, zVAD-cmk (1 μM) (Fig. 1A). However, a smaller proportion of the cells also died in the presence of zVAD-cmk, and this caspase-independent cell death might be attributed to necrosis. Consistently, FITC-annexin V and PI staining indicate a time-dependent increase in FITC-annexin V-positive cells, and a smaller fraction of PI-positive cells was only observed after 6-h infection (Fig. 1B). Taken together, these data suggest that in the early phase of infection, P. aeruginosa induces primarily caspase-dependent apoptotic cell death in freshly isolated mouse alveolar macrophages.

P. aeruginosa-induced apoptosis of alveolar macrophages is Asm dependent

To further define mechanisms that mediate apoptosis of macrophages upon P. aeruginosa infection, we investigated the role of the Asm, which has been recently shown to play a critical role in host-pathogen interactions (9, 20). To this end, we infected alveolar macrophages with P. aeruginosa and determined the enzymatic activity of the Asm and the formation of ceramide. The results reveal a very rapid activation of the Asm upon P. aeruginosa infection, peaking at ~15 min after infection (Fig. 2A). Enhanced activity of the Asm correlated with increased ceramide concentrations in WT macrophages after P. aeruginosa infection (Fig. 2B). This decrease might be mediated by consumption of ceramide by host cell enzymes, but also by the ceramidase activity from P. aeruginosa (21). Furthermore, surface ceramide release was detected in WT macrophages, as determined by FACS using anti-ceramide mAbs. Deficiency of the Asm in macrophages isolated from Smpdl−/− mice abrogated both the surface release of ceramide (Fig. 2C) and the induction of apoptosis in macrophages upon infection (Fig. 2D), indicating the importance of the Asm for the induction of macrophage death by P. aeruginosa.

Asm mediates P. aeruginosa-induced ROS production

Previous studies showed a critical role of the Asm in ROS production in macrophages, hepatocytes, and endothelial cells (18, 22, 23). Because ROS are critical factors for host-pathogen interactions, but also a mediator of cell death, we tested whether Asm mediates P. aeruginosa-triggered ROS production and whether Asm-mediated redox signaling initiates death signaling upon infection. As shown in Fig. 3A, P. aeruginosa induced ROS burst in WT cells, whereas no ROS release was observed in Smpdl−/− cells after 30-min infection. Furthermore, as shown in Fig. 3B, P. aeruginosa time-dependently increased intracellular ROS in WT macrophages, whereas no increase was observed in Smpdl−/− cells. It has been well documented that NADPH oxidase-derived superoxide production is a primary source of ROS generation in macrophages and neutrophils upon bacterial infection (24, 25). Consistently, diphenyleneiodonium chloride (DPI) (50 μM; Sigma-Aldrich), a NADPH oxidase inhibitor, significantly attenuated P. aeruginosa-induced ROS production (Fig. 3B) and cell death (Fig. 3C). Likewise, apocynin (100 μM; Calbiochem), a more specific inhibitor for NADPH oxidase, also prevented P. aeruginosa-induced cell death. In phagocytes, once membrane NADPH oxidase is assembled and activated, it generates superoxide extracellularly. Superoxide dismutase (100 U/ml; Calbiochem), a membrane-impermeable enzyme that catalyzes dismutation of superoxide to hydrogen peroxide and oxygen, significantly attenuated P. aeruginosa-induced cell death (Fig. 3C). Therefore, NADPH oxidase-derived extracellular superoxide is importantly involved in P. aeruginosa-induced cell apoptosis. Taken together, these studies demonstrate that Asm mediates P. aeruginosa-induced ROS production via activation of NADPH oxidase, events that are critical for the induction of cell death upon infection.

**FIGURE 3.** P. aeruginosa-induced cell death involves NADPH oxidase-derived ROS generation. A. To determine ROS burst, WT or Smpdl−/− macrophages were incubated with a fluorescence probe, H2DCFDA. Cells were infected for 30 min, and fluorescence was determined by a fluorescence microplate reader. RFU over time (ΔRFU/min) was used to represent ROS release. B. Intracellular ROS accumulation is dependent on Asm and NADPH oxidase activity. WT or Smpdl−/− macrophages were loaded with H2DCFDA, in the absence or presence of DPI (50 μM), a NADPH oxidase inhibitor. Cells were infected for the indicated time and analyzed by FACS. Relative ROS levels were used to indicate intracellular ROS accumulation. C. TUNEL analyses indicate that DPI (50 μM), apocynin (100 μM), or superoxide dismutase (SOD) (100 U/ml) blocked apoptosis. Data are mean ± SD of four independent experiments. (⁎, Δ as above, p < 0.05).

Asm mediates P. aeruginosa-induced activation of NADPH oxidase via ceramide-enriched membrane platforms

NADPH oxidase consists of two membrane-bound subunits, gp91<sup>pox</sup> and p22<sup>pox</sup>, which form a flavocytochrome b<sub>558</sub> complex, and cytosolic subunits p47<sup>pox</sup>, p40<sup>pox</sup>, p67<sup>pox</sup>, and the small GTPase Rac, which translocate to the membrane to assemble the active complex following cell activation (26). To explore the mechanism regarding how Asm activation leads to NADPH oxidase-derived ROS production, we examined whether ceramide-enriched membrane platforms are involved in aggregation of gp91<sup>pox</sup>, a major membrane subunit of NADPH oxidase. Fluorescence microscopy studies reveal that P. aeruginosa infection (15 min) induced formation of ceramide-enriched platforms in WT cells (Fig. 4A). Ceramide-enriched platforms were observed as early as 1 min after infection, peaking at ~5 min (ceramide clustering in ~80% of all cells). In marked contrast, ceramide-enriched membrane platforms were absent in Asm-deficient cells after infection (Fig. 4, A and B), indicating that Asm is essential for...
P. aeruginosa-induced formation of ceramide-enriched membrane platforms. Costaining of ceramide and gp91phox demonstrates that gp91phox aggregates in and colocalizes with ceramide-enriched platforms (Fig. 4C), events that were also absent in Smpd1−/− cells, revealing a critical role of the Asm for the formation of active NADPH oxidase. These data indicate that, in P. aeruginosa-induced Asm-mediated redox signaling, ceramide-enriched membrane platforms serve to aggregate NADPH oxidase, leading to activation of this enzyme and subsequent ROS production.

Redox regulation of Asm activation and membrane platform formation

The studies presented above indicate that Asm controls ROS generation upon P. aeruginosa infection. However, recent studies have suggested that Asm is also subject to redox regulation (18, 27–29). Therefore, we first examined whether there is also a feedback regulation of ROS on Asm activation upon P. aeruginosa infection. As shown in Fig. 5A, we detected that P. aeruginosa-induced Asm activation was inhibited in the cells by treatment either with the NADPH oxidase inhibitor DPI (50 μM), or a H2O2 degrading enzyme catalase (10 U/ml), for 30 min. Cells were infected with P. aeruginosa for 15 min and applied for Asm activity assay or confocal microscopy. In parallel, effects of H2O2 (1 mM for 5 and 15 min) on Asm activity and ceramide-enriched membrane platform formation were examined. A, Asm activation was blocked by NADPH oxidase inhibition (DPI + PA) or ROS scavenging (catalase + PA), whereas enhanced oxidative stress by H2O2 increased Asm activity. B, Formation of ceramide-enriched membrane platforms was prevented by DPI and catalase. Furthermore, Asm activity is required for oxidative stress-induced formation of ceramide-enriched membrane platform because H2O2-induced ceramide clustering was observed in WT, but not Smpd1−/− cells. Data for A and B are mean ± SD of three independent experiments. Significant differences between infected or H2O2-treated cells and noninfected controls were determined by Student’s t test and are indicated by *.* Significant differences between infected cells treated with DPI or catalase and left untreated were determined by Student’s t test and indicated by Δ (p < 0.05).
Asm-mediated generation of ROS amplifies \textit{P. aeruginosa}-induced \textit{Asm} activation in a positive feedback manner.

Next, we determined whether ROS has a similar feedback effect on \textit{Asm}-mediated formation of ceramide-enriched membrane platforms. \textit{P. aeruginosa}-induced membrane platform formation was diminished by DPI or catalase, whereas H2O2 dramatically increased ceramide-enriched platform formation in WT, but not in Smpd1\textsuperscript{-/-} cells, suggesting that \textit{Asm} is essential for ROS-induced formation of membrane platforms (Fig. 5\textsuperscript{B}).

These data raise the question as to whether \textit{P. aeruginosa} activate \textit{Asm} first, which then triggers ROS that maintain the activity of the \textit{Asm} high or vice versa. DPI or catalase reduced, but not completely blocked \textit{P. aeruginosa}-induced \textit{Asm} activation after 15-min infection (Fig. 5\textsuperscript{A}; DPI vs DPI plus \textit{P. aeruginosa} and catalase vs catalase plus \textit{P. aeruginosa}), whereas early release of ROS was completely prevented in Smpd1\textsuperscript{-/-} cells (Fig. 3\textsuperscript{A}). These data indicate that an initial ROS-independent activation of the \textit{Asm} results in generation of ceramide-enriched membrane platforms that serve to release ROS upon infection. ROS keep the activity of the \textit{Asm} high, leading to enhanced formation of ceramide-enriched membrane platforms and further ROS release in a positive feed-forward loop.

\textbf{Role of JNK in \textit{P. aeruginosa}-induced apoptosis}

Next, we aimed to identify target molecules or signaling pathways downstream of \textit{Asm}-mediated redox amplification signaling, which trigger cell death. Ceramide and ROS have been reported to activate JNK, which has been previously shown to trigger cell death (30–33). Therefore, we examined whether JNK serves as a target for \textit{Asm}-mediated redox signaling in \textit{P. aeruginosa}-induced cell death. JNK activation upon \textit{P. aeruginosa} infection was detected in WT cells as early as 5 min after infection, with maximum levels reached \textit{30 min} after initiation of the infection (Fig. 6\textsuperscript{A}), whereas genetic deficiency of the \textit{Asm} prevented JNK activation by \textit{P. aeruginosa} infection. Likewise, WT cells treated with the NADPH oxidase inhibitor DPI (Fig. 6\textsuperscript{A}) failed to activate JNK after \textit{P. aeruginosa} infection. Furthermore, \textit{P. aeruginosa} infection induced phosphorylation and nuclear translocation of c-Jun in WT cells, which was...
also inhibited by DPI (Fig. 6B). Finally, the specific JNK inhibitor SP60012 prevented \textit{P. aeruginosa} and H$_2$O$_2$-induced cell death (Fig. 6C), indicating that JNK activation, downstream of Asm-mediated redox amplification signaling, is an essential step for \textit{P. aeruginosa}-triggered apoptosis.

**Discussion**

In the present study, we show that \textit{P. aeruginosa} induces redox signaling via stimulation of the Asm. This pathway, which is required for cell death by the pathogen \textit{P. aeruginosa}, induces rapid formation of ceramide-enriched membrane platforms that serve to aggregate NADPH oxidase and, thus, to produce ROS. \textit{P. aeruginosa}-induced Asm activation and ceramide-enriched platform formation are in turn regulated by NADPH oxidase-derived ROS in a positive feedback manner. ROS activate JNK that triggers \textit{P. aeruginosa}-induced cell death. These events were all absent in \textit{Smpd1}$^{-/-}$ mice that are deficient in Asm activity, indicating the critical role of the Asm in \textit{P. aeruginosa}-triggered ROS release and induction of apoptosis. The findings in the present study also highlight a central role of ceramide-enriched platforms in the amplification of Asm-mediated redox signaling and provide a novel function of these redox-signaling platforms, i.e., induction of apoptosis in alveolar macrophages infected with \textit{P. aeruginosa}.

The present study provides a novel view as to how Asm-dependent NADPH oxidase-derived ROS modulate the fate of the macrophage and the course of the infection. Upon infection, macrophages use ceramide-enriched platforms to rapidly and efficiently activate NADPH oxidase via assembly and aggregation of its subunits, resulting in initiation of redox signaling. In the early phase of infection, the intracellular ROS level is well controlled by up-regulation of antioxidant enzymes, such as superoxide dismutase via NF-$\kappa$B activation. The duration of JNK activation depends on intracellular ROS that oxidize and inhibit MAPK phosphatases (34). Therefore, no apoptosis occurs at this stage, and bacteria can be efficiently removed by innate immunity. During prolonged infection, accumulation of ROS in macrophages promotes strong and sustained JNK activation, which may result in cell apoptosis mediating an anti-inflammatory role of NADPH oxidase via the well-known inhibition of the immune system by apoptotic cell surfaces. This may prevent overshooting of the immune reaction against the pathogen and result in a local down-modulation of the immune response to prevent an uncontrolled release of cytokines. If the bacteria are already eliminated at this time, the induction of apoptosis might also contribute to the resolution of inflammation. However, if macrophages are unable to clear all bacteria, the bacteria increase both apoptosis and necrosis of alveolar macrophages, thereby promoting the inflammation by recruitment of other immune cells such as neutrophils, monocytes, and T lymphocytes to the site of infection. Furthermore, death of macrophages should permit enhanced bacterial growth and inflammation, resulting in an uncontrolled infection.

It is well known that NADPH oxidase is required for host defense against invading pathogens because ROS are toxic to most bacteria. In addition, NADPH oxidase is importantly involved in redox regulation of host responses against pathogens, for instance, via ROS-dependent activation of NF-$\kappa$B, a crucial transcription factor that controls expression of many proinflammatory genes. Indeed, a recent study has shown that \textit{P. aeruginosa}-induced activation of NF-$\kappa$B and increase in lung TNF-$\alpha$ level were inhibited, and bacteria clearance was impaired in \textit{p47}$^{phox^{-/-}}$ mice compared with WT mice (35). In the present study, we found that \textit{P. aeruginosa} induces Asm and ROS-dependent JNK activation, indicating JNK as a link between NADPH oxidase-derived ROS and apoptotic cell death in alveolar macrophages. Therefore, it seems that NADPH oxidase plays a dual role in controlling proinflammatory responses and cell apoptotic signaling, which is suggested to be anti-inflammatory during \textit{P. aeruginosa} infection.

Membrane rafts and ceramide were identified to have an important function in infectious biology. Many pathogens target and use rafts for infection of cells, including \textit{Escherichia coli}, \textit{Mycobacterium tuberculosis}, \textit{Campylobacter jejuni}, \textit{Vibrio cholerae}, and \textit{P. aeruginosa} (16, 36). However, the molecular mechanisms of raft-mediated infection of mammalian cells with pathogens are largely unknown. In this study, we demonstrate that ceramide-enriched platforms play a critical role in a feed-forward loop in amplifying NADPH oxidase-derived redox signaling and cell death. In this feed-forward loop, a primarily weak Asm activation is enhanced by the production of ROS, resulting in higher Asm activity and efficient formation of ceramide-enriched platforms and, thus, also NADPH oxidase activation. This notion is supported by the facts that DPI or catalase (a ROS-degrading enzyme) not only inhibited \textit{P. aeruginosa}-induced Asm activation, but also prevented Asm-mediated ceramide-enriched platform formation. It is of note that NADPH oxidase inhibition did not completely block Asm activity. Furthermore, Asm activation preceded the release of ROS, indicating that the bacteria stimulate Asm via unknown mechanism resulting in ROS release, which then oxidizes Asm to promote further stimulation of the enzyme. Previous studies have shown that ROS activate Asm by dimerization upon oxidation of a cysteine residue to form disulfide bonds (28). Consistently, previous studies showed that Asm activation by CD95 ligand or TRAIL was compromised by antioxidants or ROS scavengers (18, 27). The above notion is further supported by the fact that H$_2$O$_2$, a stable and cell-permeable ROS, activates Asm and induces formation of ceramide-enriched platform in WT macrophages, whereas no platforms formed in \textit{Smpd1}$^{-/-}$ cells. Taken together, Asm, ceramide-enriched membrane platform, NADPH oxidase, and ROS constitute a feed-forward loop resulting in a rapid and efficient amplification of NADPH oxidase activity and ROS production, finally triggering cell death.

In summary, our studies demonstrate that \textit{P. aeruginosa} initiates an Asm-mediated redox signaling network that is strongly amplified via a positive feedback mechanism. Activation of Asm results in formation of ceramide-enriched membrane platforms, in which NADPH oxidase is activated to produce ROS. ROS derived from these membrane platforms further enhance Asm activation and formation of ceramide-enriched platforms and finally induce apoptosis via a JNK-dependent pathway. The present study provides a novel pathway initiated in primary alveolar macrophages by \textit{P. aeruginosa}, and suggests that modulation of this redox signaling by targeting Asm may determine the fate and functions of these macrophages.

**Acknowledgments**

We thank Christoph Böhmer for assistance on fluorescent microplate reader, and Siegfried Moyer for administrative and graphical assistance.

**Disclosures**

The authors have no financial conflict of interest.

**References**


