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Review

Acid Sphingomyelinase-Ceramide System in Bacterial Infections

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Abstract
Acid sphingomyelinase hydrolyzes sphingomyelin to ceramide and phosphorylcholine. Ceramide molecules spontaneously interact with each other and generate ceramide-enriched membrane domains. These ceramide-enriched domains further fuse, forming large ceramide-enriched platforms that participate in the organization of receptors and in the amplification of signaling molecules. Recent studies have suggested several bacteria and bacterial toxins that stimulate the activation and the translocation of acid sphingomyelinase, which leads to the release of ceramide. The acid sphingomyelinase/ceramide system also regulates the internalization of bacteria into the host cell, the subsequent cytokine release, inflammatory response, and initiation of host cell apoptosis. In addition, ceramide has been implicated in the fusion of phagosomes and lysosomes upon bacterial infection. Thus, this system modulates the reorganization of cell membrane receptors and intracellular signaling molecules during bacteria-host interactions. The acid sphingomyelinase and ceramide system may thus serve as a novel therapeutic target for treating infections.

Introduction

Although sphingolipids have long been considered as structural components of cell membranes, recent studies have revealed their crucial functions in the regulation of physiological and pathological processes. In host-bacteria interactions, sphingolipids play an important role in the regulation of the balance between the host and the microbe [1, 2]. Sphingomyelin is the most abundant sphingolipid, comprising 2%-15% or even higher of the total phospholipid in specific tissues [3]. Sphingomyelin can be hydrolyzed

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by sphingomyelinases, which breakdown the phosphodiester bond to generate ceramide. Sphingomyelinases are characterized and identified as acid, neutral and alkaline sphingomyelinases based on the optimal pH of their activity. Acid sphingomyelinase (abbreviated here uniformly as ASM for the human and murine protein), the most studied of these 3 enzymes, is critically involved in many aspects of cell signaling [4]. ASM is characterized for its central role in the re-organization of molecules within the cell upon diverse stimuli and upon the induction of apoptosis, as well as cellular differentiation, proliferation, tumor presentation, cardiovascular disease, and bacterial infections [for review see for instance ref. 4].

Antibiotics have long been preferred and effective treatments for bacterial infections. However, antibiotic resistance has become a severe threat to global public health. The lack of efficacious strategies for treating bacterial infections leads to a worsened clinical outcome, including death, and marked financial costs. Thus, it is important and urgent to identify novel therapeutic targets to fight against bacterial infections. In this review, we will focus on ASM and provide an overview of the regulation and activation of ASM and ASM-generated lipid domains in the process of bacterial infections. We will also discuss the regulation of the host immune system by ASM.

Acid sphingomyelinase

Acid sphingomyelinase plays an important role in sphingolipid metabolism and is responsible for hydrolyzing sphingomyelin to ceramide and phosphorylcholine. The ASM gene is 5-6 kb long, localizes to chromosome 11p15.1–11p15.4 and contains six exons and five introns [5, 6]. Human ASM cDNA encodes a polypeptide of 629 amino acids [7, 8], which shares approximately 82% amino acid identity with murine acid sphingomyelinase [9]. ASM consists of three main domains: the N-terminal saposin domain, the proline-rich connector, and the catalytic domain [10, 11]. ASM deficiency results in the accumulation of sphingomyelin and causes lysosomal storage diseases, i.e., the fatal neuropathic and visceral disease Niemann-Pick type A and the visceral anomalies disease Niemann-Pick type B [6, 12-14].

It was reported that ASM has an optimal pH of 4.5-5.0 for activity [15], however, the ASM also catalyzes the hydrolysis of LDL-sphingomyelin on the plasma membrane at a higher, or almost neutral, pH [16, 17]. The single ASM gene generates two distinct enzymes: a lysosomal form of ASM (L-ASM) and a secretory form of ASM (S-ASM). The generation of two forms of ASM results from alternative modification and trafficking. The mutation of N-glycosylation sites affects the catalytic activities and intracellular processes of L-ASM and S-ASM [18]. Previous studies have shown that the mannose-6-phosphorylation (M6P) receptor system mediates lysosomal trafficking of ASM [19, 20]. Additional studies have been reported indicating that the trans-Golgi network (TGN) transmembrane protein sortilin plays a critical role in L-ASM trafficking along a Golgi-dependent route [19, 21, 22]. The pre-pro-form of ASM with a 75 kDa (65 kDa protein core) molecular weight enters the Golgi, thereby generating the pro-form of ASM with a 72-75 kDa (63-64 kDa protein core) [23]. L-ASM translocates to the lysosome as a 57 kDa (43 kDa protein core) [24] or a 65 kDa (55 kDa protein core) enzyme [24-26]. The activation of S-ASM is dependent on exogenous Zn^{2+}, whereas L-ASM binds to Zn^{2+} ions on its way to lysosomal compartments, resulting in the independence of exogenous Zn^{2+} [17]. Although several groups have studied the ASM, only a few studies have discussed the precise molecular mechanism in the regulation of the lysosomal and secretory forms of ASM.

Various stimulations can regulate the activation of ASM, i.e., reactive oxygen species (ROS), proteases, death receptors, irradiation, phosphorylation, and pathogen infections. For example, hydrogen peroxide induces an activation of ASM in different cells [27-29]. In accordance, inhibition of ROS by several ROS scavengers such as TIRON, N-acetylcysteine (NAC), superoxide dismutase (SOD), catalase or the nicotinamide adenine dinucleotide

phosphate (NADPH)-oxidase inhibitor diphenyleneiodonium chloride (DPI) blocked the activation of ASM induced by different stimulations [29-33]. Genetic silencing of NADPH oxidase subunit $gp91^{phox}$ also inhibited ASM activation [34]. *In vitro* studies indicated that the direct oxidation of ASM at the C-terminal cysteine residue 629 results in activation of enzymes [35]. These findings indicate that ROS is required for the activation of ASM, although it remains unclear whether ROS regulates the enzyme activity directly or indirectly [4, 36].

ASM-generated ceramide formation

Ceramide is commonly considered as the backbone of sphingolipids, which are one of the main components of the plasma membrane. Ceramide can be generated from several pathways, including the hydrolysis of sphingomyelin by sphingomyelinase, *de novo* synthesis, the salvage pathway and the hydrolysis of complex glycosylated lipids [37]. There are more than 28 distinct enzymes regulating ceramide metabolism as a substrate or product [38-44].

Ceramide is composed of a D-erythro-sphingosine backbone and fatty acid-containing acyl chains of different lengths connected via an amide ester bond [45, 46]. It contains a hydroxyl functional group, an amide linkage and an OH group on a sphingosine backbone, which forms hydrogen bonds. The hydrophilic hydrogen bonds and hydrophobic moieties result in the spontaneous separation of ceramides from other phospholipids and the formation of distinct micro lipid domains [47, 48]. Upon activation of ASM, ceramide molecules generated from sphingomyelin spontaneously interact with each other, forming small ceramide-enriched membrane domains. These small ceramide-enriched domains function in signaling transduction and can further fuse to large ceramide-enriched domains, termed platforms [49-51].

The generation of ceramide by ASM alters the physiological properties of the biological membranes. The ceramide-enriched platforms re-organize receptors and signaling molecules upon various stimulations. The formation of ceramide-enriched domains can be visualized by fluorescence staining [47, 48]. Ceramide-enriched platforms occur in cells upon diverse receptor or non-receptor stimuli, including CD95 [52-55], FcγRII [56], DR5 [33], CD40 [57], platelet-activating factor receptor (PAF) [58], viral infection [59-61], *N. gonorrhoeae* [62], *S. aureus* [31], *P. aeruginosa* [29, 63]; pyocyanin [27], cisplatin [64], Cu^{2+} [32], irradiation [65], and UV-light [66, 67]. CD95 and DR5 belong to the family of death receptors and induce programmed cell death, i.e. apoptosis. PAF receptor present in platelets, but also in other cells of the cardiovascular system, such as endothelial cell, that plays an important role in coagulation, endothelial dysfunction and shock. FcγRII. CD40 are receptors that are present in immune cells, mainly B-lymphocytes, and serve as important co-receptors. Pyocyanin is produced by *P. aeruginosa* and functions as a toxin that regulates redox sensitive targets in mammalian cells. Cisplatin is a commonly used chemotherapeutic drug. The interaction of ceramide-enriched platforms with CD95 is one of the best-studied stimulations. CD95 induces the activation and translocation of ASM onto the outer leaflet of the plasma membrane, thus generating ceramide [52, 54]. Ceramide generation and aggregation forms a ceramide-enriched membrane platform, which results in the clustering of CD95 within a confined area of the membrane and amplification of CD95 signaling [52-54]. The disruption of these ceramide-enriched membrane domains abolishes CD95 assembly with downstream signaling molecules [68].

ASM in bacterial infections

Various studies have shown that the ASM/ceramide system plays a critical role in a wide range of cellular processes, such as cell death, proliferation, growth and differentiation [32, 69-71]. Alterations in the ASM/ceramide system are involved in several physiological and pathological processes, such as genetic diseases [72, 73], tumor development [74,

75], neurogenesis, neuronal integrity and behavior [76-79], atherosclerosis [80, 81], and infections with pathogens [31, 59-62]. Particularly, the interaction of bacterial infections and the ASM/ceramide system has emerged as a novel research direction. The studies related to ASM/ceramide and bacteria as well as bacteria-generated toxins are listed in Table 1.

Pseudomonas aeruginosa (*P. aeruginosa*)

P. aeruginosa is the most studied bacterium among all pathogens interacting with ASM. *P. aeruginosa* is a gram-negative bacterium commonly affecting immune-compromised patients and patients with cystic fibrosis, chronic wounds, sepsis, or chronic emphysema [82, 83]. Epidemiology studies have reported the high prevalence of *P. aeruginosa* with morbidity and mortality in chronic lung infections. Patients with cystic fibrosis have a particularly risk for chronic *P. aeruginosa* infections.

The infection of mammalian cells with different strains of *P. aeruginosa* induces the rapid activation of ASM, particularly, the translocation of ASM from intracellular compartments to the extracellular leaflet of the plasma membrane, where the ASM colocalizes with *P. aeruginosa* at the infection site [63]. Other studies have confirmed the activation of ASM and the generation of ceramide triggered by *P. aeruginosa* in various cells as well as mice [29]. *P. aeruginosa* activation results in generation of ceramide-enriched microdomains that spontaneously form ceramide-enriched platforms and that initiate lipid raft reorganization. These ceramide-rich rafts are essential for the internalization of *P. aeruginosa* into mammalian cells, which is prevented by the disruption of these rafts via pharmacological inhibitors of the ASM or by ASM-deficiency. This effect is consistent with the finding that clustering of cystic fibrosis conductance regulator (CFTR) in ceramide-enriched domains correlates with internalization of *P. aeruginosa* into respiratory epithelial cells [63, 84]. Currently, the mechanisms how ceramide-enriched platforms regulate these signaling events are unknown.

ASM and the ceramide system in *P. aeruginosa* infection are also critically involved in cell apoptosis. ASM deficiency results in the failure of the formation of ceramide-enriched membrane platforms, which correlates with a lack of apoptosis *in vitro* and *in vivo* [63]. The exogenous addition of recombinant ceramide is sufficient to restore apoptosis in ASM-deficient epithelial cells. Ceramide is involved in either extrinsic (by stimulation of receptors for proteins from the TNF-α and CD95/Fas families) or intrinsic (mitochondria and ER metabolic stress mediated apoptosis) apoptotic pathways, which have been reviewed in detail [85, 86]. CD95 receptor molecules are concentrated in ceramide-enriched membrane platforms and likely induce cell apoptosis upon *P. aeruginosa* infection [63]. Another potential mechanism underlying ASM-mediated *P. aeruginosa*-induced cell death may be the amplification of redox signaling [29]. In freshly isolated macrophages, ASM is required for the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and for the release of reactive oxygen

Table 1. ASM/ceramide system in bacterial infection

Bacteria/toxin	Mechanism	References
Pseudomonas aeruginosa	IL-1 release, septic death	[63]
	NADPH oxidase activation, ROS production	[29]
	$gp91^{phox}$ clustering, ROS production	[98]
	IL-8 release	[89]
	Pulmonary inflammation, epithelial cell death	[70]
	CD95 clustering, cell death	[173]
	Integrin accumulation, sphingosine depletion	[95]
Staphylococcus aureus	Cytochrome C release, endothelial cell apoptosis	[107]
	Superoxide formation, tight junction degradation	[31]
	CD44 interaction, phagolysosome maturation	[109]
mycobacteria	Granuloma formation	[132]
	Macrophage necrosis	[133]
	Phagosome maturation	[21]
Listeria monocytogenes	Fusion of late phagosomes with lysosomes	[129]
	Cytokine release, reactive nitrogen release	[140]
Neisseria gonorrhoeae	CEACAM receptor-mediated phagocytosis	[144]
	Activation of PC-PLC	[62]
Neisseria meningitidis	Internalization of bacteria	[145]
Escherichia coli	Dendritic cells apoptosis	[147]
	Cytokine release	[174]
Salmonella enterica	ROS generation	[154]
Propionibacterium acnes	Hijacking host ASM	[158]
LPS	Dendritic cell apoptosis	[147]
	TNF-α and MIP-2 release	[162]
	Pulmonary inflammation	[154]
	Endothelial cell apoptosis	[165]
Pyocyanin	Neutrophil cell death, ROS release	[27]
α-toxin	α-toxin binding, host cell necrosis	[71]
	Tight junction degradation	[117]
	Cathepsin release, inflammation	[121]

species (ROS), thereby activating c-Jun N-terminal kinase (JNK) triggered apoptosis. Two subunits of NADPH oxidase $p47^{phox}$ and $gp91^{phox}$ are required for ASM-regulated apoptosis in mammalian cells [29, 87].

In addition, ceramide is accumulated in the lungs of CFTR-deficient mice prior to any infection and is normalized by the heterozygosity of ASM. Increased ceramide concentrations mediate clustering of CD95 in the plasma membrane and apoptosis of lung epithelial cells [52, 55, 88].

Deficiency or dysfunction of ASM plays a role in the release of cytokines in response to *P. aeruginosa* challenge. *P. aeruginosa* infection leads to an uncontrolled release of IL-1β from infected cells or from the lungs of ASM-deficient cells [63], whereas the addition of exogenous ceramide is sufficient to rescue the phenotype of ASM-deficient epithelial cells. Blocking ASM with multiple approaches, i.e., silencing, pharmacological inhibitors, or specific antibodies, significantly increased IL-8 release upon *P. aeruginosa* infection in epithelial cells [89]. On the other hand, apoptosis mediated through the ASM/ceramide system may physically limit the control of cytokine release [63]. This system might also influence cytokine release through regulating the synthesis or through interfering with gene transcription or protein expression of cytokines.

The ASM/ceramide system is essential for host defenses against *P. aeruginosa* infection. Mice with genetic ASM deficiency fail to clear *P. aeruginosa* pulmonary infections, and 90% of these animals died in 7 days [63]. Death might be caused by an over-activation of the immune system, since intravenous injection of neutralizing antibody against IL-1β successfully rescued ASM-deficient mice from lethal pneumonia caused by *P. aeruginosa*. Consistently, the decrease or absence of endogenous IL-1 activity suppresses pulmonary inflammatory responses, thereby improving the host defense against *P. aeruginosa* infection in the lungs [90]. The regulation of ROS production by the ASM/ceramide system is another mechanism involving bacteria killing [29]. NADPH oxidase is required for host defenses against invading pathogens because ROS are toxic to most bacteria. The deficiency of ASM and the absence of ceramide-enriched platforms abolish ROS production via NADPH oxidase in freshly isolated alveolar macrophages upon *P. aeruginosa* infection. Previous studies have indicated that JNK functions between NADPH oxidase-derived ROS production and apoptotic cell death in *P. aeruginosa* infection in phagocytes, which is consistent with recent studies [91]. ROS production is importantly involved in the redox regulation of host responses against *P. aeruginosa*, but this pathogen can actively block the ROS burst via the PI3K pathway using the two type III secreted effector proteins, ExoS and ExoT [92]. Particularly, ExoS interferes with the signaling cascade that mediates NADPH oxidase assembly by ADP-ribosylating Ras. Most of the events described in the internalization and elimination of intracellular bacteria require expression of the type III secretion system in *P. aeruginosa* [93, 94]. Notably, it is unknown whether ceramide-enriched membrane platforms play a role in the transfer of bacterial proteins and delivery of bacterial cells into mammalian host cells via the type III secretion system.

The cleavage of the fatty acid moiety from ceramide by ceramidase produces sphingosine, a bioactive lipid that plays a prominent role in the pulmonary defense against *P. aeruginosa*. In both humans and mice with cystic fibrosis, an increase in ceramide and in the formation of ceramide platforms leads to an ectopic trapping and clustering of β1-integrins on the luminal pole of bronchial epithelial cells [95]. β1-integrin impairs the acid ceramidase (aCDase) activity and expression, consequently resulting in the accumulation of ceramide and the decrease in surface sphingosine. Reducing ASM activity with ASM pharmacological inhibitors amitriptyline or fluoxetine normalizes ceramide and sphingosine levels and β1-integrin expression and prevents *P. aeruginosa* infection in CF mice. Sphingosine is abundantly expressed on the luminal surface of human nasal epithelial cells in healthy individuals and in the trachea of mice, but is almost undetectable in CF patients and in Cftr-deficient mice. In contrast, ceramide levels are elevated in CF mice. Inhalation of sphingosine eliminates existing *P. aeruginosa* infections and clears *P. aeruginosa* or *S. aureus* infections in CF mice [96, 97], whereas the pharmacologic or genetic normalization of ceramide prevents *P. aeruginosa*

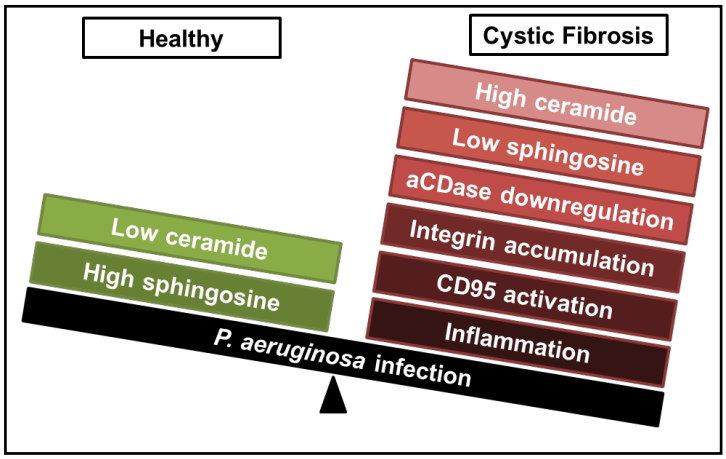

Fig. 1. Disruption of ceramide-sphingosine balance in cystic fibrosis. In healthy cells and in individuals, a low ceramide and high sphingosine level maintains a balance. In contrast, abnormally high ceramide and low sphingosine levels activate several signaling pathways, such as integrin accumulation, CD95 activation, and proinflammatory and anti-inflammatory cytokines, in the airways of cystic fibrosis patients or cells.

infection in CF mice [30, 70, 98-100]. In healthy cells, the ratio of ceramide to sphingosine is relatively stable and balanced. However, if this balance is disrupted as in CF, cells are highly susceptible to bacterial infections. Ceramide elevation induces acid ceramidase dysfunction and reduces sphingosine, integrin accumulation, and CD95 activation and eventually reduces uncontrolled inflammation (Fig. 1). This balance has been further demonstrated in *S. aureus*; the ASM/ceramide/acid ceramidase/sphingosine system in CF lungs and the correction of ceramide and sphingosine levels in bronchial epithelial cells prevent pulmonary infection [101].

Pyocyanin is a redox-active compound produced by *P. aeruginosa*, which easily accepts and donates electrons and crosses biological membranes, acting as a mobile electron carrier for *P. aeruginosa* [102]. The pyocyanin-induced rapid death of neutrophils depends on ASM, a novel mechanism for the activation of mitochondrial ASM in the generation of ROS in mitochondria [27]. Whether pyocyanin induces a change in mitochondrial membrane lipids has not yet been determined.

Currently, the function and regulation of mitochondrial ASM has not been extensively studied, although studies clearly showed mitochondrial expression of the enzyme, its association with pro-caspase 3-induced apoptosis [103] or the glutamate-induced regulation of necrosis [104]. In addition, ASM regulates the lipid composition of membranes.

Staphylococcus aureus (*S. aureus*)

S. aureus is a commensal opportunistic bacterium that colonizes approximately 30% of human populations. This bacterium frequently causes diseases from mild skin and soft tissue infections to life-threatening diseases, such as pneumonia, endocarditis, sepsis, and toxic shock syndrome [105]. The methicillin-resistant *S. aureus* (MRSA) has become a major pathogen and critical problem in both community and hospitals worldwide, particularly resulting from the lack of effective therapeutic approaches to control multiple antibiotic-resistant *S. aureus* infection [106].

In 2001, a study showed that *S. aureus* infection triggers ASM activation and ceramide production in human endothelial cells [107]. Genetic deficiency of ASM significantly inhibits the death of human fibroblasts triggered by *S. aureus*, which is mediated by stimulation of the JNK signaling pathway as well as alterations in mitochondrial function. The functional inhibition of JNK by Tam67 gene transfection prevents *S. aureus*-induced cell apoptosis. These results are consistent with the finding that ASM and ceramide-enriched platforms mediate macrophage apoptosis via the stimulation of JNK upon *P. aeruginosa* infection [29]. The endothelial cell death mediated by the ASM/ceramide system may cause the degradation of tight junction proteins and the breakdown of the endothelial cell barrier, enabling the

bacterium to infect other tissues or organs. In cystic fibrosis airways, the accumulation of ceramide results in the membrane recruitment of inflammasome proteins and the activation of caspase 1 as well as the phosphorylation of JNK [108].

A recent study showed that genetic deficiency or pharmacological inhibition of ASM protects mice against pneumonia and lethal *S. aureus* sepsis [31]. ASM is activated by *S. aureus* in endothelial cells; subsequently, ceramide-enriched platforms are generated. ASM activation triggers the release of superoxide, whereas ASM activation is inhibited by antioxidants. The ASM/ceramide system and ROS act as a positive feedback loop mechanism upon *S. aureus* infection, which is similar to the findings of previous studies [29]. Another mechanism in *S. aureus* activated ASM involves CD44, a glycoprotein that interacts with Ezrin/Radixin/Moesin (ERM) and links the actin cytoskeleton to the plasma membrane and extracellular matrix [109]. Macrophages are activated via CD44 upon infection with *S. aureus*, thereby stimulating the activation of ASM and the release of ceramide. In addition, ASM/ceramide-triggered superoxide production induces the degradation of tight junction proteins ZO1, ZO2, occludin and E-cadherin upon *S. aureus* infection *in vitro* or *in vivo*, an effect that was ameliorated by the inhibition of ASM via amitriptyline or antioxidants. Several studies have demonstrated that superoxide is responsible for the degradation of tight junctions via proteolytic matrix metalloproteinases (MMP) [110-112]. Moreover, ASM was shown to positively regulate the mRNA transcription and protein expression of MMP [113-115].

S. aureus is the primary cause of sepsis and lethal lung edema, even with the clinical administration of antibiotics. Mice treated with the ASM inhibitor amitriptyline or lacking ASM expression show reduced lung edema, because degradation of tight junctions was decreased and thereby myeloid cell trafficking was inhibited [31]. However, on the other hand the bactericidal capacity is also reduced since ASM dysfunction leads to the failure of clustering and activation of NADPH oxidase, resulting in the susceptibility and high mortality of mice to *S. aureus* infection. ASM inhibition was achieved using functional inhibitors, such as imipramine, desipramine and amitriptyline, which displace the ASM from the lysosomal membrane resulting in degradation of the enzyme and, thus, a functional inhibition [4, 79]. Functional ASM inhibitors also improve endothelial stresses response during sepsis [116]. The activity and expression of plasma ASM increased depending on the severity of sepsis in patients. Freshly isolated serum from patients with sepsis instantaneously induces the breakdown of sphingomyelin and the elevation of ceramide in endothelial cells, an effect that is abrogated by desipramine. The inhibition of ASM by pre-incubation with desipramine or NB6 blocks the clustering of receptor complexes, such as the CD95L/Fas-receptor, as well as the formation of ceramide-enriched microdomains.

The precise mechanism of ASM activation upon *S. aureus* infection is far from understood; extended studies have demonstrated that *Staphylococcal* alpha-toxin (α-toxin) is one of the factors mediating the activation of ASM and leading to detrimental effects of the pathogen on endothelial cells [117]. The α-toxins, a class of β-barrel pore-forming cytotoxins, are major host injurious toxins secreted by *S. aureus,* and these molecules function by forming pores in cell membranes, damaging membrane permeability, and eventually triggering cell death [118-120]. ASM is rapidly activated by the wild-type *S. aureus* strain as well as by purified α-toxins, whereas the α-toxin-deficient JE2-Δhla mutant strain has no effect on ASM activity. Pre-incubation with the ADAM10 inhibitor GI254023X or β-cyclodextrin, which blocks toxins by binding to toxin heptamers, markedly decreases ASM activation upon cellular stimulation with α-toxin. *S. aureus* α-toxin induces the degradation of tight junctions in endothelial cells, which is abrogated by the inhibition of ASM or ADAM10. Furthermore, while infection with *S. aureus* JE2 results in severe pneumonia in CF mice, the deletion of α-toxin by β-cyclodextrin reduces the pathogenicity of *S. aureus*. Also, the inhibition of ceramide generation by the application of amitriptyline is beneficial to prevent infection [101]. These studies reveal a central role for α-toxin and ASM in *S. aureus* infection. Another study from the same group demonstrates a novel signaling pathway, in which *α*-toxin activates ASM and triggers the formation of ceramide in lysosomes of macrophages [121]. Activation of the ASM/ceramide

system in macrophages induces the release of cathepsin B from lysosomes into the cytoplasm, associated with Nlrc4 and Asc, as well as eventual activation of the inflammasome and release of IL-1β. These studies connect the lysosomal ASM/ceramide system with the regulation of inflammation, which is central for the control of infections and the immune system.

Further studies reveal, that the ASM/ceramide system protects against staphylococcal α-toxin-induced keratinocyte death [71, 122].

S. aureus is one of the major causes for the pathogenesis of sepsis, and although a series of appropriate antibiotics are used to clear the bacteria burden, many patients still die from fatal lung edema [123-125]. A combination of antibiotics with genetic ASM deficiency or pharmacological inhibition of the ASM successfully rescues mice from the lethality of *S. aureus* infection. This combination is sufficient to clear the bacteria and to prevent tight junction protein degradation on endothelial cell layers, as well as it prevents an uncontrolled over-activation of inflammation, which is harmful to the host immune system. A potential mechanism of the ASM/ceramide system in combination with antibiotics against *S. aureus* infection is shown in Fig. 2. Amitriptyline, a drug routinely used to treat major depression, in combination with the appropriate antibiotics, might be a novel therapeutic target to treat systemic *S. aureus* and most notably MRSA infections.

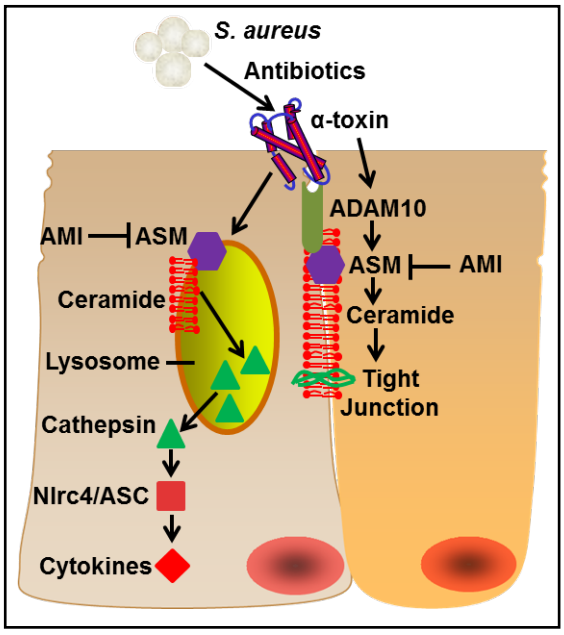

Fig. 2. Interaction of *S. aureus* with the ASM/ceramide system. 1. Antibiotics are often insufficient to cure severe *S. aureus*, respectively MRSA infection. A-toxin induces the activation of ASM and the release of ceramide via ADAM10, which is linked to the degradation of tight junctions (right cell). However, the α-toxin-activated ASM/ceramide system also mediates the trafficking of cathepsins from lysosomes to the cytoplasm, followed by the formation of the Nlrc4/ASC complex and the production of inflammatory cytokines. The functional ASM inhibitor amitriptyline (AMI) can prevent tight junction degradation and cytokine release. A combination of ASM inhibitors with antibiotics provides a novel approach to treat *S. aureus* and MRSA infection.

Pathogenic mycobacteria

Pathogenic mycobacteria, including *Mycobacterium marinum* (*M. marinum*), *Mycobacterium tuberculosis* (*M. tuberculosis*), and *Mycobacterium avium* (*M. avium*), often cause several diseases in humans, such as skin lesion, respiratory illness, fever, and tuberculosis [126, 127]. Among these diseases, tuberculosis is a main health problem, having caused 1-5 million deaths in 2014 [128]. The host fights against pathogenic mycobacteria involve both innate and adaptive immune systems, which often wall off the pathogen enclosed in granuloma. Pathogenic mycobacteria survive and replicate in host immune cells, particularly macrophages. These pathogens prevent phagosome-lysosome fusion and evade killing, thereby persisting in the host cells.

ASM interact with the proneurotrophin receptor sortilin, which is required for the infection of *M. tuberculosis* in macrophages [21]. Sortilin mediates ASM trafficking from the Golgi complex into mycobacteria-containing phagosomes. Once delivered to the

phagosome, ASM localizes with lysosomal-associated membrane protein (LAMP) 2 for the growth restriction and elimination of *M. tuberculosis* in bone marrow-derived macrophages. Moreover, depleting ASM with the pharmacological inhibitor desipramine increases the survival of *M. tuberculosis* [21]. Furthermore, ASM hydrolyzes sphingomyelin to ceramide, which is involved in the regulation of phagosome maturation, the fusion of phagosomes and lysosomes and mycobacterial killing [129-131]..

ASM deficiency protects mice against *M. avium* infection through a cell-cell fusion mechanism [132]. Interestingly, this effect is controversial with many studies showing that ASM deficiency confers susceptibility to bacterial infection *in vitro* and *in vivo*. In a recent study [132], after intravenous infection with *M. avium*, ASM-deficient mice survived 120 days, while the wild-type mice died between 70 and 80 days. The histology results revealed that, in ASM-deficient mice *M. avium* was found within minor and restricted granulomas. In contrast, wild-type mice have large granulomas with massive mycobacteria. Multinucleated giant cells containing an overload of bacteria were formed in wild-type, but not in ASM-deficient mice. ASM modulates the formation of giant cells, which provide an environment for *M. avium* survival and replication. The authors indicate a mechanism in which extracellular surface ASM changes the biophysical properties of the plasma membrane, therefore affecting the fusogenicity of macrophages with granulomas.

The ASM/ceramide system contributes to macrophage necrosis upon *M. marinum* infection [133]. Tumor necrosis factor (TNF) excessively triggers ROS generation-induced cell necrosis in macrophages infected with *M. marinum,* while this necrosis is prevented by the knockdown of ASM or by the long-term clinical use of desipramine, which inactivates ASM. The blocking of necrosis resulting from ceramide reduction is reproduced by the overexpression of acid ceramidase. However, the precise mechanism how the ASM/ceramide system mediates macrophage necrosis is poorly known. The combination of gene knockdown of ASM and Cyclophilin D (CYPD), a mitochondrial matrix protein and component of the permeability transition pore, synergistically prevents the cell death of macrophages, prolongs bacterial clearance, significantly reduces bacterial burden, and consequently reverses susceptibility to *M. marinum* infection [133, 134]. In another scenario, the infection of macrophages with *M. tuberculosis* induces cell necrosis via lysosomal membrane permeabilization and via the release of lysosomal sphingomyelinase [135]. After infection, the release of hydrolases results in a 10-fold decrease in the sphingomyelin concentration. Thus, these findings suggest that the ASM/ceramide system mediates the bioprocess of mycobacterial infection and the survival of host cells.

***Listeria monocytogenes* (*L. monocytogenes*)**

L. monocytogenes-induced listeriosis is a severe disease, particularly for specific populations, such as the elderly, newborns, pregnant women, and immunocompromised patients. Innate immunity is also responsible for bactericidal activity against *L. monocytogenes*. Several studies have reported macrophage phagocytosis of *L. monocytogenes* and neutrophil infiltration of tissues and organs, which contribute to the clearance of the pathogen [136, 137]. However, after internalization into host cells, the pore-forming toxin listeriolysin O secreted from *L. monocytogenes* mediates lysis of the phagosomal membrane and bacterial escape into the cytoplasm, thereby causing replication in the cell [138, 139].

ASM-deficiency highly impairs the bactericidal capacity of mice challenged with *L. monocytogenes* due to the failure of macrophage intracellular bacteria killing [140]. The LD50 of ASM-deficient mice is lower than 10^2 colony forming units (CFU), while the LD50 of wild-type is approximately 10^4 CFU upon i.p. infection with *L. monocytogenes*. ASM-deficiency induces a high bacterial load and a massive necrotic decay of the liver. The uptake of *L. monocytogenes* by macrophages is independent of ASM; however, ASM-deficient macrophages are unable to clear the bacteria or to restrict the replication of bacteria at later time points due to a lack of efficient phago-lysosomal fusion [129]. Instead, *L. monocytogenes*

rapidly escape from the phagosome into the cytosol in ASM-deficient macrophages. ASM-deficiency reduces co-localization of intracellular *L. monocytogenes* with the late endosome/lysosome marker Lamp1 and the listericidal proteases cathepsin D, B and L [129], while in wild-type cells ASM-generated ceramide targets and enhances the activation of the lysosomal cathepsin D [141]. Interestingly ASM does not interfere with the production of reactive oxygen intermediates upon *L. monocytogenes* infection, indicating that the oxidative listericidal pathway is not impaired in ASM-deficiency [129, 140].

Pathogenic *Neisseria*

Pathogenic *Neisseria,* including *Neisseria meningitidis* (*N. meningitidis*) and *Neisseria gonorrhoeae* (*N. gonorrhoeae*), are gram-negative pathogens often recognized as commensal bacteria on human mucosal surfaces. Previous studies have demonstrated that *Neisseria* species employs multiple strategies to interact with various host cell receptors during the bioprocess of infection. A phase-variable outer membrane protein called opacity-associated (Opa) proteins, encoded by 4 genes in *N. meningitidis* and 11 genes in *N. gonorrhoeae*, facilitates their survival in hosts. Opa proteins interact with cellular receptors to generate a tight connection between bacteria and host cells, thus mediating bacterial invasion [142, 143].

In 1997, a study showed that ASM mediates the invasion of *N. gonorrhoeae* in non-phagocytic cells [62]. ASM is activated by *N. gonorrhoeae* in both epithelial cells and fibroblasts. Pharmacological inhibition of ASM by imipramine prevented the invasion of *N. gonorrhoeae*. Moreover, ASM-deficient Niemann–Pick disease type A (NPDA) cells showed reduced internalization, which is restored by the reconstitution of the enzyme.

Additional studies from the same group by using human phagocytic cells suggest that the ASM/ceramide system is critical for the invasion of *N. gonorrhoeae* via carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) receptors [144]. Only the infection of *N. gonorrhoeae*-expressing virulent Opa proteins 35 and 55 leads to the activation of ASM. Pharmacological inhibition results in the reduction of bacterial internalization, whereas reconstitution of C_{16}-ceramide completely restores bacterial internalization. The ASM inhibitor imipramine abolished the pronounced induction of JNK activity and Src-like tyrosine kinases during infection.

An additional study indicated that the ASM/ceramide system determines the internalization of Opc-expressing *N. meningitidis* into endothelial cells [145]. The integral outer membrane protein Opc is expressed by various virulent *N. meningitidis* lineages and mediates the adhesion and invasion of a wide range of host cells. Infection of *N. meningitidis* rapidly triggers activation of ASM and induces the formation of extracellular ceramide-enriched platforms to which the bacteria adhere. The regulation of ASM upon *N. meningitidis* infection [145] is induced by the PC-PLC-mediated release of DAG, which is also observed in the related species *N. gonorrhoeae* [62]. ASM pharmacological inhibition, gene knockdown, or gene deficiency reduces the invasion of bacteria but does not affect the adhesion of human endothelial cells. Furthermore, the infection of different strains of *N. meningitidis* with or without Opc gene expression into endothelial cells demonstrates that Opc protein enhances the bacterial invasion driven by the ASM/ceramide system. The formation of ceramide-enriched platforms upon *N. meningitidis* exposure mediates the recruiting and clustering of tyrosine kinase ErbB2 to the bacterial sites on host cells.

Taken together, these findings suggest that ASM and ceramide play a central role in the regulation of host receptor interactions with Opa or Opc proteins in the infection of human cells with pathogenic *Neisseria*.

Escherichia coli (*E. coli*)

Although most *E. coli* are harmless and are important members of the healthy human intestine, pathogenic strains consists of diverse subtypes, including diarrheagenic *E. coli* and extraintestinal *E. coli*, which can cause diseases, including diarrhea, urinary tract infections, meningitis, pneumonia, and septicemia. *E. coli* disrupts host biophysical processes with bacteria effectors and toxins in various strategies, such as modifying host cell apoptosis, altering the actin cytoskeleton, regulating autophagy process, and targeting the multiple kinase signal transduction [146].

The ASM/ceramide system is necessary for cellular apoptosis in dendritic cells (DCs) during the course of *E. coli* infection [147]. The infection of human monocyte-derived immature DCs with a high numbers of *E. coli* results in cell apoptosis, which is inhibited by pharmacological inhibitor, either imipramine or D609. Importantly, the exogenous reconstitution of ceramide reverses these inhibitory effects. Compared with mature DCs, immature DCs express a significantly higher level of ASM, resulting in cell death in response to infection. Nitric oxide (NO) is a free radical and is one of the most versatile factors mediating various bioprocesses in the immune response [148], including the targeting of ASM [149]. ASM activation and immature DCs apoptosis induced by *E. coli* is inhibited by DETA-NO, constantly releasing NO. This mechanism of apoptosis inhibition by NO involves the activation of guanylate cyclase, the formation of cyclic guanosine monophosphate (cGMP), and the activation of G kinase. Taken together, the activation of ASM and the generation of ceramide as well as their exposure to NO depends on cGMP formation during the *E. coli* infection process.

Notably, recent studies have shown that the activation of ASM by platelet-activating factor-receptor (PAF-R) generates the formation of a ceramide-enriched-platform inside of a signalosome complex, including eNOS, producing NO in endothelial cells. Moreover, mechanically, the platform mediates the phosphorylation/dephosphorylation of the serine, threonine, and tyrosine residues of eNOS [150]. Further studies indicate that the inhibition of ASM decreases NO production in a NF-κB-regulated manner and that the exogenous addition of ceramide induces the biogenesis of inducible NO synthase (iNOS) and apoptosis [151].

Salmonella enterica serovar Typhimurium

S. enterica is a rod-shaped gram-negative bacterium causing a series of infectious illnesses from localized gastroenteritis to systemic severe typhoid fever, which is a global health problem [152]. *S. enterica* serovar Typhimurium (*S. typhimurium*) is one of the most-studied types that specifically infects humans, although the mechanism of its selectivity remains unknown [153]. As a facultative intracellular pathogen, *S. typhimurium* promotes its invasion into host cells residing in a distinct membrane bound compartment, the *Salmonella*-containing vacuole (SCV). Additionally, effector proteins of this bacterium can interfere with immune cell functions, can block the activation of the immune response and can evade the bactericidal effect.

Studies have shown that the genetic deficiency of ASM dramatically enhances the susceptibility of mice to the facultative intracellular bacterium *S. typhimurium* but not to the extracellular bacterium *S. aureus*, suggesting that ASM is involved in host defenses against intracellular pathogens [140]. Subsequent studies have also shown a key role for ASM in the killing of *S. typhimurium* in macrophages [154]. The reduction of bacteria killing in macrophages is dependent on ASM. This study also showed that the activation of ASM in *S. typhimurium* elimination is linked to NADPH-mediated ROS release, consistent with the studies of ASM regulating host defense against *Pseudomonas* [29]. The supernatant of infected macrophage contained a significantly increased ASM enzymatic activity compared with the uninfected group, which indicates that *S. typhimurium* infection leads to the redistribution of ASM from the cytosol to the cell membrane or to the extracellular environment. This

trafficking of ASM does not only exist in *S. typhimurium*, as *E. coli* induces a similar effect. According to the extracellular translocation of ASM in response to different bacteria species, the trafficking of ASM may be required for bactericidal activity against various types of pathogenic bacteria.

Propionibacterium acnes (*P. acnes*)

P. acnes, an opportunistic pathogen, is critically associated with the pathogenesis of acne vulgaris, which is the most prevalent skin disease, persisting by up to 85% among individuals 12-24 years old [155, 156]. The *P. acnes* genome encodes the Christie-Atkins-Munch-Petersen (CAMP) factor, which binds to immunoglobulins G and M and acts as a pore-forming toxin. In addition, *P. acnes* induces the release of inflammatory cytokines involved in the activation of Toll-like receptor 4 (TLR-4), thereby manipulating the host immune response. The invasive and chronic implant infections are closely related to the biofilm formation of *P. acnes* [157].

ASM is involved in *P. acnes* virulence-induced inflammation [158]. The co-culture of *P. acnes* with HaCaT keratinocytes and RAW264.7 macrophages stimulates the secretion of ASM into the culture supernatant. *P. acnes* induces cell death in host cells, which is blocked by addition of the ASM inhibitor desipramine *in vitro*. Intradermal injection of ICR mice with bacteria significantly increases ASM expression. Host ASM is responsible for recruiting of $CD11b^+$ macrophages. Importantly, the combination of CAMP factor vaccination with anti-ASM IgG injection alleviates bacteria-induced inflammation, indicating a cross talk between CAMP factor and ASM. The results indicate a mechanism that *P. acnes* resists against phagocytosis by taking advantage of the host L-ASM. Additionally, desipramine or other ASM inhibitors may be potential therapeutic compounds for treating the cytotoxicity of *P. acnes* infection.

Lipopolysaccharide

Many pathogenic bacteria initiate infection and mediate their toxicity to hosts by producing virulence factors called toxins. Lipopolysaccharide (LPS), a main component of the gram-negative bacteria cell membrane, acts as a most efficacious microbial intermediator, which is responsible for the pathogenesis of sepsis and septic shock [159, 160]. LPS provokes intense proinflammatory and microbicidal activation of host cells, including macrophages, followed by a release of cytokines, such as TNF-α, interleukins, and NO. The sudden release of a significant amount of LPS into the blood stream is detrimental, often causing endothelial injury, tissue hypoperfusion, and refractory shock. Several receptors of LPS have been identified: CD14-MD2-TLR4 molecules, β2-integrins, scavenger receptors and serum LPS-binding protein [161]. Studies have shown that cellular exposure to LPS induces activation of ASM and a release of ceramide in dendritic cells [147], macrophages [162], monocytes [163], lung tissues [164], intestinal mucosa [165] and serum [166].

LPS activates ASM not only within the cells but also stimulates the extracellular secretion into the blood and intestines *in vivo* [165, 166]. Mechanically, the activation of the ASM/ceramide system by LPS is inhibited by immune-modulating messenger NO through the formation of cGMP and through the activation of the cGMP-dependent protein kinase, therefore inhibiting dendritic cell death [147]. Inhibiting the NF-κB pathway by a cell-penetrating peptide sufficiently suppresses ASM activation. The ceramide-mediated production of TNF-α, IL-6, CXC chemokine CXCL8, and MCP-1, as key regulators of inflammation, is also reduced upon NF-κB inhibition [164]. Studies have also shown that the effect of LPS on ASM activation involves the production of IL-1β and TNF-α [165, 166]. Vice versa, ASM activation by LPS is required for the release of TNF-α [163]. Exposure to LPS leads to the generation of ceramide-enriched microdomain assembly and the activation of

the TLR4 receptor, mediating the phosphorylation of atypical PKC-ζ and the activation of the MAPK family, composed of ERK 1/2, p38, and JNK/SAPK.

In conclusion, these findings suggest that the ASM/ceramide system modulates the inflammatory response and the death of host cells upon LPS challenge.

Summary

Although multiple studies implicate the ASM/ceramide system in bacteria-host interactions, mechanisms regarding the roles and regulations of ASM in cellular processes still require further definition. Whether bacteria directly regulate ASM activity, trafficking, and localization or indirectly regulate other cellular pathways remains to be characterized. The role of lysosomal ASM in bacterial killing or immune evasion as well as the mechanisms of secretion of ASM on the plasma membrane during bacterial internalization requires further characterization. The subsequent biological consequences of ASM-generated ceramide in different parts of the cell also remain unclear at present.

Amitriptyline, a tricyclic antidepressant (TCA), is a drug used for the treatment of a number of mental diseases. Importantly, amitriptyline is recognized as a functional ASM inhibitor [167]. The application of amitriptyline to cystic fibrosis mice normalizes pulmonary ceramide levels and abolishes pathological outcome, including susceptibility to infection [70]. Moreover, the inhibition of ASM by amitriptyline and other tricyclic antidepressants prevents *P. aeruginosa* infection and pulmonary inflammation in mice and in patients with cystic fibrosis [168-170]. Importantly, clinical trials indicate that treatment of cystic fibrosis patients with amitriptyline results in a decrease in ceramide levels in lung cells and an increase in lung function [171, 172]. With regard to ASM inhibitors treating bacterial infections, the dose and administration in experiments and clinical trials are shown in Table 2. Amitriptyline may be a novel, safe and effective medicine to treat CF patients.

ASM is critical in the regulation of host interactions with bacteria, including *P. aeruginosa*, *S. aureus*, mycobacteria, *L monocytogenes*, *Neisseria* spec., *E. coli*, *S. enterica*, *P. acnes* and bacterial toxins or LPS. Fig. 3 shows the potential role of the ASM/ceramide system in bacterial infections. The infection of mammalian cells with bacteria triggers the activation of ASM and the secretion of ASM onto membranes as well as the extracellular

Table 2. ASM inhibitors in treating infection or infection-related diseases in human or mice

ASM inhibitors	Usage	Species	References
Amitryptyline	4 mg/L in saline, inhalation	Mice	[168]
	10 mg/kg, intraperitoneal injection	Mice	[87, 95]
	120 mg/L in drinking water	Mice	[79, 95]
	25, 50, 75 mg/d, oral	Human	[171, 172]
Fluoxetine	4.5 mg/L in saline, inhalation	Mice	[168]
	120 mg/L in drinking water	Mice	[95]
Trimipramine	12.5 mg/L in saline, inhalation	Mice	[168]
Sertraline	35 mg/L in saline, inhalation	Mice	[168]
Desipramine	9 mg/L in saline, inhalation	Mice	[168]
Chlorprothixene	8 mg/L in saline, inhalation	Mice	[168]

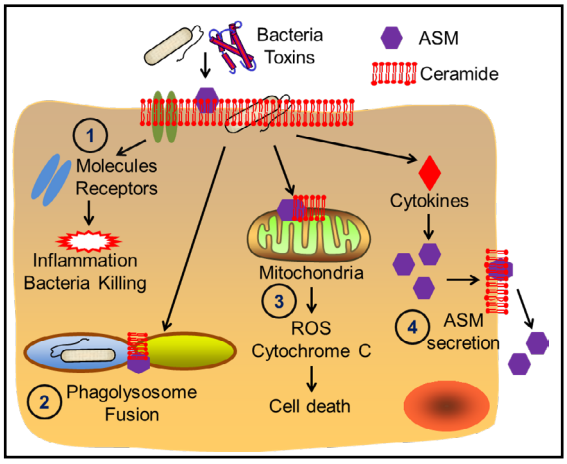

Fig. 3. ASM/ceramide system in bacteria-host interactions. 1. Bacteria induce activation of ASM and release of ceramide, which recruits signaling molecules and receptors, such as NADPH oxidase, ErbB2, JNK, CFTR, CD95, JNK, and p38 kinase, therefore modulating ROS generation, cytokine release, host cell death, and bacterial killing. 2. ASM-generated ceramide recruits molecules and receptors and mediates bacterial internalization. In addition, the ASM/ceramide system is needed for the fusion of phagolysosomes. 3. The ASM/ceramide system is involved in mitochondria-induced cell death upon infection. 4. Inflammatory cytokines stimulate the secretion of ASM from the cytoplasm to the extracellular environment.

environment. ASM-generated ceramide initiates lipid domain organization, thereby mediating interactions of bacteria with host cells. ASM facilitates the activation of NADPH oxidases, which involves the generation of superoxide, responsible for bacteria killing and regulating cell apoptosis. Ceramide platforms also mediate the internalization of bacteria into host cells. Moreover, ASM-generated ceramide modifies the membrane biophysical properties and recruits receptor molecules, thereby modulating the fusion of phagosomes and lysosomes. In addition, ASM influences cytokine release and inflammatory responses. Taken together, although the detailed mechanisms of the ASM/ceramide system acting on bacterial infection remain unknown, strong evidence shows the central role of this system in bacteria-host interactions. Targeting the ASM/ceramide system might be a novel and potential therapeutic approach for treating bacterial infection.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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