Staphylococcus aureus α-toxin induces inflammatory cytokines via lysosomal acid sphingomyelinase and ceramides

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1. Introduction

1.1 *staphylococcus aureus* (*s.aureus*)

1.1.1 Components and Products of *S. aurues*

In an elegant series of clinical observations and laboratory studies published in 1880 and 1882, Ogston described *staphylococcal* disease and its role in sepsis and abscess formation (Ogston A 1882a; Ogston A 1882b; Ogston A 1984). More than 100 years later, *Staphylococcus aureus* remains a versatile and dangerous pathogen in humans, because of its bility to adapt to adverse environmental conditions，its ability to cause a diverse array of diseases ranging from mild skin and wound infections to fatal sepsis or multiple organ failure (Yang et al., 2017; Hudson A.J et al, 2017; H. Patel et al., 2012; Xiaopeng Zhang et al., 2017). Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Despite much research and development there is no approved vaccine for *S. aureus* (Hudson AJ et al., 2017).

About 30% of the populations are colonized with this bacterium. *S. aureus* is among the leading causes of superficial lesions such as skin inflammations and ulcer infections such as boils, styes and furunculosis; more serious infections is deep-seated and systemic infections such as osteomyelitis, endocarditis, pneumonia, and bacteremia; and toxemic syndromes such as toxic shock syndrome (TSS) and *staphylococcal* scarlet fever (both due to toxic shock syndrome toxin-1 (TSST-1) and *staphylococcal* enterotoxins (SEs), *staphylococcal* scalded-skin syndrome (SSSS; due to exfoliations), and *staphylococcal* food poisoning (Tong SY et al.,2015; Justyna Bien et al., 2011; N Alizadeh et al., 2017) (Fig 1.1.1A). *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices (Marimuthu et al., 2014; Trine K et al, 2015).

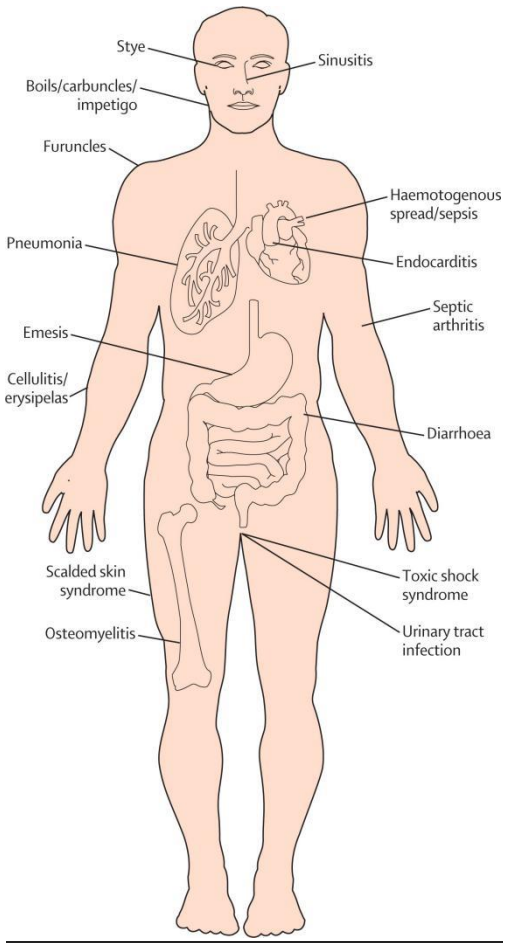


Figure 1.1.1 A Sites of infection and diseases caused by *Staphylococcus aureus*

The *staphylococcus aureus* constituted of capsule (slime layer), cell wall, membrane and cytoplasm (Figure 1.1.1B). Capsule production by *S. aureus* was first described in 1931 by Gilbert (Gilbert, 1931). At least 18 strains of capsule of *S. aureus* have been described and partially characterized (Wergeland, 1989). Most clinical isolates of *S. aureus* produces type 5 and type 8, which respectively accounts for 25% and 50% of human infections. Purified serotype 5 and 8 capsular polysaccharides offer promise as target antigens for a vaccine to prevent *staphylococcal* infections, although the genetics and mechanisms of capsule biosynthesis are complex (O'Riordan and Lee, 2004).

The cell wall of *S. aureus* shows the typical features of gram-positive bacterial cell walls. Under the electron microscope it appears as a relatively thick (about 20 to 40 nm) homogeneous structure. The chemical structure of its major component is peptidoglycan by weight and teichoic acid binds to the peptidoglycan through a phosphodiester bond (Salton 1994; Peter G 1998). Penicillin binding proteins (PBPs) are critical components of the cell wall synthesis machinery in bacteria. The beta-lactam antibiotics inhibit PBPs by competing with the precursor for binding to the active site of the enzyme. PBPs enzymes are associated

with reduced bacteria susceptibility to oral cephalosporins and recently, they have been implicated in the emergence of "MRSA superbugs" (Kshetry et al 2016). One of the most worrisome evidence on MRSA is related to high mortality rates among concentrations patients. It is estimated that for MRSA patients, the mortality rate is two to three times higher than for patients infected with *S. aureus* constling steps (K öck et al 2010).

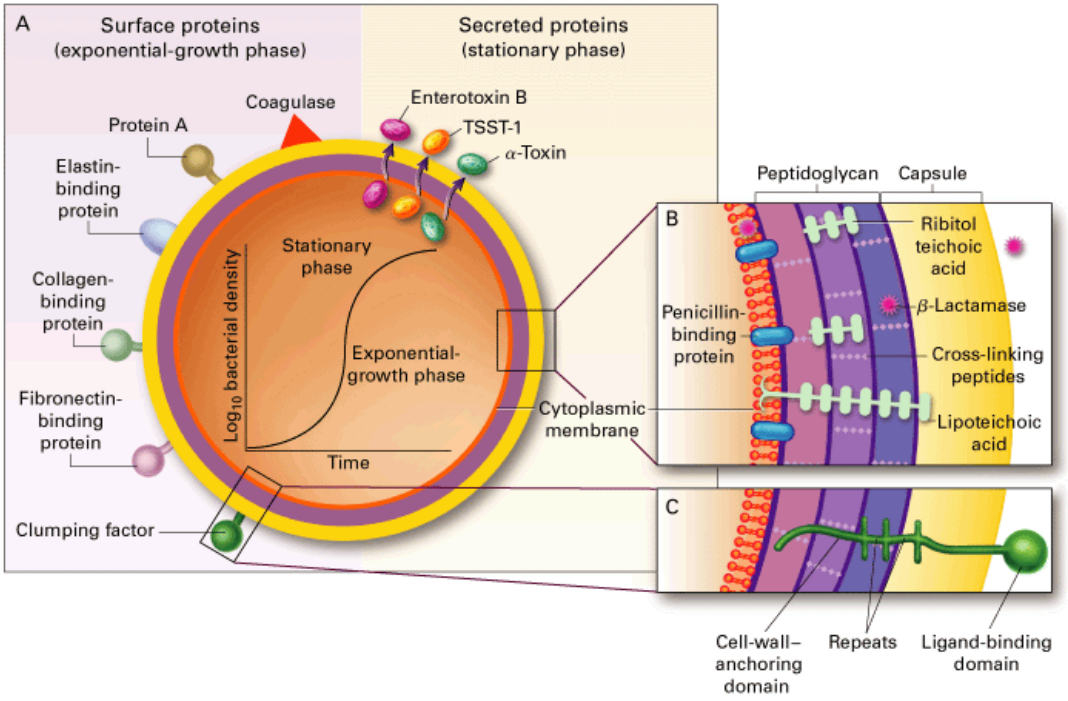


Figure 1.1.1 B Structure of *S. aureus* (Lowy, 1998).

Panel A: The surface and secreted proteins. The synthesis of these proteins is dependent on the growth phase and is controlled by regulatory genes such as agr. Panels B and C Cross sections of the cell envelope. Many of the surface proteins and peptides have a similar structural organization as clumping factor, including repeated segments of amino acids. TSST-1 denotes toxic shock syndrome toxin.

Staphylococcal surface proteins are covalently attached to peptidoglycan. The structure of these proteins is composed of a signal sequence at the N terminal which is cleaved during secretion, and a wall-spanning region and sorting signal at the carboxyl terminal facilitating the covalent anchorage to peptidoglycan. Ligand-binding domain at the N terminal on the bacterial surface functions as adhesins of some proteins (Foster and McDevitt 1994). Protein A has an ability binds to the Fc region of immunoglobulin resulting antiphagocytic properties (Fig. 1.1.1 B). In addition, protein A mediates the activation of host intracellular signaling and increase of inflammation, leading severe pneumonia (Normark et al., 2004; Soong et al., 2011). Several surface proteins bind extracellular-matrix molecules and function in invasion of host cells and evasion of elimination of host cells, of which microbial surface component

recognizing adhesive matrix molecules (MSCRAMMs) are the largest class (Patti et al., 1994).

S. aureus can produce numerous toxins which are classified into three families: pore-forming toxins, exfoliative toxins (ETs) and superantigens (SAgs) (Grumann et al., 2014). Pore-forming toxins damage the membranes of host cells, which can ultimately lead to cell lysis. At sublytic concentrations, these pore-forming toxins are potent cell stressors. These toxins trigger membrane permeabilization and damage by pore formation, induce inflammation characterized by increased vascular permeability, infiltration of neutrophils and macrophages and cytokine production or mediate cell death resulting in typical pus formation (Bhakdi 1984; Mattix ME 1995; Diep BA 2010; Ménoret A 2012). In synergy with other danger signals such as lipoproteins that activate the toll-like receptor 2 the toxins trigger the NALP3-inflammasome response resulting in release of cytokines IL-1, IL-18 and IL-33 (Franchi et al., 2012). Hla, hemolysin-γ (Hlg) and PVL have been shown to exert strong pro-inflammatory effects in this manner (Holzinger et al., 2012; Kebaier et al., 2012; Munoz-Planillo et al., 2009; Perret et al., 2012).

Functionally, ETs are isoforms of enzymes with high species-specificity. They have glutamate-specific serine protease activity and selectively cleave a single peptide bond in the extracellular region of human and mouse desmoglein 1 (Dsg1; desmosomal intercellular adhesion molecule), a keratinocyte cell–cell adhesion molecule. In this way, the ETs act as "molecular scissors" facilitating bacterial skin invasion (Nishifuji et al., 2008). By loosening the keratinocyte junctions they cause blistering diseases known as bullous impetigo and staphylococcal scalded skin syndrome.

The *staphylococcal* SAgs belong to the most potent T-cell mitogens known. Some of these toxins stimulate human T-cells at femtomolar concentrations. The superantigens interact with major histocompatibility complex (MHC) class II proteins by binding to the α-chain or to a conserved histidine in the β-domain, and trigger extensive T-cell proliferation and cytokine release (Fraser and Proft, 2008; Marrack and Kappler, 1990).

1.1.2 alpha-toxin

Alpha-toxin, also known as alpha-hemolysin (Hla, α-toxin), is the major cytotoxic agent released by bacterium *Staphylococcus aureus* and the first identified member of the pore

forming beta-barrel toxin family (Bhakdi S 1991). The alpha-toxin of *Staphylococcus aureus* is a pore forming toxin that penetrates host cell membranes causing osmotic swelling, rupture, lysis and subsequently cell death. Haemolysin alpha is toxic to a wide range of different mammalian cells; i.e., neurotoxic, dermonecrotic, haemolytic, and it can cause lethality in a wide variety of animals. Its toxic effects include activation of the arachidinic metabolism in endothelial cells due to $Ca2^{+}$ influx, activation of cellular nucleases (Inoshima I et al., 2011) and resulting apoptosis (Nygaard TK et al., 2012), activation of the autophagic pathway in case of intracellular presence of S. aureus (Mestre MB et al., 2010), release of procoagulatory factors due to $Ca2^{+}$ influx in platelets, vasoconstriction associated with a liberation of thromboxane A(2) and prostacyclin (Sibelius U et al., 2000), and an increase of vascular permeability that might lead to pulmonary oedema and adult respiratory distress syndrome. Experimental studies indicated alpha toxin to be an important virulence factor in both rabbit and murine models of keratitis (McCormick CC et al., 2009) as well as in pneumonia (Bubeck Wardenburg J et al., 2007) and superinfection of influenza (Lee MH et al., 2010).

Toxin Structure and Regulation of Production The gene coding for α-toxin was present in a single copy on the staphylococcal chromosome, the hla locus is rather invariant across sequenced *S. aureus* strains, with almost complete conservation of primary amino acid sequence. The hla locus encodes a 319 amino acid protein containing a 26 amino acid leader peptide predicted to be α-helical in structure (Gray G.S et al., 1984). The polypeptide is processed to yield a mature extracellular protein of 293 amino acids weighing approximately 33kDa water-soluble monomer (Tweten RK et al., 1983). Circular dichroism studies revealed the mature toxin is composed almost entirely of β-strands with little to no α-helical structure (Tobkes N et al., 1985; Y.Q. Xiong et al., 2006). α-toxin monomers aggregated into an oligomeric structure on the host cell surface，forming heptameric toxin structure which ring-like structures 10 nm in diameter with 6–7 subunits and a central pore of approximately 2–3 nm (Arbuthnott J.P et al., 1973; Freer J.H et al., 1973). The holotoxin is described to encompass three broad domains: (1) the cap domain on the extracellular face of the toxin, exposed to the aqueous environment, defining the entry of the pore; (2) the rim domain that is juxtaposed to the outer leaflet of the host plasma membrane; and (3) the stem domain that forms the membrane-perforating β-barrel pore (Fig 1.1.3 A) (Song L et al., 1996).

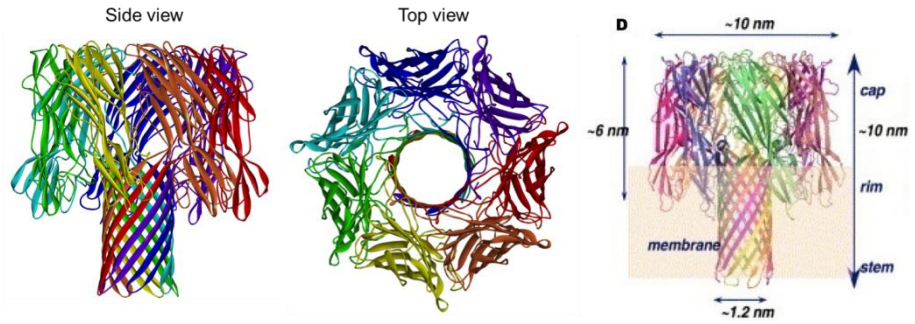


Figure 1.1.2 A Molecular model of the of α-toxin heptameric pore.structure.
Molecular modeling was performed using the 3D structure of α-hemolysin from *S. aureus*. Noting the regions of the toxin that demarcate the entry of the pore(Cap), the membrane-interfacing region (Rim), and the membrane perforating stem. Molecular model of the cereus haemolysin II heptameric pore.

Oligomerization and Pore Formation The molecular mechanism by which α-toxin binds to the surface of host cell membranes had been a longstanding subject of debate in the field (Cassidy P et al., 1976; Hanada K et al., 2006), as experimental evidence provided by multiple investigators either supported the ability of the toxin to bind to membrane lipids or to interact with host cells in a specific fashion consistent with proteinaceous receptor binding. Few years ago ADAM10 was defined as a candidate proteinaceous receptor for α-toxin (Wilke G.A et al., 2010). ADAM10 is not only the eukaryotic receptor for α-toxin, but also mediates the proper assembly of the toxin, which is a pre-requisite of the integration of α-toxin into cell membrane (Inoshima N et al., 2012). Binding of α-toxin to ADAM10 is necessary for α-toxin-induced cytotoxicity and the disruption of epithelial and endothelial barriers (Powers ME et al, 2012).

Contribution of α-Toxin to *S. aureus* Disease The Bundaberg accident and related investigations were the first of multiple studies to suggest that α-toxin may play an important role in the pathogenesis of human disease (Kolata J et al., 2011). Adhikari and Fritz proved a statistically significant increase in anti-Hla titer correlated with protective immunity against recurrent infection (Fritz S.A et al., 2013; Adhikari R.P et al., 2012). Bacterial genetic and protein profiling analysis provides a second line of evidence implicating α-toxin in the pathogenesis of human disease. Researches indicate that people with severe *S. aureus* disease, Shows skin/soft tissue infection, pneumonia, and sepsis/bacteremia in clinical (Roundtree P.M et al., 1958; Hassall J.E et al., 1959). Analysis of the hla and agr loci in these strains revealed the capability for α-toxin expression, confirmed by a highly virulent phenotype of these isolates in animal studies of Hla-mediated disease (DeLeo F.R et al., 2011). While early

studies alluded to the triad of lethal disease, hemolysis, and dermonecrosis as the chief manifestations of α-toxin-induced host injury (Burnet F.M, 1929), clinical and disease modeling data highlight a considerable complexity of the role of α-toxin in pathogenesis consistent with the ability of the toxin to cause injury and elicit cellular responses in a wide array of cell types (Fig 1.1.3 B). The discovery of ADAM10 as a cellular receptor for α-toxin has allowed for a more thorough examination of the molecular mechanisms by which α-toxin contributes to disease at the epithelial and endothelial tissue barriers (Inoshima I et al., 2011; Inoshima N et al., 2012; Powers M.E et al., 2012). These findings along with substantial recent advances in our understanding of toxin-mediated regulation of host immunity (Craven R.R et al., 2009; Frank K.M et al., 2012; Kebaier C et al., 2012; Yarovinsky T.O et al., 2008; Hruz P et al., 2009; Lizak M et al., 2012) will be the subject.

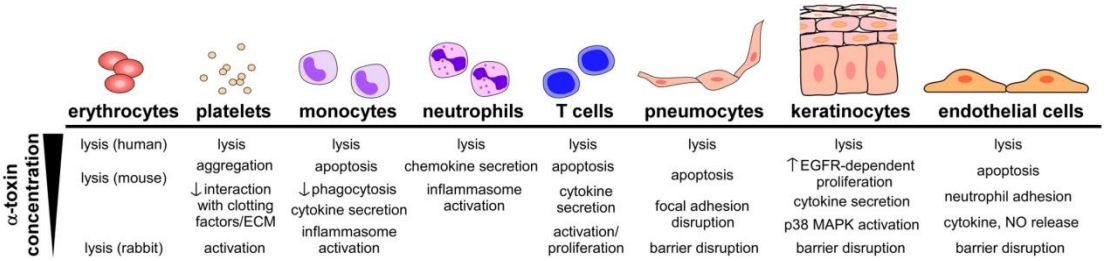


Figure 1.1.2 B Cellular responses to intoxication by Hla.

Multiple cell types are targeted by α-toxin, each displaying unique effects that are dependent on the relative concentration of toxin to which the cell is exposed.

Toxin-Induced Tissue Injury ADAM10, a cellular receptor for α-toxin, is a zinc-dependent metalloprotease expressed as a type I transmembrane protein on the surface of a wide array of host cells (Seals D.F 2003; Edwards D et al., 2008). ADAM10 is responsible for the cleavage of the ectodomains of a large number of host proteins including members of the cadherin family, epithelial growth factor, betacellulin, syndecans, chemokines, amyloid precursor protein (APP), platelet glycoprotein VI, notch, and ephrin, with substrate specificity varying by cell type (Allinson T.M et al., 2003; Maretzky T et al., 2005; Janes P.W et al., 2005; Gardiner E.E et al., 2007; Schulte A et al., 2007; Schulz B et al., 2008; Gibb D.R et al., 2010; Saftig P et al., 2011; Horiuchi K et al., 2006). ADAM10-mediated cleavage yields discrete biologic outcomes, as many extracellular and intracellular cleavage products are active signaling moieties that facilitate both physiologic and pathophysiologic processes.The requirement for ADAM10 as a cellular receptor for α-toxin in *S. aureus* pathogenesis was recently demonstrated utilizing conditional knockout approaches in the alveolar epithelium and the mature epidermis (Inoshima I et al., 2011; Inoshima N et al., 2012). The loss of

ADAM10 expression in the lung led to a marked improvement in the outcome of *S. aureus* pneumonia (Inoshima I et al., 2011). Similarly, ADAM10 knockout in the skin was associated with a reduction in the size of *S. aureus* skin lesions and abrogation of the severe dermonecrotic tissue injury that is a hallmark of the action of α-toxin (Inoshima N et al., 2012).

A number of studies indicate that α-toxin induces signaling events in the target cell. The small pore formed by the toxin permits the rapid release of ATP, K^+ ions (Cassidy P et al., 1976; Lizak M et al., 2012; Bhakdi S et al., 1989). However, restricts the movement of macromolecules across the cell membrane. One of the early and perhaps most important, cellular events following toxin pore formation is the influx of extracellular calcium into the cell. As a central trigger of cell signaling pathways, increased intracellular calcium stimulates hydrolysis of membrane phospholipids and metabolism of arachadonic acid to leukotrienes, prostanoids, and thromboxane A2 (Grimminger F et al., 1997; Bhakdi S et al., 1989; Suttorp N et al., 1985). Toxin treatment also leads to the generation of nitric oxide in endothelial and epithelial cells, activation of protein kinase C, and induction of NF-κB nuclear translocation (Grimminger F et al., 1997; Rose F et al., 2002; Suttorp N et al., 1985).

Together, these events signify the pro-inflammatory stimulus evoked by intoxication, also evident by cellular production of IL-1β, IL-6, and IL-8(Bhakdi S et al., 1989; Rose F et al., 2002). These inflammatory stimuli, as well as associated cell death via pyroptosis, can exert a marked impact on the local tissue microenvironment, stimulating immune cell recruitment, increasing reactivity of the vasculature, promoting tissue edema, and modulating host immunity (Craven R.R et al., 2009; Seeger W et al., 1990; Buerke M et al., 2002; Suttorp N et al., 1988). These studies indicate that α-toxin caused a primary disturbance of the epithelial barrier in the lung and the skin, manifesting as proteinaceous pulmonary edema and dermonecrotic injury, respectively.

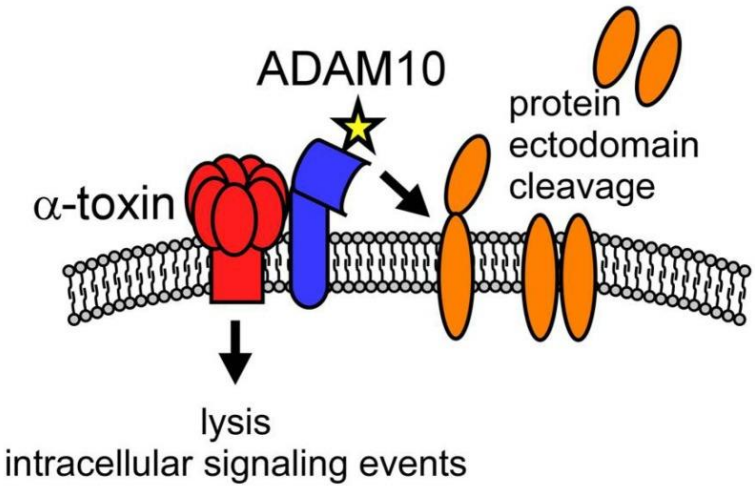


Figure 1.1.2 C Dual mechanism of action of α-toxin on susceptible host cells.
Model illustrating key functions of the α-toxin(red)-ADAM10(blue) complex, facilitating membrane binding of the toxin with subsequent oligomerization and pore formation. The formation of the toxin pore leads to two functionally linked outcomes—induction of host cells signaling and/or cellular lysis (dependent on toxin concentration) and the rapid upregulation of the metalloprotease activity of ADAM10 (denoted by a star). ADAM10, in turn, acts in a cell-specific manner to cleave ectodomain-containing proteins (orange) that appear to represent important biological mediators of α-toxin action.

Toxin-Induced Immunomodulation Multiple studies have indicated that immune cells are targets of α-toxin. Pro-inflammatory signaling in the host in response to infection is a double-edged sword, affording protection from pathogens yet contributing to self-injury when overly robust. Inflammation is a key feature of *S. aureus* infection, most readily appreciated in the lungs and skin wherein the rapid infiltration of innate immune cells is observed in both human and murine hosts (McElroy M.C et al., 1999; Parker D et al., 2012; Miller L.S et al. 2011). α-toxin induces inflammatory responses in multiple cells, resulting in the release of cytokines and vasoactive agents (Grimminger F et al., 1997; Bhakdi S et al., 1989; Suttorp N et al., 1985; Rose F et al., 2002; Suttorp N et al., 1993; Suttorp N et al., 1992; Onogawa T, 2002) .

One hallmark of innate immune cell activation is the secretion of the inflammatory cytokine interleukin-1β (IL-1β), a consequence of inflammasome activation and caspase-mediated cleavage of pro-IL-1β to yield the active cytokine. Intoxication with α-toxin induces IL-1β secretion in macrophages and monocytes, implicating this lineage as a target of the toxin during infection and demonstrating the importance of inflammatory cell death in disease pathogenesis (Craven R.R et al., 2011; Kebaier C et al., 2012; Bhakdi S et al., 1989).

Craven and colleagues demonstrated that the nucleotide binding domain and leucine rich repeat containing gene family, pyrin domain containing protein (NLRP3) inflammasome was activated in monocytic cells following exposure to α-toxin, resulting in caspase-1 activation and IL-1β secretion (Craven R.R et al., 2011) .

While antagonism of the toxin in vivo through toxin-neutralizing antibodies blunted IL-1β secretion during *S. aureus* pneumonia (Bubeck Wardenburg J et al., 2008), the molecular mechanisms underlying this response in vivo had not been investigated until recently. Following on the work of Craven, Kebaier and colleagues demonstrated toxin-dependent activation of the NLRP3 inflammasome in *S. aureus* pneumonia, leading to necrotic tissue injury (Kebaier C et al., 2012).

α-toxin neutralizing antibodies significantly decrease the amount of IL-1β secreted by isolated mouse neutrophils exposed to Hla in vitro, suggesting that the toxin in part contributes to this beneficial inflammatory host response in the skin (Cho J.S et al., 2012). Taken together, these data illustrate the role of α-toxin in inflammasome activation, and highlights the dichotomy between the beneficial effect of this pro-inflammatory response to infection of the skin and the detrimental effect of toxin-mediated inflammation in the lung. Interestingly, antagonism of α-toxin by active or passive immunization affords protection in both the lung and the skin (Kennedy A.D et al., 2010; Ragle B.E et al., 2009; Tkaczyk C et al., 2012; Bubeck Wardenburg J et al., 2008), highlighting the existing challenges in the field to understand how distinct cellular responses to α-toxin are integrated in the context of the tissue microenvironment during infection.

In addition to these effects on innate immunity, there is growing evidence that α-toxin modulates the adaptive immune response. Patterning of adaptive immune responses have been noted to occur through two mechanisms: (1) direct cellular injury, wherein α-toxin induces apoptotic cell death in monocytes, B cells and T cells(Nygaard T.K et al., 2012); (2) through alteration of signaling between innate and adaptive immune cells, particularly via the cytokine interleukin 17A (IL-17A) (Niebuhr M et al., 2011).

Research over the last few years has led to a significant increase in our understanding of the role of α-toxin in the molecular pathogenesis of *S. aureus* disease. A tangible outcome of these studies is an appreciation of this toxin as a leading target for disease-modifying therapies, and has engendered an increased focus on understanding the role of the toxin in

human *S. aureus* infection. Vaccines, passive immunization strategies, small molecule inhibitors of the toxin, and most recently small molecule-based targeting of host ADAM10 have all demonstrated a degree of efficacy in combatting *S. aureus* disease in animal modeling systems (Inoshima I et al., 2011; Inoshima N et al., 2012; Powers M.E et al., 2012; Kennedy A.D et al., 2010; Foletti D et al., 2013; Tkaczyk C et al., 2012; Bubeck Wardenburg J et al., 2008; Menzies B.E et al., 1996; Rauch S et al., 2012;Ragle B.E et al., 2010;Spaulding A.R et al., 2012).

1.2 Acid sphingomyelinase and ceramide system

1.2.1 Lipid interactions and domain formation

The classic fluid mosaic model, first introduced by Singer and Nicolson in 1972, predicts free movement of proteins in the lipid bilayer; this model was based on biophysical experiments that determined the melting temperatures of lipids (Singer and Nicolson, 1972). The theory is proposed as a basic framework model for cell membranes which could interprets studies on membrane proteins and structure and dynamics of lipids at the time. The fluid mosaic model defined biological membranes as a matrix composed of fluid bilayer of phospholipids with mobile globular integral membrane proteins and glycoproteins that were intercalated into the fluid lipid bilayer, which are "protein icebergs floating in the sea of lipid". However, in the past 10 to 15 years, this concept has been revised; it currently indicates that lipids are not uniformly distributed in the cell membrane, and the membrane microdomain "lipid rafts" was proposed (Lisanti et al., 1988; van Meer et al., 1987), they are ordered into distinct membrane domains that are enriched by sphingolipids and cholesterol, which promotes selective lateral segregation in the membrane plane and serves as a basis for lipid sorting (Simons and Ikonen, 1997). Recently, the new concept description of lipid rafts: Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006).

The plasma membrane of mammalian cells consists primarily of (glycero) phospholipids, sphingolipids and cholesterol (Hakomori, 1983). Sphingolipids consisted hydrophobic ceramide moiety "tails" and a hydrophilic "head" consisting of phosphate group. Sphingolipids, particularly the most abundant sphingolipid sphingomyelin, have a higher melting temperature compared with phospholipids in the cell membrane; tend to interact with each other through hydrophilic interactions of the hydrophilic sphingolipid head groups (Brown and London, 1998; Harder and Simons, 1997; Simons and Ikonen, 1997; Xu et al., 2001). Cholesterol functions as a spacer between the bulky head groups of sphingolipids head coordinate with sphingolipids via hydrogen bonds and hydrophobic van der Waal interactions of the sterol ring system and the ceramide moiety of sphingolipids, thus interactions formed. The tight interactions induced stable domains exist in a liquid-ordered- or even gel-like phase, which are separated from other phospholipids in the cell membrane (Brown and London, 1998, Kolesnick et al 2000, Simons and Ikonen， 1997).

Studies have suggested sphingolipids and cholesterol seem to be not randomly distributed in the membrane, they existing in a liquid-ordered phase of a distinct membrane domains ,formation of distinct sphingolipid- and cholesterol-enriched very small membrane domains, termed rafts (Simons and Ikonen， 1997). Recent microscopy studies of cell membranes using the STET technique suggest that these rafts have a diameter of less than 20 nm (Eggeling et al 2009). Cholesterol and some cholesterol precursors not only interact with sphingolipids but also seem to fill the void spaces between bulky sphingolipids and, sterically, to stabilize sphingolipid- and cholesterol-enriched domains (Megha et al 2004, Xu et al 2001). This notion is consistent with the finding that extraction of cholesterol from membranes using drugs as beta-cyclodextrin, nystatin or filipin that binds cholesterol destroys membrane rafts (Keller and Simons, 1998). Many studies characterized rafts by their resistance to detergents, which are caused by the high biophysical order in this phase, although the use of detergents might be problematic to investigate the physiology of lipids and detergent-resistant membrane domains are not the same as membrane rafts (Munro, 2003; Lichtenberg et al., 2005).Ceramide, a bio-active sphingolipid, plays a specific and important role in regulating the lipid rafts (Cremesti et al, 2002). Ceramide-rich "domains" are known to be in a highly ordered state in comparison to cholesterol-containing rafts. Furthermore, ceramide shows the tendency to dislocate cholesterol from rafts which can cause a distinct property change in raft composition (Megha and London, 2004).

1.2.2 Ceramide and ceramide enriched platforms

Sphingomyelin is predominantly present in the outer leaflet of the cell membrane. Sphingomyelin can be hydrolyzed to ceramide, which dramatically alters the biophysical properties of the plasma membrane. Ceramide constitutes the hydrophobic backbone of all complex phingolipids and is composed of a sphingosine joined with an amide bond to fatty acyl chains (Sandhoff, 2010) (Fig 1.2.2 A).

Ceramide has been previously believed to be an entirely structural element found in the cell plasma membrane, but by now, its involvement in a variety of signaling pathways has been made clear (Chalfant et al 2002, Gulbins et al 2006， Huwiler et al 2001, Yabu et al 2015).

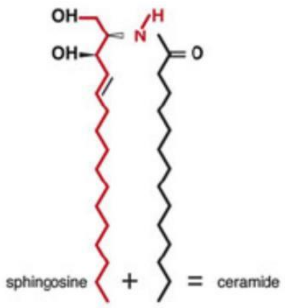


Figure 1.2.2 A Structure of ceramide.

Ceramide consists of a long chain base which is linked to a fatty acid via an amide bond. (Mencarelli and Martinez-Martinez, 2013)

The structure of ceramides with the long (C16–20) and very long (C22–24) acyl chains (Fahy et al., 2005; Sandhoff, 2010), determines their poor solubility in water and cannot exist in biological fluids or in the cytosol (Goni et al., 2005). Ceramide molecules have the tendency to spontaneously self-associate. With the hydroxyl functional group, the amide linkage and the OH group on the sphingosine backbone, ceramides form extensive hydrogen bonds, which contribute to their high bulk transition temperature and in-plane phase separation, finally promote the formation of ceramide-enriched domains, particularly solid-like condensed domains in bilayers and monolayers (Castro et al., 2014; Maula et al., 2011).

Moreover, ceramide dramatically alters membrane properties with other lipids. Low concentration as 5 mol% ceramide is sufficient to spontaneously induce ceramide-enriched membrane domains formation in model membranes (Veiga et al., 1999). A large number of biophysical techniques have been used to detect complex phase behavior of lipid mixtures with ceramide, including nuclear magnetic resonance (Hsueh et al., 2002), atomic force microscopy (Chiantia et al., 2006), X-ray diffraction (Boulgaropoulos et al., 2012), fourier transform infrared spectroscopy (Boulgaropoulos et al., 2011), fluorescence spectroscopy (Castro et al., 2007; Silva et al., 2007) and fluorescence microscopy (Staneva et al., 2009).

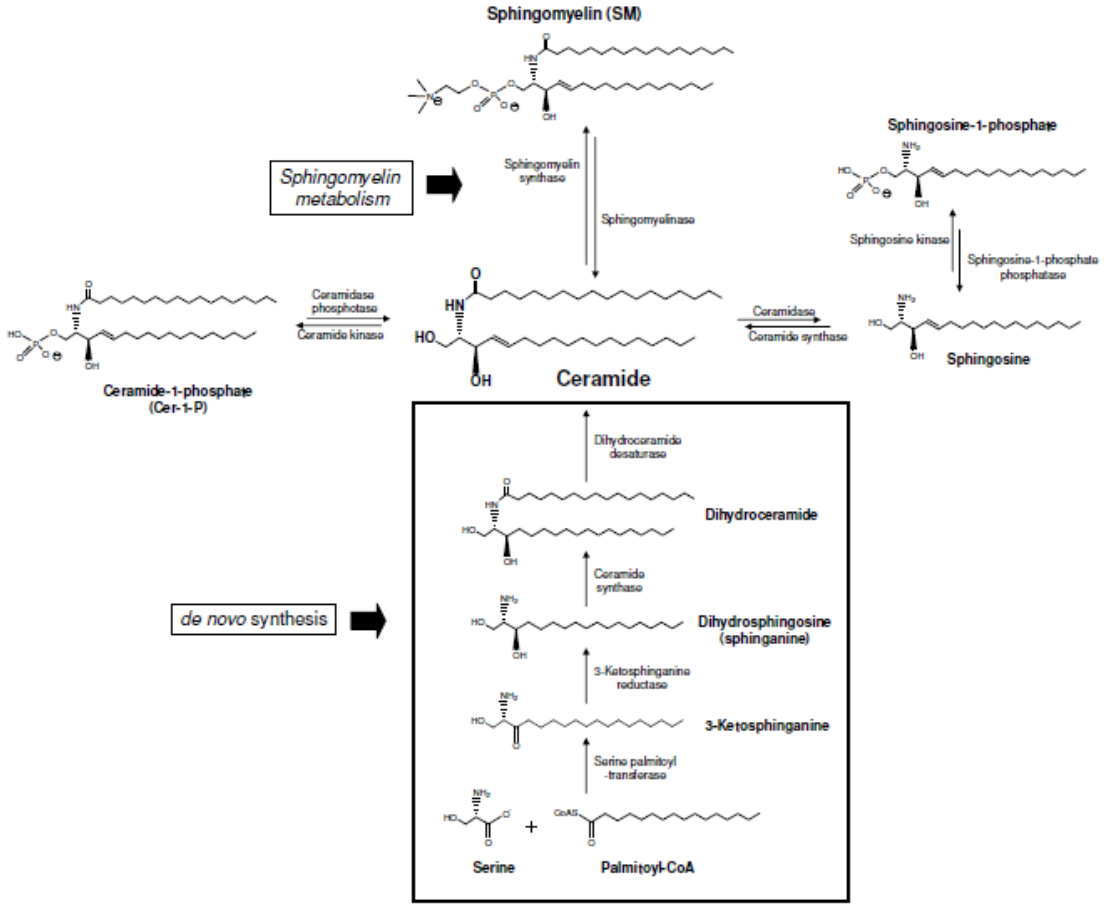


Figure 1.2.2 B Ceramide synthesis and metabolism.

Ceramide can be generated from the sphingomyelin via sphingomyelinase pathway (sphingomyelin metabolism) or via the de novo synthesis pathway where cells synthesize ceramide from serine and almitoyl-CoA (de novo synthesis). Ceramide can be further converted into other sphingolipids such as ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate.

Ceramides are generated from diverse pathways (Fig 1.2.2 B). Several enzymes located in specific subcellular compartments are involved in ceramide metabolism, and certain activation of molecule signaling can occure due to their sites of function. Various stimuli can activate different metabolic pathways result the formation of ceramides. Two major pathways are the sphingomyelinase pathway that ceramide generates from sphingomyelin by the activities of sphingomyelinases and the de novo synthesis pathway that synthesizes ceramide from serine and palmitoyl-CoA by the activity of ceramide synthase.

Sphingomyelinase pathway The sphingomyelin hydrolysis pathway proceeds with the hydrolysis of sphingomyelin by activation of the enzyme sphingomylinases (SMases) resulting in the formation of ceramide and phosphorylcholine. Three different types of

sphingomylinases (SMases) have been identified on the basis of their pH-optimum: acidic SMases, neutral SMases, and alkaline SMases. They are known to be localized in different compartments within the cells (Fensome et al., 2000; Hofmann et al., 2000; Rajagopalan et al., 2015; Stoffel, 1999; Tomiuk et al., 2000; Wu et al., 2010; Wu et al., 2005). Sphingomyelin can be regenerated from ceramide by sphingomyelin synthase. Ceramide can also be converted into other sphingolipids, such as ceramide-1-phosphate, sphingosine, and sphingosine- 1-phosphate.

Recent studies further revealed three additional pathways for the formation of ceramide, i.e. by the reverse activity of the acid ceramidase catalyzing synthesis of ceramide from sphingosine (Okino et al., 2003), by hydrolysis of complex- glycosylated lipids (Ishibashi et al., 2007) and by hydrolysis of ceramide-1-phosphate (Mitra et al., 2007). (Fig 1.2.2 B)

De nove synthesis pathway The de novo synthesis of ceramide is located in endoplasmic reticulum (Bartke and Hannun, 2009; Carpinteiro et al., 2008). Begin with the condensation of serine and palmitoyl-CoA by serine palmitoyl-transferase, which forms 3-ketosphingosine, which then undergoes reduction to dihydrosphingosine. By addition of a C16 to C24 fatty acid to the amino group of dihydrosphingosine via ceramide synthase, dihydroceramide is produced. Finally ceramide is synthesized from dihydroceramide by dihydroceramide desaturase (Chalfant et al., 2002; Wang et al., 2002; Seumois et al., 2007). Ceramide can be delivered to the Golgi by vesicular trafficking or the ceramide transfer protein CERT for further structure modification to other sphingolipids (Hanada et al., 2007; Yamaoka et al., 2004). (Fig 1.2.2 B)

In general, the accumulation of ceramide within cellular membranes leads to the formation of ceramide-enriched membrane domains, which appears to sort proteins and to provide platform for the spatial recruiting receptors and intracellular signalling molecules upon various stimuli (Fig.1.2.2 C) (Bollinger et al., 2005; Grassme et al., 2007; zhang et al., 2009). Ceramide plays important roles in a series of physiological and pathophysiological processes.

First, the tightly packed ceramides strongly stabilize lipid rafts (Kolesnick et al., 2000; Xu et al., 2001). This stabilization of lipid rafts promote the formation of cell membrane domains into a lipid ordered state as mentioned above. Further, ceramide-enriched membrane domains serve the reorganization and clustering of receptor molecules, including CD95

(Grassme et al., 2001), CD40 (Grassme et al., 2002), CD20 (Bezombes et al., 2004) and CD44 (Cao et al., 2017). Clustering of these receptors by ceramide may lead the very high receptor density, thus activating the downstream signaling molecules of the receptors, the exclusion of inhibitory molecules, conformational change of the receptor, and stabilization of the interaction of the receptor with its ligand (Grassme et al., 2007).

In addition, ceramide also function as second messenger. Ceramide has been shown interacting and activating different enzymes such as cathepsin D (Heinrich et al., 1999; Zebrakovska et al., 2011), phospholipase A2 (Bharath et al., 2015). Recent studies have identified an important function of ceramide in infectious biology. Basically, ceramides seem to be involved in the interaction of pathogens with host receptors, receptor clustering, and intracellular signaling molecules. Several studies have shown the ceramide interaction of pathogens with host, Pseudomonas aeruginosa (Becker et al., 2010; Pewzner-Jung et al., 2014), *Staphylococcus aureus* (Peng et al., 2015). In summary, ceramide and ceramide-enriched membrane domains might act as a crucial motif to reorganize the topology of a given signalosome, thus permit the stress stimulation and receptors to transmit biophysiological signals into the cell.

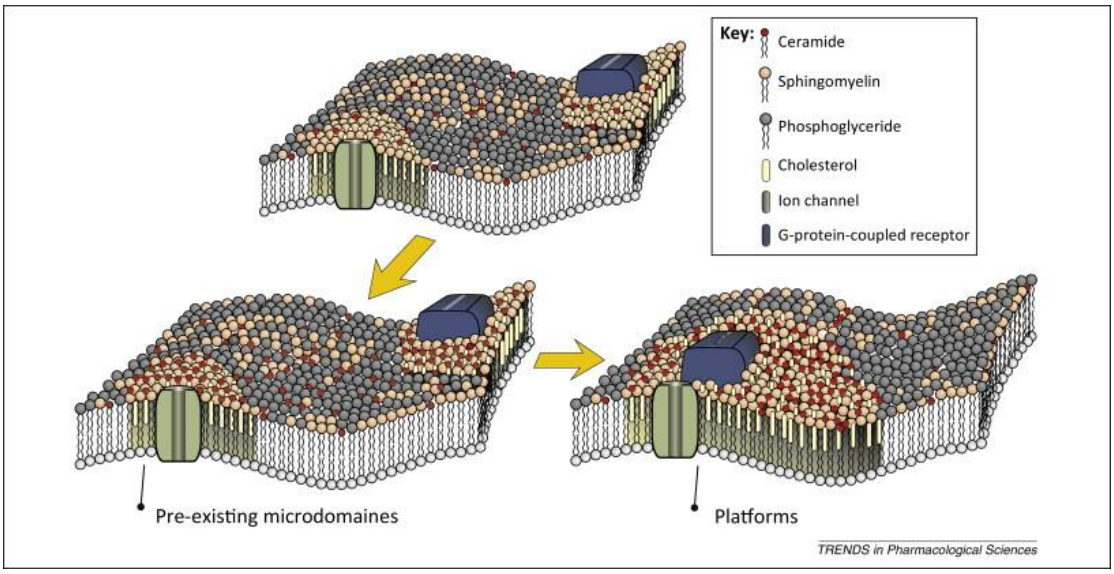


Figure 1.2.2 C Ceramide action in biological membranes.

Biological membranes consist of sphingolipids (mainly sphingomyelin), cholesterol, and phosphoglycerides. Sphingolipids and cholesterol spontaneously interact with each other and separate from other phospholipids into distinct microdomains named rafts. The accumulation of ceramide, which is present ten times less abundant than sphingomyelin under normal conditions, changes the biophysical properties of these domains in the biological membranes. Ceramide molecules tend to self-associate and

form ceramide-enriched microdomains, which can further fuse to large ceramide-enriched platforms. These ceramide-enriched membrane platforms function in recruiting and clustering of receptor molecules (Kornhuber et al., 2014).

1.2.3 Acid sphingomyelinase (ASM)

Acid sphingomyelinase (ASM), a hydrolase first identified by Gatt and colleagues in 1963 (Gatt, 1963), the enzyme was shown to be critically involved in many forms of cell activation (Samet and Barenholz, 1999; Gulbins and Kolesnick, 2003). Acid sphingomyelinase hydrolyzes sphingomyelin to ceramide and phosphorycholine, preferentially at an acidic pH. ASM is composed of three main domains: the N-terminal saposin domain, the proline-rich connector, and the catalytic domain (Gorelik et al., 2016; Xiong et al., 2016). Deficiency of ASM results in the accumulation of sphingomyelin and causes lysosomal storage diseases, which are fatal neuropathic and visceral disease Niemann-Pick type A and a visceral anomalies disease Niemann-Pick type B (Brady et al., 1966; Schuchman et al., 1992; Schuchman, 2007).

Two types of ASM are characterized based on their localization: lysosomal sphingomyelinase (L-SMase) (Fowler, 1969; Jenkins et al., 2011) and secretory sphingomyelinase (S-SMase) (Schissel et al., 1996). The name L-SMase hints at its being located in lysosomes, whereas the S-SMase is secreted into the serum (Schissel et al 1998). The two ASM forms differ in the trafficking process controlled by the same protein precursor. There exists distinct glycosylation pattern between L-SMase and S-SMase: S-SMase is transported to plasma membrane via Golgi secretory pathway in contrast to L-SMase which depends on lysosomal trafficking (Schissel et al 1998). L-SMase is known to be independent of Zinc (Zn^{2+}) for its activation in contrast to S-SMase (Schissel et al., 1996; Schissel et al., 1998). The role of L- SMase in mediating apoptosis is also been reported. Its involvement in activating Bcl-2- associated X protein (BAX) by stimulating apoptotic cascade signaling (Jin et al 2008, Kashkar et al 2005), mitochondrial dysfunction (Zeidan et al 2008), and activation of caspases with the involvement of cathepsin D (Heinrich et al 2004) has been well described.

Compared to L-SMase, the role and function of S-SMase in stimulating ceramide production and inducing cells death is unclear to date, but an increase in the level and activity of S-SMase in serum has been found in multiple diseases, for instance in chronic heart failure

(Doehner et al., 2007), hemophagocytic lymphohistiocytosis (Takahashi et al., 2002), severe sepsis (Claus et al., 2005).

Activation of several receptors, such as CD95 (Dumitru and Gulbins, 2006; Grassme et al., 2001), DR5 (Carpinteiro et al., 2008), CD40 (Grassme et al., 2002), and the platelet-activating factor (PAF) receptor (Göggel R et al., 2004), but also some bacterial and viral infections or stress stimuli, trigger the surface exposure of acid sphingomyelinase (Grassmé et al., 2001; Cremesti et al., 2001). Further, changes in the glycosylation pattern of acid sphingomyelinase result in the expression of a secretory form of acid sphingomyelinase that is released upon stimulation, for instance by activation of interleukin-1 receptors (Schissel et al., 1996 and 1998).

1.2.4 ASM and ceramide system in bacterial infection

ASM and ceramide were shown to play a crucial role in a wide range of diseases, including cancer (Zhu L et al., 2016), cystic fibrosis (Becker KA et al., 2010), diabetes(Kady N et al., 2017), Alzheimer's disease(He X et al., 2010), atherosclerosis(Deevska G.M et al., 2012), and major depression (Rhein C et al., 2017), as well as and bacterial (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*) infections (Peng, H et al., 2015; Zhang Y et al., 2008). Since ASM is located intracellular in lysosome and extracellular on the plasma membrane and ceramide contribute to the formation of the plasma membrane. Ceramide molecules may act in these diseases by the alteration of membrane biophysics, the self-association of ceramide molecules within the cell membrane and the ultimate formation of larger ceramide-enriched membrane domains / platforms.

S. aureus is a primary cause of sepsis and lethal lung edema even with the treatments of antibiotics clinically. It was reported that the mammalian acid sphingomyelinase (Asm)/ceramide system play an inportant role in the development of lung edema caused by *S. aureus*. Genetic deficiency or pharmacological inhibition of ASM protects mice against pneumonia and lethal *S. aureus* sepsis (Peng et al., 2015). ASM is activated by *S. aureus* in endothelial cells thereby the ceramide enriched platforms are generated. The Asm/ceramide system triggered the formation of superoxide, resulting in degradation of tight junction proteins (ZO1, ZO2, occluding and E-cadherin) followed by lung edema. Treatment of infected mice with amitriptyline, a potent inhibitor of Asm, protected mice from lung edema caused by *S. aureus*, but did not reduce systemic bacterial numbers. Several evidences have

demonstrated that the superoxide is responsible for the degradation of tight junctions via proteolytic enzymes matrix metalloproteinases (MMP) (Abdul-Muneer et al., 2015; Gu et al., 2011; Rochfort et al., 2014). In turn, treatment with antibiotics reduced bacterial numbers but did not protect mice from lung edema. In contrast, only the combination of antibiotics and amitriptyline inhibited both pulmonary edema and bacteremia protecting mice from lethal sepsis. This combination is sufficient to clear the bacteria with a protection of tight junction protein degradation which suggesting a novel therapeutic approach to treat lung edema and bacteremia in *S. aureus* induced sepsis.

Recently, it was found that CD44 serves as a receptor for *S. aureus* in macrophages. CD44 activates the acid sphingomyelinase upon infection and triggers the release of ceramide. Ceramide forms distinct domains in the plasma membrane that serve to cluster CD44 and thereby amplify CD44 signaling, which results in activation of small Rho family GTPases, reorganization of the actin cytoskeleton, internalization of *S. aureus* by macrophages, fusion of phagosomes with lysosomes and intracellular killing of the pathogen. Genetic deficiency of CD44 or acid sphingomyelinase abrogates these events. Moreover, acid sphingomyelinase-deficient macrophages fail to kill intracellular *S. aureus* and are highly susceptible to pulmonary *S. aureus* infections. (Li C et al., 2017)

Staphylococcal aureus alpha-toxin (α-toxin) appears to function by forming pores in cell membranes, damaging the membrane permeability, eventually triggering the cell death. α-toxin is secreted as water soluble monomer protein, once they reach the targets they are capable of binding and oligomerization into a heptameric structure, therefore inserting or translocating across the cell membrane of the host (Gouaux et al., 1994; Song et al., 1996). ASM-ceramide system is found protecting against *staphylococcal aureus* α-toxin induced keratinocyte death (Brauweiler et al., 2013). Filaggrin is a protein critical for epidermal skin barrier function (Irvine et al., 2011). α-toxin targets and damages the filaggrin deficient or undifferentiated keratinocytes. The differentiation process leads to a significant increase of expression of ASM mRNA and protein. Gene silence of filaggrin reduces the both the amount and activity of secreted ASM but not the intracellular ASM activity. ASM is sufficient to protect cell death against α-toxin by reducing expression of α-toxin receptors and the binding of α-toxin with receptors (Brauweiler et al., 2013).

ASM-ceramide system is also involved in several other host bacteria infection. Upon *Pseudomonas aeruginosa* (*P. aeruginosa*) infection, ASM is activated and translocated to the

extracellular leaflet of the plasma membrane which stumilates the ceramide generation and platform formation, thus mediates the bacteria internalization and killing, cytokine such as IL-1β release, cell death, inflammatory response, and susceptibility to bacteria challenge (Grassme et al., 2003; Teichgraber et al., 2008; Zhang et al., 2008). Further, ASM generated ceramide promotes bacteria killing and cell death in the infection of macrophages with pathogenic mycobacteria (Roca F.J and Ramakrishnan L, 2013). ASM deficiency highly impairs the bactericidal capacity of mice challenging the *Listeria Monocytogenes* (*L. Monocytogenes*), which due to the fail of macrophages intracellular bacteria killing involving the listeriocidal proteases cathepsin D, B and L (Utermohlen O et al., 2003). In addition, ASM generated ceramide mediates interaction of pathogenic Neisseria with host receptors carcinoembryonic antigen related cellular adhesion molecule (CEACAM) or tyrosine kinase ErbB2 regulated phagocytosis in phagocytic or non-phagocytic cells (Grassme H et al., 1997; Hauck C.R et al., 2000; Simonis A et al., 2014). The activation of ASM and generation of ceramide and their exposure to NO depend on the cGMP formation during the infection process of *Escherichia coli* (*E. coli*) (Falcone S et al., 2004). Salmonella triggers a significant increase in the secreted fraction of ASM (McCollister B.D et al., 2007). Recent studies have shown host ASM also involves in the *P. acnes* virulence induced inflammation (Nakatsuji T et al., 2011).

ASM-ceramide system has been shown to be crucially in the regulation of host interaction with bacteria, including *P. aeruginosa*, *S. aureus*, *mycobacteria*, *L monocytogenes*, *Neisseria*, *E. coli*, *Salmonella*, *P. acnes* and bacteria toxins. We presumable role of ASM-ceramide system in the bacterial infection process on the cells. The infection of mammalian cells with bacteria triggers the activation of ASM and secretion of ASM onto membrane and extracellular environment. The ASM generated ceramide initiate the lipid raft organization thereby mediating the bacteria acting on host cells. ASM facilitates the activation of NADPH oxidases which involves generating of superoxide, known to be responsible for bacteria killing and regulate the cell apoptosis. The ceramide platforms critically 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 phagosome and lysosome. In addition, ASM have an influence on the cytokine release, inflammatory response, and the susceptibility of mice. Taken together, although the detail mechanism of ASM-ceramide system acting on bacterial infection remains to be further studied, strong evidences shows the central role of this system in bacteria host interactions. Targeting the ASM-ceramide system is a novel potential therapeutic approach for treating bacterial infection.

1.3 The inflammasome in the host response to infection

1.3.1 Inflamasome

The inflammasomes are important platforms that account for recognition and restriction of the infection by pathogenic microbes. Key aspects of innate immunity are the detection of invaders or tissue injury and the activation of inflammation that alarms the system through the action of cytokine and chemokine cascades. Inflammasomes are multi-protein signaling platforms assembled in response to viral and bacterial pathogens as well as endogenous danger signals which ise ssential for host resistance to infections. Inflammasomes are large cytosolic multiprotein complexes, including NLRP3, NLRC4, AIM2, (Pedra, J. H et al., 2009; Jwa-Jin Kim and Eun-Kyeong Jo, 2013) these multi-protein complexes contribute to the activation of inflammatory caspases (i.e., caspase-1) (Vance RE, 2015; Man SM et al., 2016) that result in the proteolytic processing and secretion of cytokines, including interleukin (IL)-1β and IL-18 (Martinon F et al., 2009; F. Martinon et al., 2002) and initiation of an inflammatory form of cell death referred to as pyroptosis.

Inflammasome activation can be induced by a wide variety of microbial pathogens and generally mediates host defense through activation of rapid inflammatory responses and restriction of pathogen replication and mucosal immune responses. The hallmarks of inflammasome activation are the processing of caspase-1, the maturation and release of interleukin-1β (IL-1β) and the induction of pyroptosis, a lytic inflammatory cell death (Dinarello C, 2010).

IL-1β is one of the quintessential pro-inflammatory cytokines that broadly affects inflammatory processes (Dinarello C.A 2010). There is no typical singnal sequence that would allow IL-1β which is synthesized as a pro-protein, and instead its activation and cellular release are controlled by the cysteine protease caspase-1 (Cerretti D.P et al., 1992). Similarly, caspase-1 is responsible for the processing and secretion of IL-18, as well as the secretion of other proteins such as IL-1α and fibroblast growth factor-2 or TNF-α through an unconventional protein secretion pathway (Furuoka M et al., 2016; Keller M et al., 2008). Like other caspases, caspase-1 is synthesized as an inactive zymogen (pro-caspase-1) and becomes proteolytically active only after controlled dimerization in inflammasomes that are built around one of several different molecules (Kerur N et al., 2011; Agostini L et al., 2004).

In the decade since the initial description of an inflammasome, it has become clear that

there are in fact multiple distinct inflammasomes, each of which is activated by unique stimuli that can include infectious agents as well as noninfectious stimuli. The formation of each inflammasome is dictated by a unique scaffolding protein (Figure 1.3.1). Most of these scaffolding proteins contain a nucleotide-binding domain (NBD) and leucine-rich repeats (LRRs) and are thus members of the NBD-LRR (NLR) superfamily (Roberts TL, 2009; Jones JW, 2010).

Whereas the leucine-rich repeat (LRR) domain is thought to be involved in autoinhibition that is disabled on direct or indirect sensing of the activating signal, the nucleotide-binding domain (NBD) is involved in the regulation of homo-oligomerization or hetero-oligomerization, which is required for inflammasome assembly. On receiving an activating signal, inflammasome sensors recruit pro-caspase-1 (which has a caspase activation and recruitment domain (CARD) either directly through homotypic binding of CARD or indirectly through a pyrin domain (PYD) by means of the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which contains a PYD and a CARD.

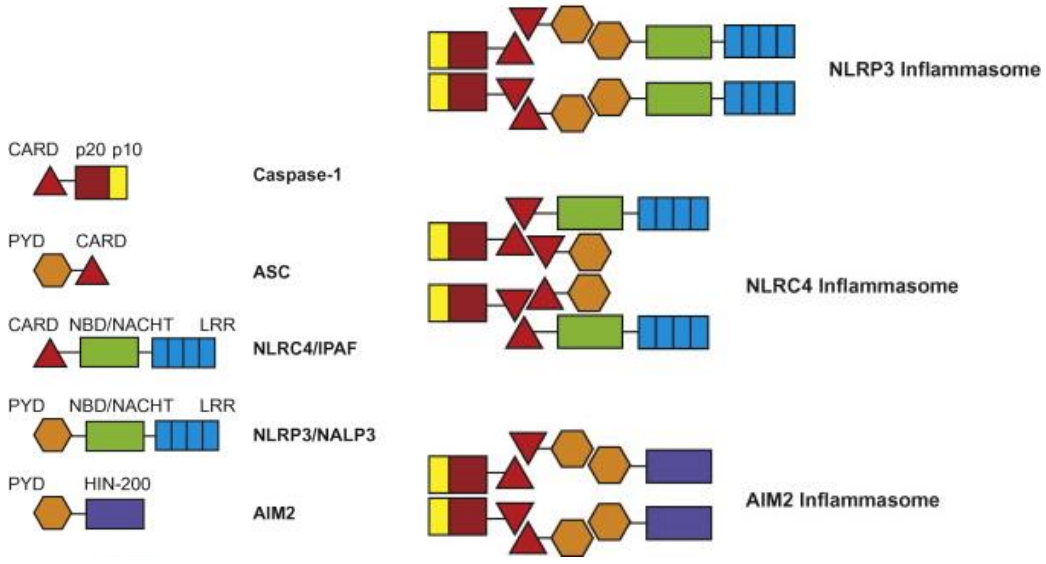


Figure 1.3.1 Inflammasome components and inflammasomes

Inflammasomes are assembled through protein–protein interactions between proteins containing Caspase recruitment domains (CARD) or Pyrin domains (PYD). Members of the NLR family, such as NLRP3 and NLRC4 contain either N-terminal PYD or CARD (respectively) as well as a central nucleotide binding domain (NBD), also known as a NACHT domain. The leucine rich repeat domain (LRR) is thought to act as an auto-inhibitory domain responsible for sensing (either directly or indirectly) stimuli that trigger inflammasome assembly. AIM2, a member of a family of proteins that contain a PYD and a DNA binding domain known as HIN-200, is an interferon-inducible protein responsible for assembly of a recently described inflammasome that responds to cytosolic DNA. (Brodsky I.E and Monack D., 2009)

1.3.2 Regulation of inflammation by inflammasomes

Inflammasome assembly is unique in its induction by a variety of both exogenous and endogenous signals. The range of activation signals sensed by each protein is distinct, but may include overlapping signals (Figure 1.3.2). Inflammasome activity needs to be tightly regulated by the host to avoid the excess production of cytokines or overt cell death. Inflammasome-regulated processes depend on the simultaneous expression of the multiple inflammasome protein components in the same cell type of inflamed tissues. It has been proved that inflammasomes have been shown to participate in the antimicrobial innate immune response (Hu B et al., 2010).

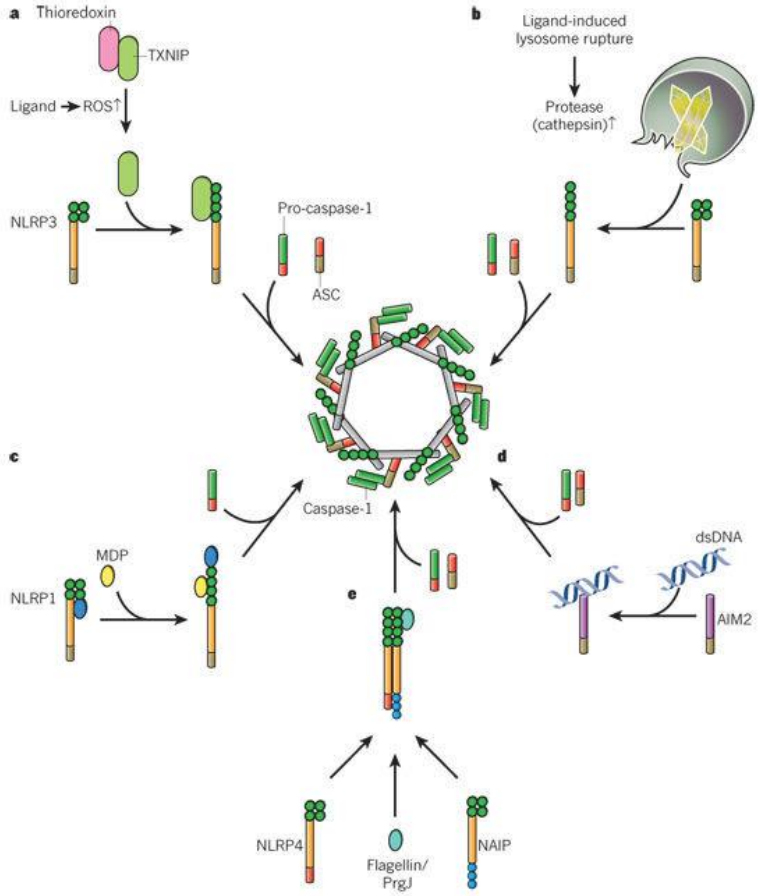
Innate immune cells employ germ line encoded pattern recognition receptors (PRRs) that are important for recognition of pathogen-associated molecular patterns (PAMPs), including macrophages, monocytes, dendritic cells, neutrophils, and epithelial cells, as well as cells of the adaptive immune system, conserved structures present in microbes and pathogens (Janeway and Medzhitov, 2002). Extensively studied PRRs are the Toll-like receptors (TLRs), Rig-like receptors (RLRs), and Nod-like receptors (NLRs); this trinity of PRR plays an important role in recognition of bacterial, viruses, fungi, and parasitic infections (Kawai and Akira, 2009; Schroder and Tschopp, 2010). TLR are transmembrane proteins that signal for expression of inflammatory genes via transcriptional factors, the NLRs are cytosolic proteins that trigger diverse host cell processes. Activation of NLRs, such as Nod1 and Nod2 turns on signaling cascades that culminate in activation of NF-κB and MAPK. Nod1 and Nod2 signaling requires the protein kinase Rip2, leading to expression of inflammatory genes, such as those of cytokines and chemokines (Franchi et al., 2008).

More over, they participate in the activation of the pro-caspase-1, which will lead to the formation of a multimeric complex called inflammasome (Schroder and Tschopp, 2010). Several inflammasomes have been described, among them the Nlrp3, Nlrc4, Nlrp1, and AMI2 inflammasomes (Pedra et al., 2009; Schroder and Tschopp, 2010). Nlrp3 inflammasome is possibly the most studied; it requires the adaptor protein Asc and leads to a strong inflammatory response mediated by the secretion of inflammatory cytokines, such as IL-1β and IL-18. The Nlrp3 inflammasome is triggered in response to diverse stimuli including several crystals, skin irritants, microbial PAMPs, microbial toxins, etc (Schroder and Tschopp, 2010).

Despite the evidence linking the NLRP3 inflammasome to the immune response to infection, only in a minority of cases has inflammasome activation by direct recognition of the pathogen been documented; many studies have indicated inflammasome activation through induction of signals related to cellular stress and damage (Schroder and Tschopp, 2010). The influenza a virus is an example of an indirect viral NLRP3 inflammasome activator. On infection, recognition of viral RNA by means of Toll-like receptor 7 (TLR7) induces transcription of the NLRP3 inflammasome components (Ichinohe et al., 2010). Subsequently, the activity of the viral ion-channel protein M2 induces pH neutralization of the trans-Golgi network, leading to potassium efflux and ROS formation, which in turn induce NLRP3 inflammasome assembly.

NLRC4 inflammasome activation is driven by type III and type IV secretion systems (T3SS and T4SS) of bacteria such as *Salmonella*, *Pseudomonas*, *Legionella* and *Yersinia*, which allow the cytoplasmic entry of the NLRC4 ligand flagellin, leading to activation of the NLRC4 pathway (Mariathasan et al., 2004; Marcelo et al., 2011). In addition, NLRC4 activation during *Legionella* infection is dependent on NAIP5, but only partial dependence on NAIP5 was demonstrated for NLRC4 activation during Pseudomonas and Salmonella infection (Miao et al., 2010; Lamkanfi et al., 2007). This indicated that different pathogen receptors therefore assist NLRC4 in the recognition of microbial ligands, broadening the diversity of sensed structures. The recently discovered protein AIM2 recognizes bacterial and viral dsDNA, resulting in an antimicrobial response to intracellular pathogens, such as Francisella tularensis, Listeria monocytogenes and some DNA viruses (Rathinam et al., 2010)

During microbial infection, distinct effector mechanisms of inflammasomes seem to be important for host defence. Although inflammasome-induced IL-1β and IL-18 are essential for the clearance of influenza virus and Shigella, respectively, pyroptosis has been suggested to be essential for caspase-1-mediated effects in vivo during infection by *Salmonella enterica Typhimurium*, L*egionella pneumophila* or *Burkholderia thailandensis* (Miao et al., 2010). Altogether, it seems that the cooperative activity of distinct inflammasomes is pivotal in the host's ability to raise a protective immune response. This cooperation is illustrated during infection by *S. Typhimurium*, in which deficiencies in either NLRP3 or NLRC4 in vivo do not lead to increased bacterial infection, whereas mice deficient in both NLRC4 and NLRP3 have a similarly increased susceptibility to infection to that of caspase-1-deficient mice (Broz et al., 2010)



Fiugre 1.3.2 Models for inflammasome activation.

Inflammasomes are assembled after sensing a structurally diverse repertoire of PAMPs and DAMPs. Several models have been proposed to explain how these signals are sensed, including models based on recognition of general cellular stress (Fig. a and b) or on direct and indirect recognition of activation signals (Fig. c–e). NLRP3 senses the reactive oxygen species (ROS), which is produced in the cell (potentially by mitochondria) directly or indirectly by activators of the NLRP3 inflammasome. Increased amounts of ROS are sensed by a complex of thioredoxin and thioredoxin-interacting protein (TXNIP), leading to the dissociation of this complex. Subsequent binding of TXNIP to NLRP3 leads to the activation of NLRP3, the recruitment of ASC and pro-caspase-1, and formation of the active inflammasome complex (a). NLRP3 is activated after lysosome destabilization. The phagocytosis of specific crystalline and particulate structures can lead to lysosome destabilization and the release of lysosomal content, including proteases. These proteases could lead to proteolytic inactivation of a negative regulator or to proteolytic activation of a positive regulator of NLRP3, resulting in inflammasome assembly (b). NLRP1 and AIM2 sense the ligand directly. The direct binding of specific ligands (muramyl dipeptide (MDP) and double-stranded DNA (dsDNA)) can lead to conformational changes in NLRP1 and AIM2, resulting in inflammasome activation. Inflammasome formation in NLRP1 is independent of ASC (c, d). NAIP proteins sense bacterial proteins resulting in the recruitment of NLRC4 and assembly of the NLRC4 inflammasome (e).

Cytokines are key mediators of the immune system. The cytokines IL-1β and IL-18 are produced in response to inflammasome activation and induce a powerful pro-inflammatory response with pleiotropic effects. IL-1β and IL-18 have amino terminal pro-domains that require cleavage by caspase-1 to generate the bioactive form. Upon cleavage, IL-1β and IL-18 are released from the cell through an unidentified mechanism and signal through the receptors IL-1 receptor (IL-1R) and IL-18R, respectively. The IL-1 receptors trigger a MyD88- and TRAF6-dependent signaling pathway that ultimately activates signal transduction pathways such as NF-κB and MAPK resulting in the release of additional pro-inflammatory cytokines, such as TNFα and IL-6 (Arend WP et al., 2008). In addition, IL-18 stimulates IFNγ production from T and NK cells promoting the antimicrobial activity of macrophages by inducing nitric oxide production (Sahoo M et al., 2011). IL-1β can also provide protection against infections by activating several host responses including neutrophil recruitment and stimulating the Th17 response (Sahoo M et al., 2011).

Inflammasome function and pyroptotic cell death are key events in the host response to bacterial pathogens. However, this is a double-edged sword as dysfunction and dysregulation can drive human inflammatory diseases, and there is a need for balance between resolution of infection and excessive inflammation. Continuing investigation into inflammasome activation mechanisms, including proposed upstream activators such as cathepsins, ROS, GBP5, PKR and PKC will drive our understanding of inflammation and hopefully elucidate novel drug targets for both antimicrobial and anti-inflammatory uses.

1.4 Aims of the study

Staphylococcus aureus is one of the major human pathogen which causes a variety of clinical infections including pulmonary infections (Lowy, 1998; Tong et al., 2015). *S. aureus* has evolved multiple comprehensive mechanisms to avoid the killing by human immune system and produce several toxins which damage not only destroy the body function but also increase replicate within the host (Scherr T.D, 2015). Studies with a number of animal models have also suggested that alpha-toxin is a key virulence factor in the pathogenesis of S. aureus infections, including pneumonia (Bubeck Wardenburg J et al., 2007), skin and soft tissue infections (Kennedy et al 2010), and bloodstream infections (Powers et al., 2012).

It was reported that the binding of α-toxin to its eukaryotic receptor A-disintegrin And Metalloprotease 10 (ADAM10) leads to the upregulation of ADAM10 activity, which is necessary for α-toxin-induced cytotoxicity (Parkin E et al., 2009). Increased ADAM10 activity in epithelial and endothelial cells disrupts the cell barrier function, and this disruption contributes to the pathogenesis of lethal lung edema.

Recent studies have implicated diverse functions of ceramide in infections. Our group has revealed that ASM-ceramide system regulates the interaction of several pathogens with host cell. In particular, our group firstly demonstrated in 2001 that *S. aureus* infection triggers ASM activation and ceramide production in human endothelial cells (Esen et al., 2001). We also identified ASM-ceramide triggered superoxide production induces degradation of tight junction protein in endothelial cells in vitro and in vivo and genetic deficiency or pharmacological inhibition of ASM protects mice against pneumonia and lethal *S. aureus* sepsis (Peng et al., 2015). Further we demonstrate that CD44 serves as a receptor for *S. aureus* in macrophages. CD44 activates the acid sphingomyelinase upon infection and triggers the release of ceramide (Li et al., 2017). However, the role of the acid sphingomyelinase/ceramide in the cellular response to bacterial toxins and the intracellular signaling pathways triggered by ceramide is largely unknown.

The present study was performed to investigate the role of the Asm-ceramide system in the infection of macrophages with *S. aureus* α-toxin. We demonstrate that *S. aureus* α-toxin activates the acid sphingomyelinase in ex vivo bone marrow-derived macrophages and triggers a release of ceramides mainly in lysosomes. The release of ceramides in lysosomes upon α-toxin treatment results in an increase of lysosomal permeability and a translocation of

cathepsin B and D from lysosomes into the cytoplasm. Cathepsin B associates with the inflammasome protein Nlrc4, activates the inflammasome and mediates the release of IL-1.

2 Materials

2.1 Chemicals

Aceticacid(C2H4O2)	Merck,Darmstadt
Acetone	Sigma-AldrichChemieGmbH,Steinheim
Amitriptyline	Sigma-AldrichChemieGmbH,Steinheim
Chloroform($CHCl_3$)	Applichem GmbH, Darmstadt, Germany
Ethylenediamine Tetraacetic Acid (EDTA)	Serva Electrophoresis GmbH, Heidelberg
Evan's Blue Dye	Sigma-Aldrich Chemie GmbH, Steinheim
Ethanol(C_2H_5OH)	Sigma-Aldrich Chemie GmbH, Steinheim
HEPES	Carl-Roth GmbH & Co, Karlsruhe
Hydrochloric acid (HCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium chloride (MgCl2)	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium sulphate (MgSO4)	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol (CH3OH)	Fluka Chemie GmbH, Buchs
Mowiol	Kuraray Specialities Europe GmbH, Frankfurt
NP-40(Igeppal)	Sigma-Aldrich Chemie GmbH, Steinheim
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium dihydrogenphosphate(KH2PO4)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium acetate (CH3COONa)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium chloride (NaCl)	Carl-Roth GmbH & Co, Karlsruhe
Sodium dodecyl sulphate (SDS)	Serva Electrophoresis GmbH, Heidelberg
Sodium fluoride (NaF)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium hydroxide (NaOH)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium phosphate (Na_2HPO_4)	Merck, Darmstadt
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma-Aldrich Chemie GmbH, Steinheim
Tris-HCl and Tris-Base	Carl-Roth GmbH & Co, Karlsruhe
Triton X-100	Sigma-AldrichChemie GmbH, Steinheim
Tween-20	Sigma-Aldrich Chemie GmbH, Steinheim
Alpha-Hemolysin (α-toxin)	Sigma-Aldrich Chemie GmbH, USA

Cathepsin B Inhibitor (CA-074Me)	Sigma-Aldrich Chemie GmbH, USA
Cathepsin D inhibitor (Pepstatin A)	Sigma-Aldrich Chemie GmbH, USA

2.2 Tissue culture materials

Cell dissociation buffer enzyme-free	Gibco/Invitrogen, Karlsruhe
MEM	Gibco/Invitrogen, Karlsruhe
Fetal Calf Serum (FCS)	Gibco/Invitrogen, Karlsruhe
L-Glutamine	Gibco/Invitrogen, Karlsruhe
MEM non-essential aminoacids	Gibco/Invitrogen, Karlsruhe
Penicilin/Streptomycin	Gibco/Invitrogen, Karlsruhe
Sodium pyruvate	Gibco/Invitrogen, Karlsruhe
Trypsin	Gibco/Invitrogen, Karlsruhe

2.3 Antibodies

Anti ASC rabbit IgG	Alexis-biochemical, CA, USA
Anti ceramide (clone 15B4) mouse IgM	Glycobiotech, Kükels, Germany
Anti Caspase-1 rabbit IgG	Santa Cruz Biotechnology, CA, USA
Anti cathepsin B goat IgG	R&D Systems, Wiesbaden-Nordenstadt, Germany
Anti cathepsin D goat IgG	R&D Systems, Wiesbaden-Nordenstadt, Germany
Anti Lamp-1 rat IgG	Abcam, Cambridge,UK
Anti Nlrp3 mouse IgG	Adipogen life sciences, CA, USA
anti-Nlrc4 rabbit IgG	lifeSpan Biosciences,Seattle, WA , USA
FITC-anti-goat IgG	Jackson Immunoresearch, West Grove, PA, USA
Cy3-anti-mouse IgM F(ab')2	Jackson Immunoresearch, West Grove, PA, USA
Cy3-anti-rat IgG	Jackson Immunoresearch, West Grove, PA, USA
FITC-anti-mouse IgG	Jackson Immunoresearch, West Grove, PA, USA

FITC-anti-rat IgG	Jackson Immunoresearch, West Grove, PA, USA
FITC-anti-rabbit IgG	Jackson Immunoresearch, West Grove, PA, USA
IL-1β ELISA kit	R&D Systems, Wiesbaden-Nordenstadt, Germany
TNF-α ELISA kit	R&D Systems, Wiesbaden-Nordenstadt, Germany

2.4 Animals

Acid sphingomyelinase (Asm)-deficient mice (Horinouchi K et al., 1995) (sphingomyelin phosphodiesterase 1 knockout; *Smpd1−/−*) and wildtype littermates (WT) mice were maintained on a C57BL/6 background. The genotype was determined by polymerase chain reaction. Mice were used at an age between 6 to 8 weeks to avoid sphingomyelin accumulation (Carpinteiro A et al., 2015). Mice were bred in the animal facility of the University of Duisburg-Essen under specific pathogen-free (SPF) conditions, according to the criteria of the Federation of Laboratory Animal Science (FELASA). All experiments were done according to the rules of the FELASA.

2.5 Equipment

Fluorescence Microplate Reader	BMG Labtech, Offenburg, Germany
Cell culture incubator	ThermoFisher scientific, MA, USA
Cell culture flask	Coring inc., NY, USA
Cell scraper	TPP, Trasadingen, Switzerland
Cell culture,6, 24 well plate	Coring inc., NY, USA
Hybond ECL nitrocellulose membrane	GE Healthcare, USA
Leica TCS SP5 confocal microscope	Leica Microsystems, Wetzlar, Germany
SpeedVac (Vacuum Concentrator)	Bachofer, Reutlingen, Germany
Portable Datalogging Spectrophotometer	Bachofer, Reutlingen, Germany
Rotary agitator	Neolab Migge Laborbedarf-Vertriebs GmbH,Germany
Parafilm	Peckiney, Chicago, IL, USA
Thermomixer	Eppendorf, Germany
Microscopic slides	Engelbrecht Medizin und labortechnik

TYPHOO 950Typhoon FLA 9500 laser scanner	GE Healthcare Life Sciences, USA

2.6 Buffer and Solutions

10 ×Alkalder wasch puffer	100mM Tris/HCl pH 9.5
	100 mM NaCl
HEPES	132mM NaCl
	20mM Hepes pH 7.4
	5mM KCl
	1mM CaCl2
	0.7 mM MgCl2
	0.8 mM MgSO4
Mowiol	6 g Glycerol
	2.4 g Mowiol
	6 ml ddH2O
	12 ml 0.2 M Tris-Base, pH 8.5
	0.1% DABCO
Phosphate buffered saline (PBS), pH 7.4	137mM NaCl
	2.7 mM KCl
	10 mM Na2HPO4
	1.8 mM KH2PO4
Running buffer	25 mM Tris
	192 mM glycine
	0.1 % SDS
Sample buffer (5X)	250 mM Tris pH 6.8
	20 % Glycine
	4 % SDS
	8 % β-mercaptoethanol
	0.2 % bromophenol blue
TBS-T	20 mM Tris
	150 mM NaCl
	0.05 % Tween 20
Transfer buffer	10 mM NaHCO3
	3 mM Na2CO3

	10 % Methanol
Trypsin	0.25% Trypsin
	5 mM Glucose
	1.3 mM EDTA
HEPES/Saline (H/S)	200mM HEPES
	1.32mM NaCl
	10mM $CaCl_2$
	7mM $MgCl_2$
	8mM $MgSO_4$
	54mM KCl
0.1%SDS-Lysis buffer (RIPA)	25mM Hepes pH7.3
	0.1% SDS
	0.5%Sodium Deoxycholate
	1% triton
	10mM EDTA
	10mM NaPP(Na-pyrophasphate)
	10mM NaF
	125mM NaCl
	10 μg/mL aprotinin/leupeptin
Digitonin lysis buffer	150mM NaCl
	50mM HEPES
	25~50mg
	10 μg/mL aprotinin/leupeptin
PFA	2.5mlPFA stocksolution
	7.5 ml PBS
PFA stock solution	8 g PFA
	100 ml PBS
ASM-Assay Lysis buffer	250mM CH_3COONa
	1.3mM EDTA
	1%NP40
	pH5
ASM Assay buffer	250mM CH_3COONa
	1.3mM EDTA
	pH5

3 Methods

3.1 Isolation and cultivation of murine bone marrow derived macrophages

Bone marrow derived macrophages (BMDMs) were fresh lyisolated by MEM medium from 6-8 week old C57BL6 wild-type (WT) mice Legs or sphingomyelin phosphodiesterase 1 knockout (*Smpd1*$^{-/-}$) mice legs, which are deficient in Asm activity. Briefly, mice were sacrificed, femurs and tibias were flushed with minimum essential medium (MEM; Gibco, UK) supplemented with 10% fetal bovine serum (Gibco), 10 mM HEPES (Roth GmbH; pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). The samples were passed through a 23G needle to obtain single cells and cultured for 24 h in small tissue-culture flasks. Cells were washed and 3×10^4 or 1.5×10^5 non-adherent cells were cultured in 24- or 6-well plates in MEM with 20% L-cell supernatant as a source of macrophage colony-stimulating factor.

We used 1×10^5 cells for all microscopy studies, 1×10^6 cells for measurement of the acid sphingomyelinase and ceramides and western blot studies and 2×10^6 cells for immunoprecipitations, respectively. If the cells were used for immunofluorescence and confocal microscopy studies, we cultured them on small glass slides fitting into the wells of a 24-well plate. For all other experiments, macrophages were directly cultured in the 6-well plate. Fresh MEM/L-cell supernatant media was applied after 4 days of culture. Macrophages mature within the next 6 days and were used on day 10 of culture.

3.2 Infection experiments

S. aureus α-toxin was obtained from Sigma, dissolved in sterile PBS and used at a final concentration of 10 μg/mL to stimulate the cells for the indicated times. Cells were pre-treated with amitriptyline (25 μM, Sigma) for 60 min in MEM supplemented with 10 mM HEPES (pH 7.4) or with the cathepsin B inhibitor CA-074Me (50 μM, Sigma) for 30 min, or with the cathepsin D inhibitor Pepstatin a (25 μM, Sigma) for 20 min.

3.3 Assay for acid sphingomyelinase activity

Acid sphingomyelinase activity was measured with green fluorescent BODIPY-FL_{C12}-sphingomyelin (Thermo Fisher Scientific) as a substrate. Briefly, cells

were infected or left untreated, harvested and lysed in 250 mM sodium acetate (Sigma) and 1% Nonidet P-40 (pH 5.0; Sigma) for 5 min on ice. Cells were further disrupted by sonification for 10 min in an ice bath sonicator (Bandelin Electronic). The protein concentration was measured by a Bradford protein assay (BioRad), and 5 μg of protein in 20 μL lysis buffer was added to 250 mM sodium acetate (pH 5.0) containing 100pmol BODIPY-FL_{C12}-sphingomyelin. The samples were incubated at 37℃ for 1 h with shaking at 300 rpm. The reaction was stopped by the addition of 1 mL chloroform: methanol (2:1, v/v) followed by centrifugation for 5 min at 14 000 rpm. The lower phase was dried in a SpeedVac Concentrator (Thermo Fisher Scientific) and resuspended in chloroform: methanol (2:1, v/v). The samples were spotted on a thin-layerchromatography (TLC) plate (Merck, Germany), separated with chloroform: methanol (80:20, v/v), scanned with a Typhoon FLA 9500 laser scanner (GE Healthcare Life Sciences, USA), and analyzed with ImageQuant software (GE Healthcare Life Sciences).

3.4 Immunostainings

Macrophages were cultured on coverslips, washed and stimulated with *S. aureus* α-toxin as above or left untreated in MEM supplemented with 10 mM HEPES, pH 7.4. The medium was removed and cells were immediately fixed in 4% paraformaldehyde (PFA; Sigma) in PBS (pH 7.4) for 10 min. Cells were then washed 3 times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were washed again with PBS and blocked for 15 min with 5% FCS in HEPES/Saline (H/S) buffer consisting of 132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM CaCl2, 0.7 mM MgCl2, and 0.8 mM MgSO4 to block non-specific binding. Cells were washed again and then incubated for 60 min with anti-ceramide antibodies (diluted 1:100, mouse monoclonal IgM; Glycobiotech), anti-Lamp-1 (1:200, Abcam #24245), anti-cathepsin D (1:100, R&D #AF1024), anti-cathepsin B (1:100, R&D #AF965), anti-Nlrc4 (1:50, Lifespan Biosciences #Ls-c148271-50), anti-NLRP3 (1：200，Adipogen life sciences. #AG-20B-0014-C100)or anti-Asc (1:100, Santa Cruz Inc. #sc30153), respectively. All antibodies were diluted in PBS, 5% FCS. Samples that were co-stained with two antibodies were incubated consecutively with the antibodies and the samples were washed 3 times with 0.05% Tween-20/PBS (PBST) for 5 min between the incubations. After the last incubation, cells were washed again 3 times for 5 min each and incubated with FITC- or Cy3-conjugated $F(ab')_2$fragments of secondary antibodies (final

concentration of all antibodies, 1.5 μg/mL; diluted in 5% FCS/PBS) corresponding to the primary antibodies. All antibodies were from Jackson Immuno Research.

Finally, the samples were washed again 3 times with 0.05% Tween 20/PBS and once in PBS and were mounted with Mowiol (Kuraray Specialities Europe GmbH). Samples were analyzed with Leica TCS SP5 confocal microscope and Leica LCS software (Leica Microsystems).

3.5 Western blot and immunoprecipitation studies

Whole cell lysates: Cells (1×10^6) were lysed in ice-cold 0.1% sodium-dodecyl sulfate (SDS), 25 mM HEPES, 0.5% deoxycholate, 0.1% Triton X-100, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 10 μg/mL aprotinin/leupeptin (all from Sigma). Lysis was allowed to complete for 5 min on ice. Samples were then centrifuged at 22,000 ×g for 5 min at 4℃ to pellet insoluble material; the supernatants were collected and added to 5x SDS Laemmli sample buffer. Samples were incubated at 95^{o}C for 5 min and proteins were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.05% Tween 20. Blots were incubated overnight at 4℃ withanti-cathepsin D (diluted 1:1000, R&D #AF1024) or anti-cathepsin B (1:1000, R&D #AF965), anti Caspase-1(1:1000, Alexis ALX-210-804) respectively. Blots were washed and developed with alkaline phosphatase-coupled secondary antibodies (1:20 000; Santa Cruz Biotechnology Inc.) using the Tropix chemoluminescence system (Amersham Pharmacia Biotech Inc.).

Separation of cytoplasmic from lysosomal proteins: Cells (1×10^6) were lysed in ice-cold 50 μg/mL Digitonin, 150 mM NaCl, 50 mM HEPES (pH 7.4), 10 μg/mL aprotinin/leupeptin (all from Sigma), incubated on ice for 10 min and centrifuged at 2,000 × g for 5 min at 4℃ to pellet insoluble material. The supernatants were collected, processed as above and blotted with anti-cathepsin B or anti-cathepsin D antibodies.

Co-immunoprecipitations: Cells (2×10^6) were stimulated with *S. aureus* α-toxin or left untreated, lysed in 0.1%SDS, 25 mM HEPES, 0.5% deoxycholate, 0.1% Triton X-100, 10

mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 10 μg/mL aprotinin/leupeptin (all from Sigma) for 5 min and centrifuged at 22,000 × g, 4^{o}C for 5 min. Samples were incubated on a nutator for 4 hrs with anti-Nlrc4 antibodies (1μg/mL, LifeSpan BioSciences #Ls-c148271-50) or anti-NLRP3 (1μg/mL, Adipogen life sciences #AG-20B-0014-C100) followed by incubation with 30 μL protein A/G agarose (Santa Cruz Inc.) for 45 min. The immune-complexes were washed 6 times in lysis buffer as above and proteins were eluted in 1x SDS Laemmli sample buffer. Samples were processed as above and blotted for cathepsin B or cathepsin D.

3.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA assays for IL-1β and TNF-α were performed as described by the vendor (R&D). We used cell pellets and the culture supernatants for ELISA assays.

3.7 Statistics

Data are expressed as arithmetic means ± standard deviation (SD) unless otherwise indicated. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was used to test between-group and within-group differences; pairwise comparisons were performed with Students t-test. Comparisons of survival variables were performed with log-rank tests. Statistical significance was set at the level of $p < 0.05$. All data were obtained from independent measurements. The GraphPad Prism statistical software program (GraphPad Software, USA) was used for the analyses.

4 Results

4.1 *S. aureus* α-toxin activates the acid sphingomyelinase

In order to test whether *S. aureus* α-toxin activates the acid sphingomyelinase / ceramide system to induce inflammation, we infected WT and Asm-deficient BMDMs (Bone Marrow-derived Macrophages) with *S. aureus* for 5, 10, 30, 60, or 120 min or left them uninfected and then determined acid sphingomyelinase activity. These biochemical studies demonstrate a sustained activation of the acid sphingomyelinase upon treatment of wildtype (WT) macrophages with α-toxin but no Asm activity in Asm-deficient macrophages (Fig. 4.1A and B).

Fig. 4.1A

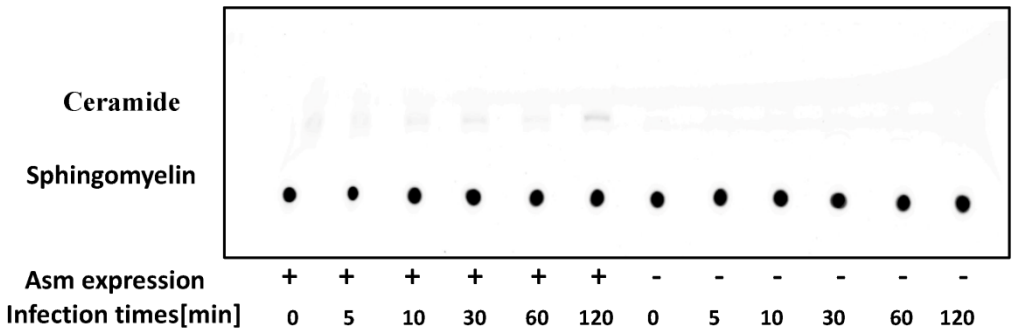


Fig. 4.1B

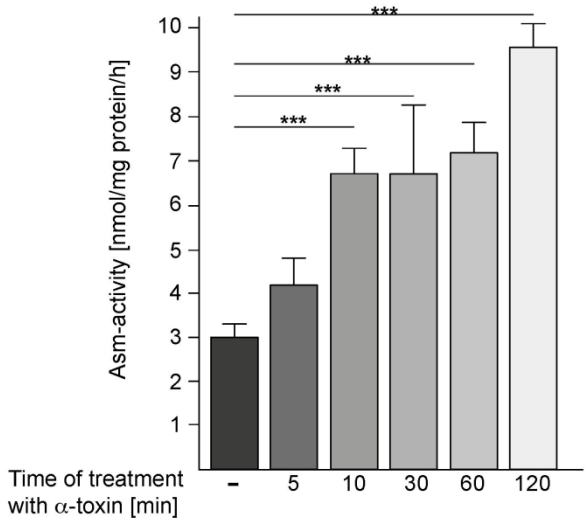


Figure 4.1 *S. aureus* α-toxin activates the acid sphingomyelinase

Bone marrow-derived macrophages were activated with 10 µg/mL *S. aureus* α-toxin for the indicated time,

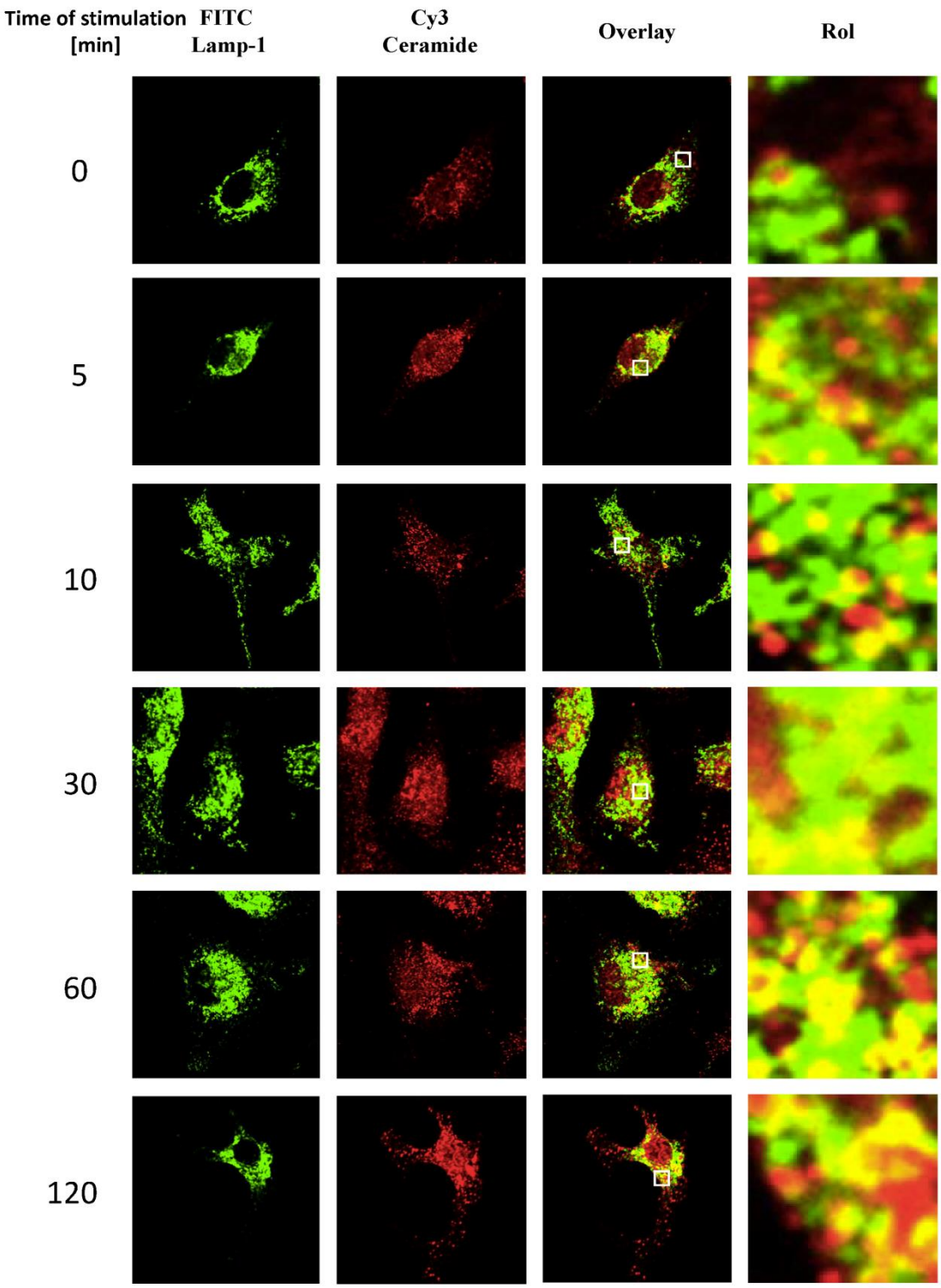
lysed and the activity of the acid sphingomyelinase (Asm) was determined by the consumption of BODIPY-FL_{C12}-sphingomyelin. Samples were extracted and separated on thin-layer chromatography (TLC) plates, which were then scanned with a Typhoon laser scanner. Panel A shows a representative result. Panel B shows the mean ± standard deviation (SD) of 3 independent experiments using Image Quant, *$p < 0.05$, ANOVA.

4.2 *S. aureus* α-toxin induces formation of ceramides within lysosomes.

Confocal microscopy studies on macrophages stained with Cy3-anti-ceramide and FITC-anti-Lamp-1 antibodies (the latter one served to detect lysosomes) revealed that most of the ceramides triggered by treatment of WT macrophages with α-toxin localized to lysosomes (Fig. 4.2A). the increase of lysosomal ceramides upon treatment with α-toxin was absent in macrophages lacking the acid sphingomyelinase (Fig. 4.2B).

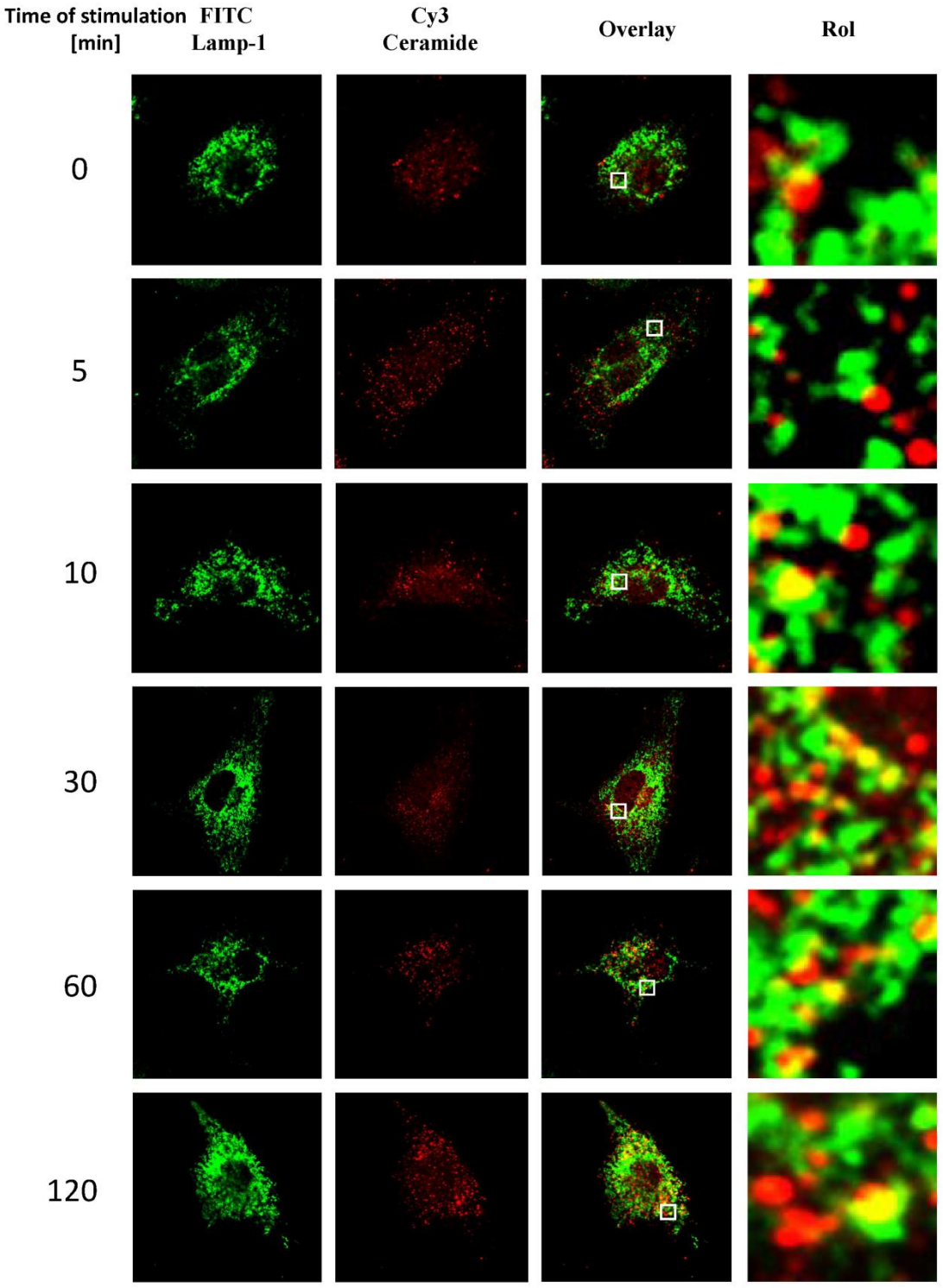
To confirm the notion of lysosomal release of ceramides upon treatment of macrophages with α-toxin, we co-stained macrophages with anti-ceramide, anti-cathepsin B and anti-cathepsin D antibodies. Cathepsin B and D also localize to lysosomes. The studies confirm a release of ceramides in lysosomes of WT macrophages, which was absent in lysosomes of cells lacking the acid sphingomyelinase (Fig.4.2 C, D and E, F). However, careful inspection of the confocal microscopy results revealed that a fraction of cathepsin B and D did not localize with ceramides after stimulation, leading us to investigate whether α-toxin induces a release of cathepsins from lysosomes.

FIG 4.2 A



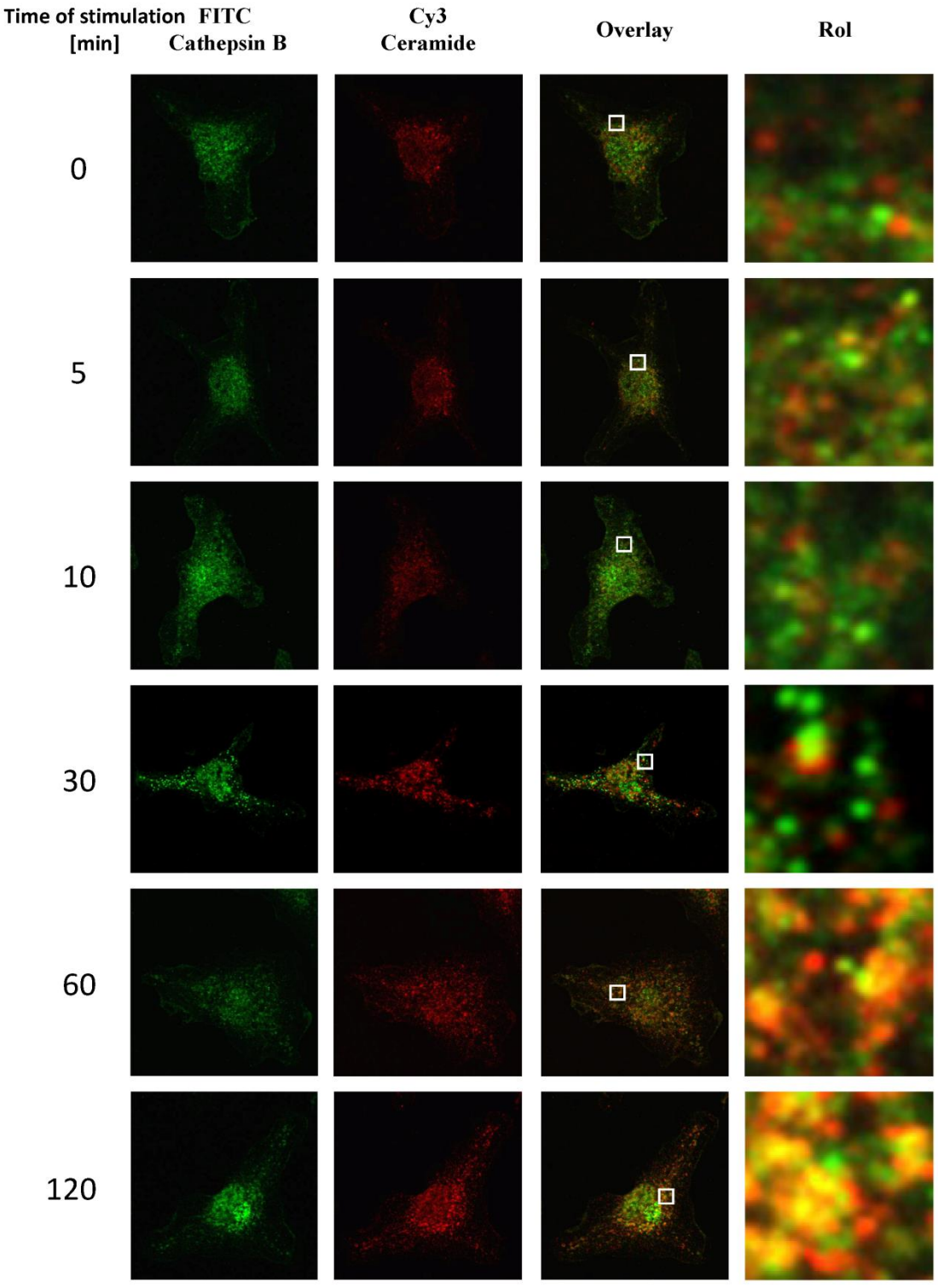
Wildtype

FIG 4.2 B


Time of stimulation [min]
FITC Lamp-1
Cy3 Ceramide
Overlay
Rol
0
5
10
30
60
120

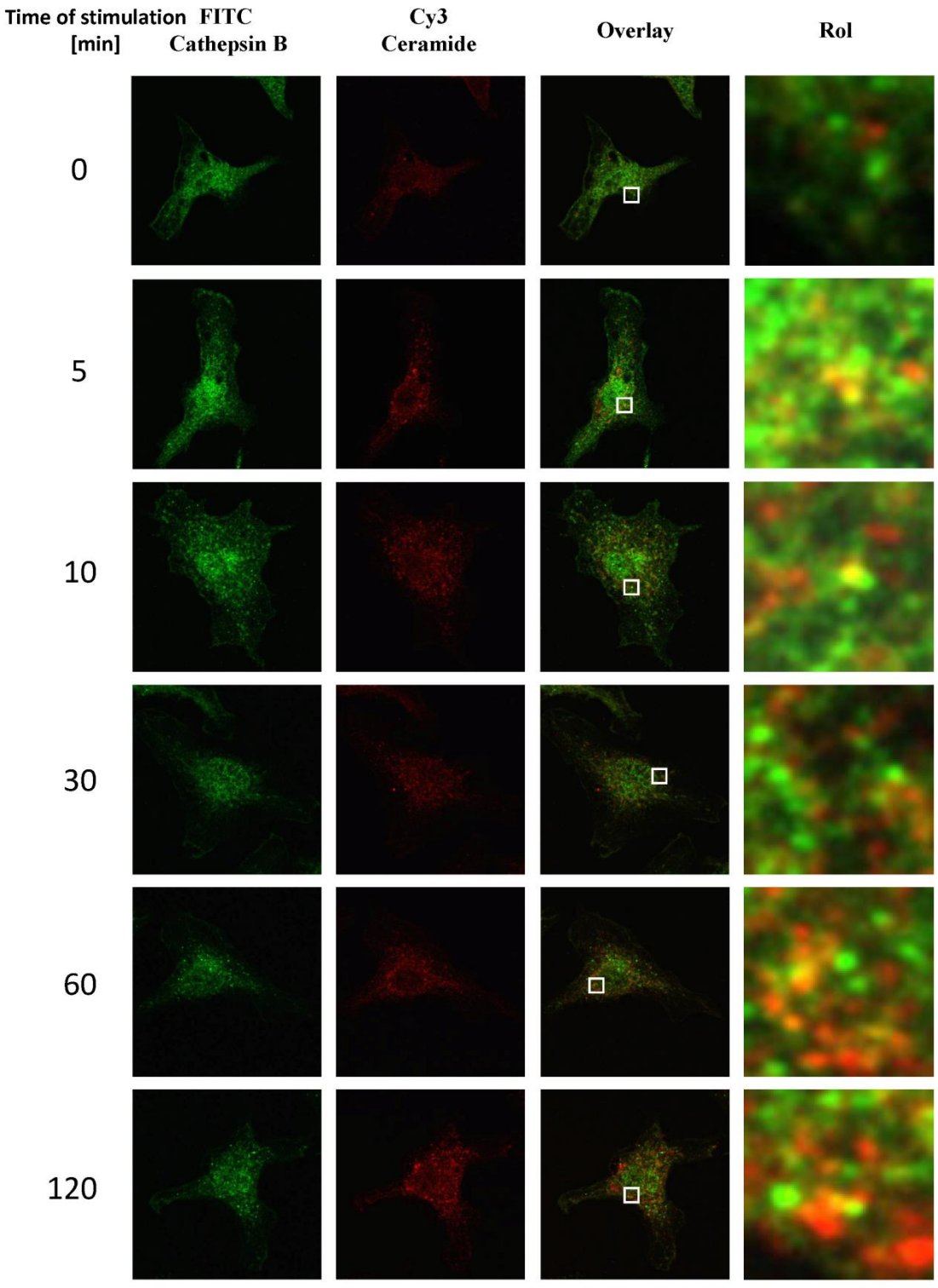
Asm$^{-/-}$

FIG 4.2 C



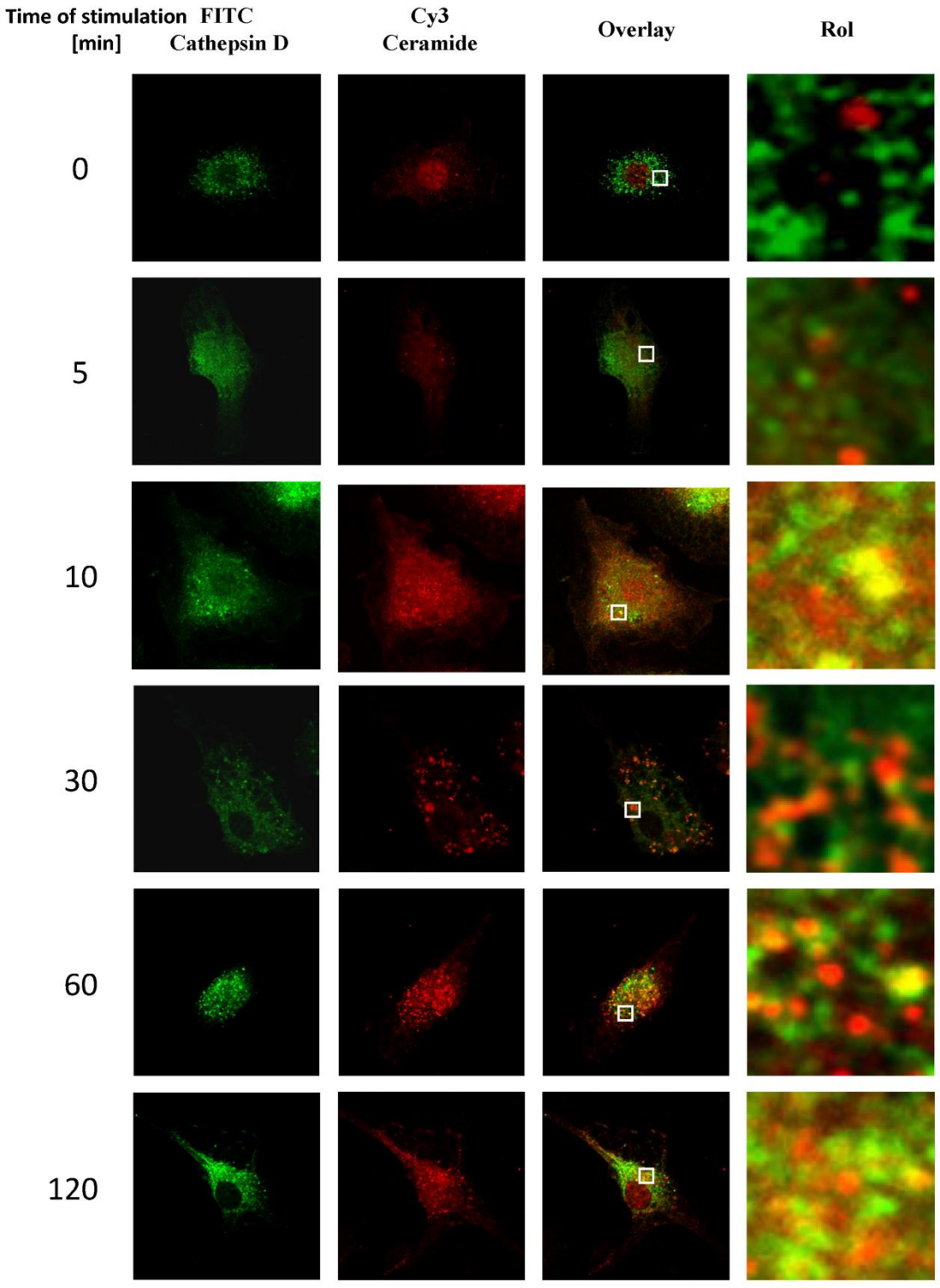
Wildtype

FIG 4.2 D


Time of stimulation [min]
FITC Cathepsin B
Cy3 Ceramide
Overlay
Rol
0
5
10
30
60
120

Asm$^{-/-}$

FIG 4.2 E



Wildtype

FIG 4.2 F

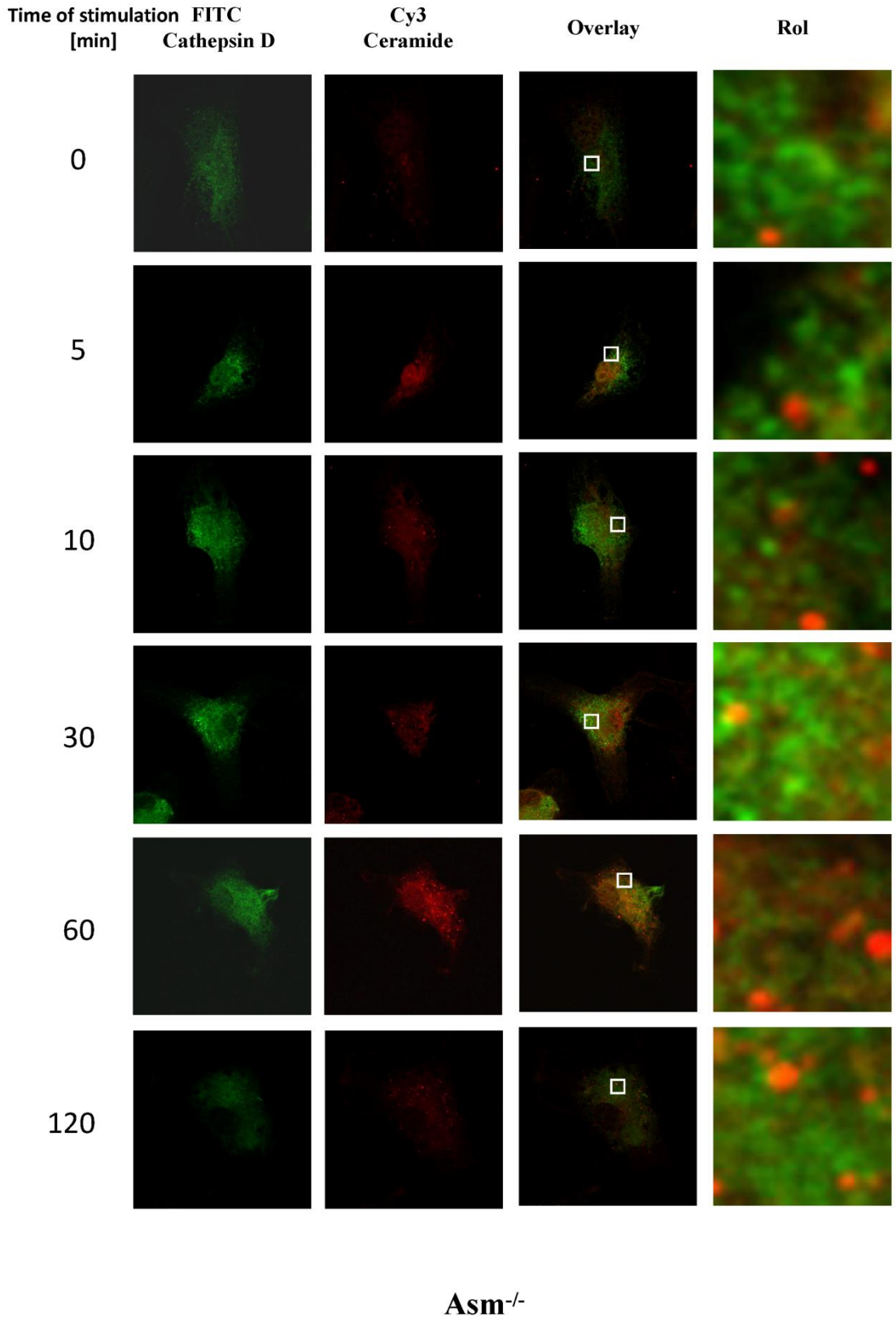


Figure 4.2 *S. aureus* α-toxin induces formation of ceramides within lysosomes.

Wild-type (WT) or Asm-deficient bone marrow-derived macrophages (BMDMs) were left untreated or were infected with *S. aureus* α-toxin. Cells were stained with FITC-labeled anti-Lamp1 antibodies and Cy3

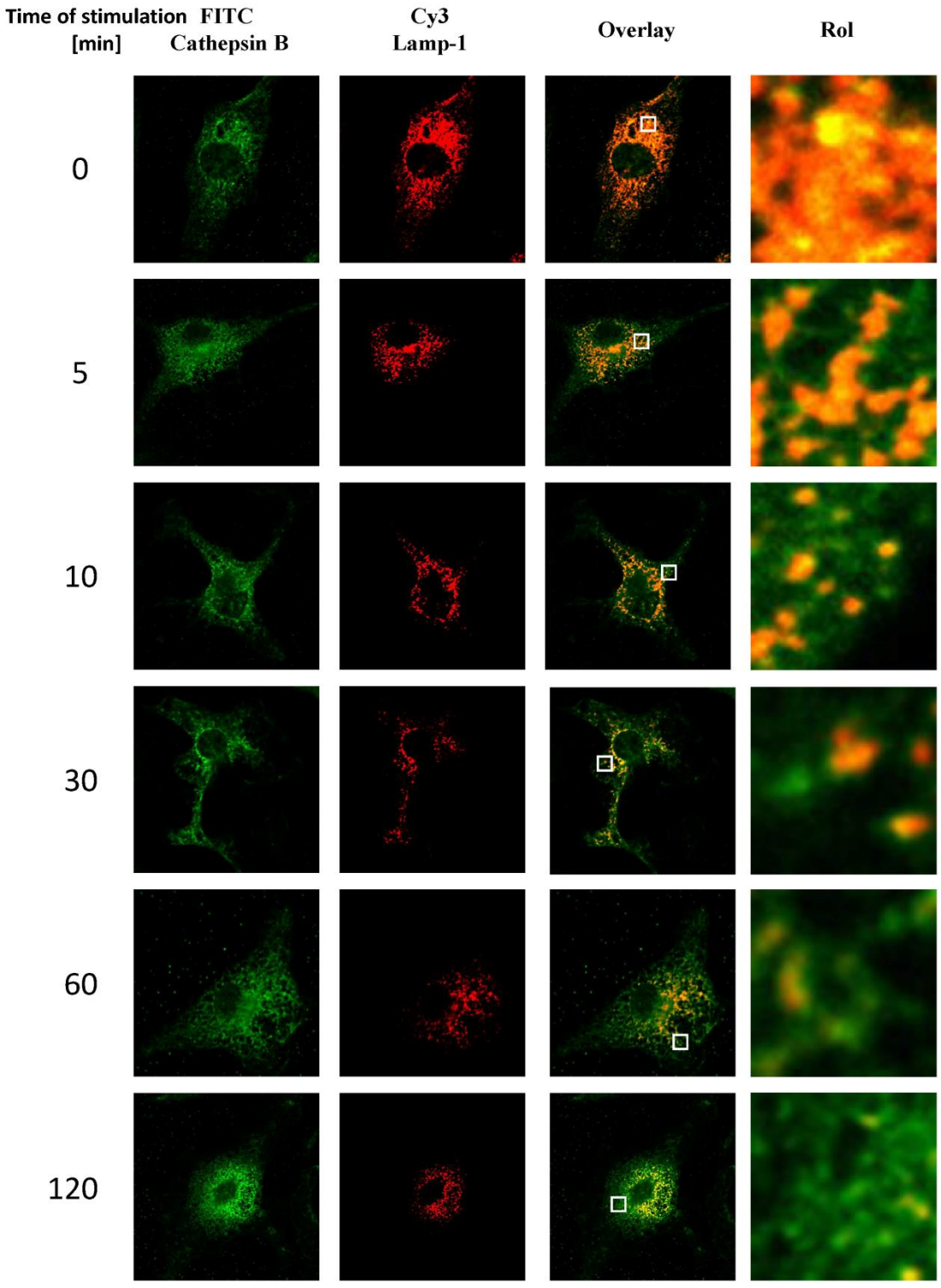
labeled anti-ceramide and were analyzed by fluorescence microscopy.

Confocal microscopy studies staining for Cy3 anti-ceramide and FITC anti-Lamp-1 (A, B), FITC anti-cathepsin B (C,D) and FITC anti-cathepsin D (E,F) as lysosomal markers reveal a lysosomal release of ceramides after stimulation with *S. aureus* α-toxin, which is absent in cells lacking the acid sphingomyelinase (Asm-/-). Shown are typical confocal microscopy results from each 4 independent studies.

4.3 *S. aureus* α-toxin triggers a release of cathepsin B and D from lysosomes into the cytoplasmvia the acid sphingomyelinase

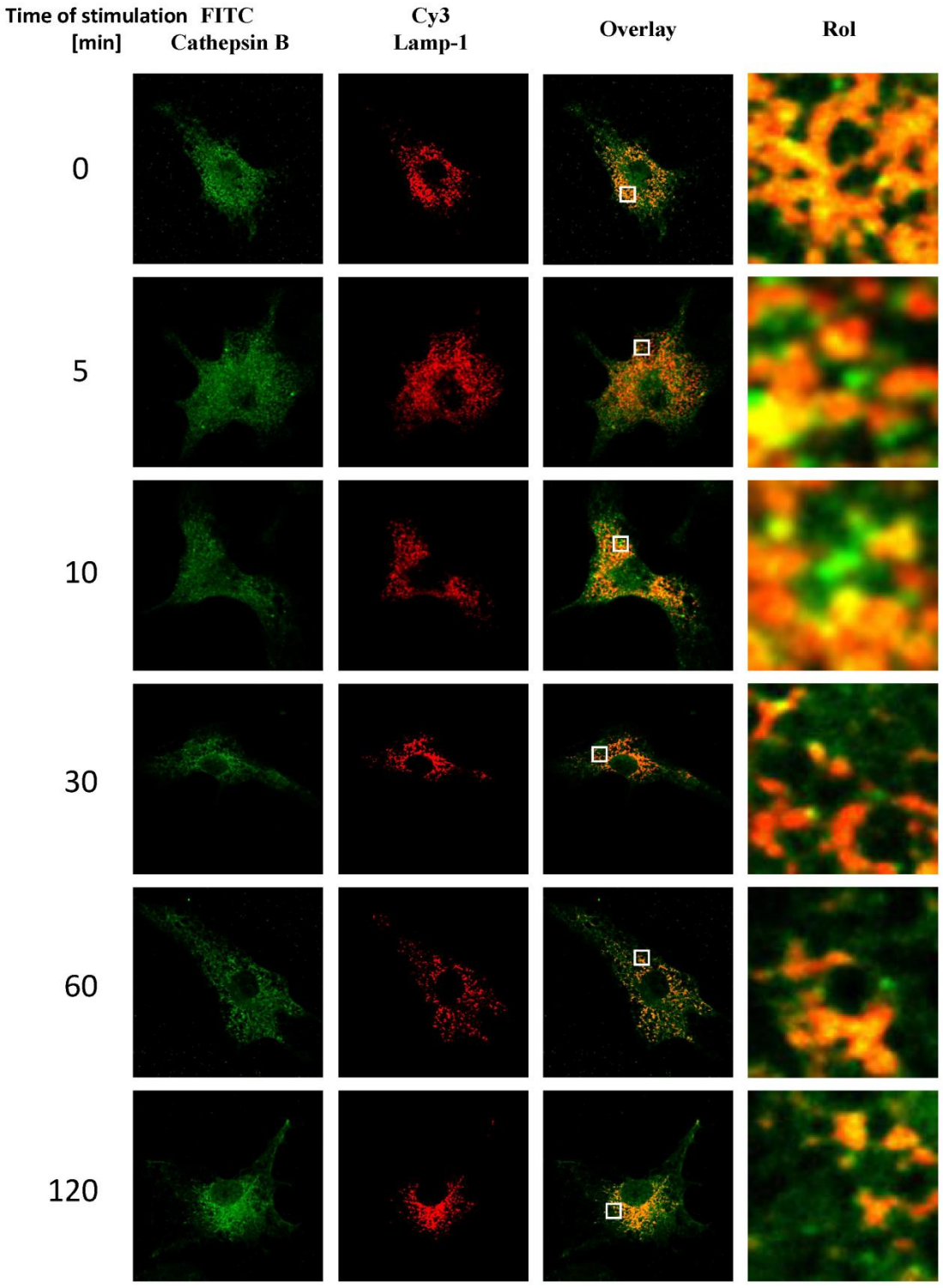
In order to investigate whether α-toxin induces a release of cathepsins from lysosomes, we co-stained macrophages prior and after stimulation with α-toxin for cathepsin B and D and Lamp-1, as marker for lysosomes. While, FITC anti-cathepsin B and D co-localized with Cy3 anti-Lamp-1 in WT and acid sphingomyelinase-deficient macrophages prior to stimulation with α-toxin (Fig. 4.3 A and C), stimulation of the cells with α-toxin resulted in a partial dissociation of the signal for cathepsin B and D and Lamp-1 in WT macrophages. This indicates a release of cathepsin B and D from lysosomes upon stimulation with α-toxin. In contrast, in acid sphingomyelinase- deficient macrophages most of the cathepsin B and D remained colocalized with Lamp-1 indicating that α-toxin had almost no effect on the localization of the two proteins in acid sphingomyelinase-deficient cells (Fig.4.3 B and D). However, a small amount of cathepsins was also released in acid sphingomyelinase-deficient cells. To confirm this notion and to quantify the release of cathepsin B and D from lysosomes upon treatment with α-toxin, we separated lysosomes from the cytoplasm and performed western blot studies for cathepsin B and D. Controls, analyzing total cell lysates, show a similar total expression of cathepsin B and D in WT and acid sphingomyelinase-deficient macrophages (Fig. 4.3 E, F and I, J). Western blot studies with cells that were lysed with digitonin to separate cytoplasmic from lysosomal proteins revealed that α-toxin induced a marked release of cathepsin B and D from lysosomes in WT cells, while much less cathepsin B and D were released from lysosomes in acid sphingomyelinase-deficient cells upon treatment with α-toxin (Fig. 4.3 G, H and K, L).

FIG 4.3 A



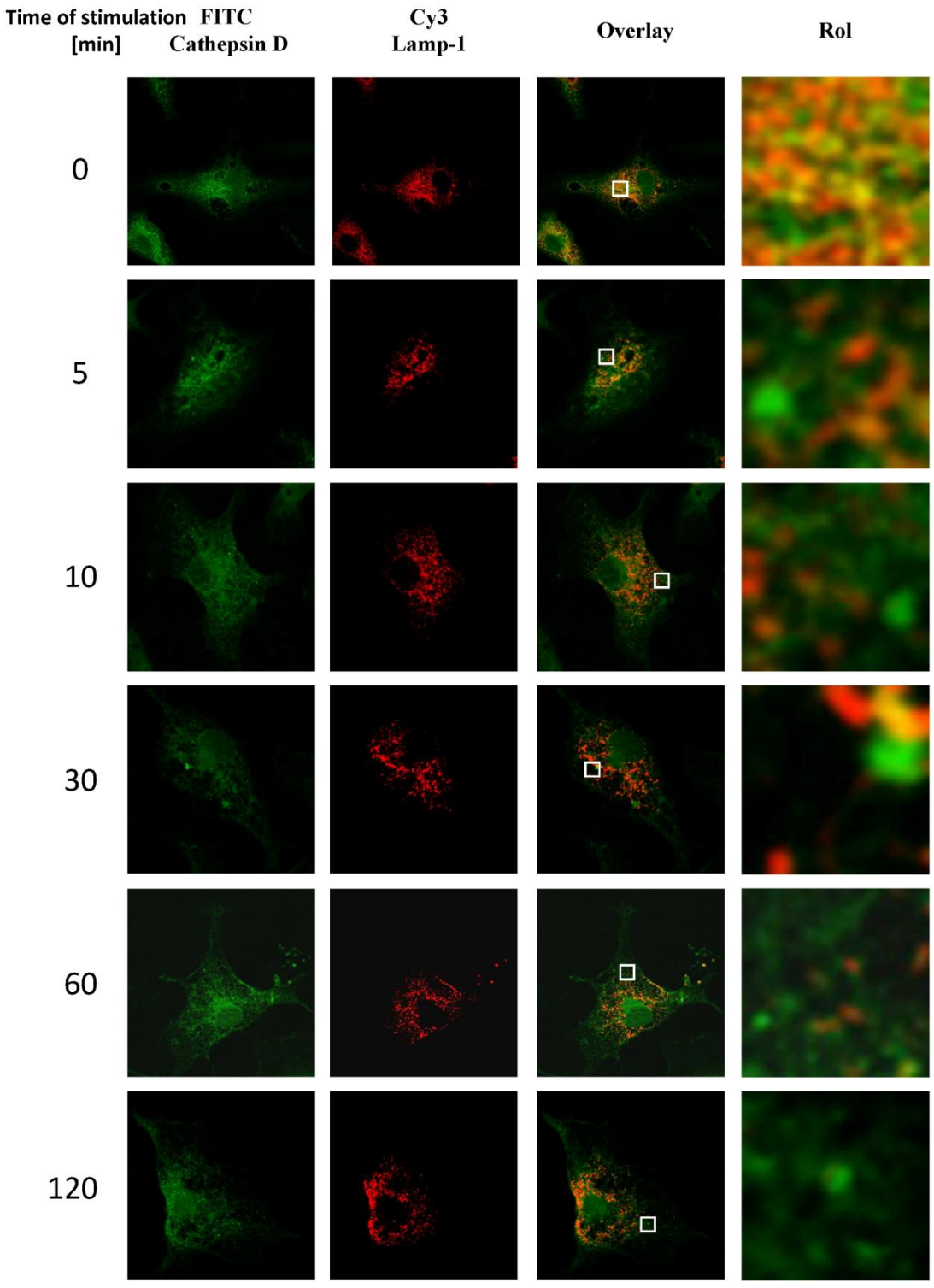
Wildtype

FIG 4.3 B


Time of stimulation [min]
FITC Cathepsin B
Cy3 Lamp-1
Overlay
Rol
0
5
10
30
60
120

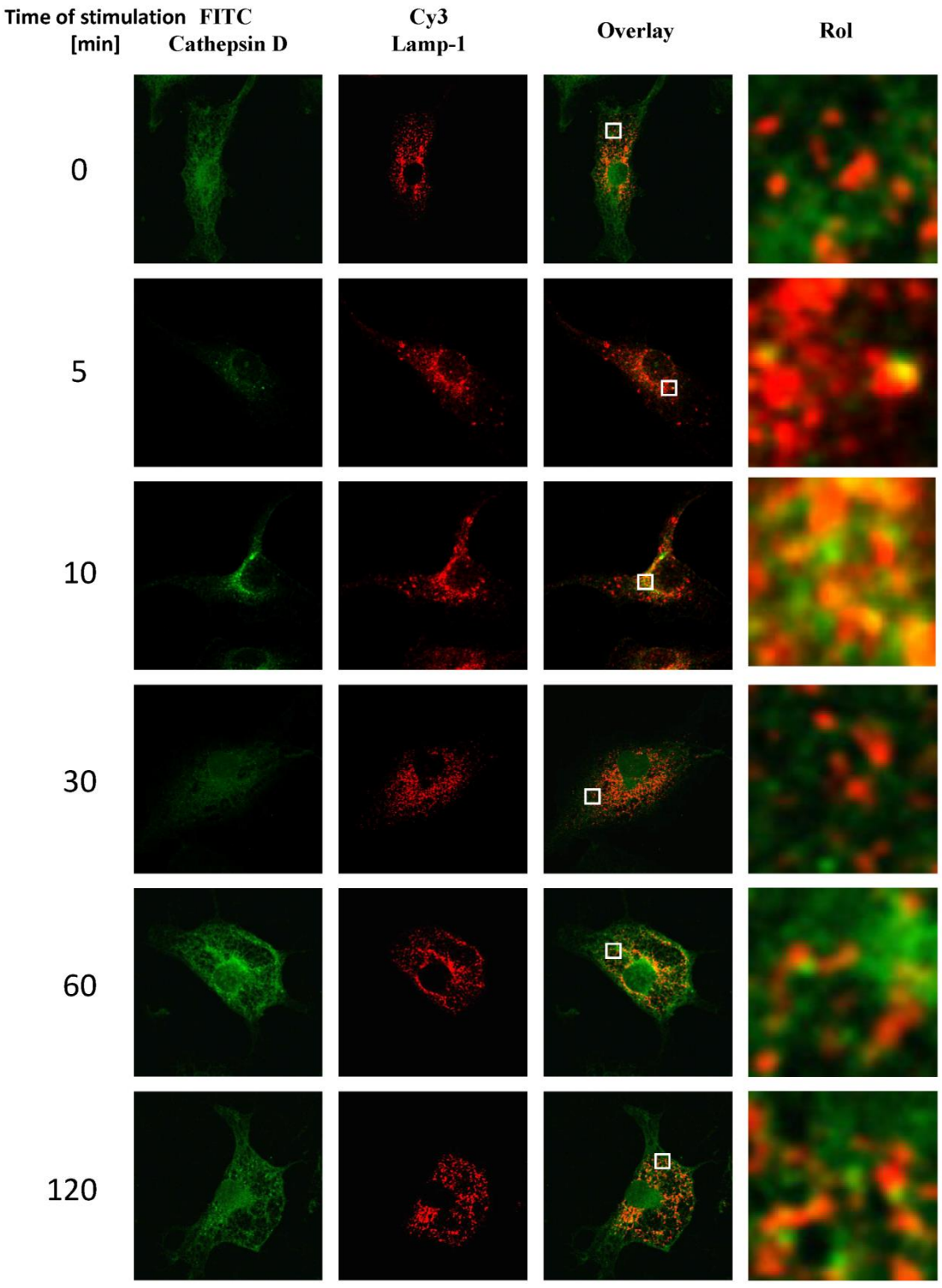
Asm$^{-/-}$

FIG 4.3 C



Wildtype

FIG 4.3 D


Time of stimulation [min]
FITC Cathepsin D
Cy3 Lamp-1
Overlay
Rol
0
5
10
30
60
120

Asm$^{-/-}$

FIG 4.3 E

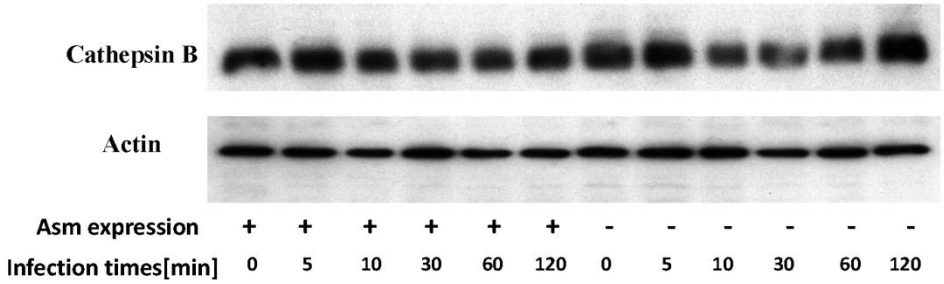


FIG 4.3 F

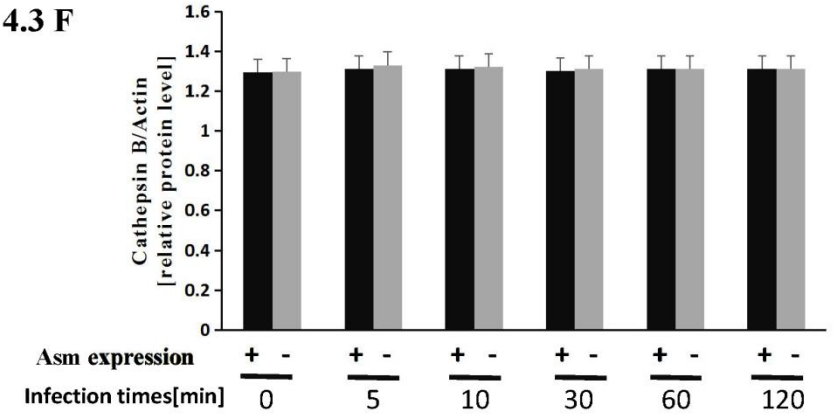


FIG 4.3 G

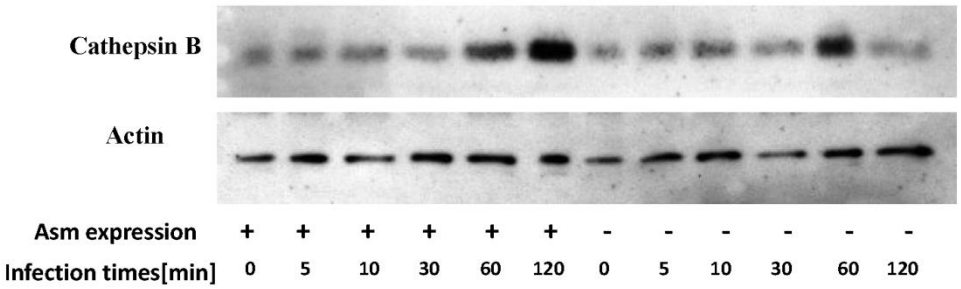


FIG 4.3 H

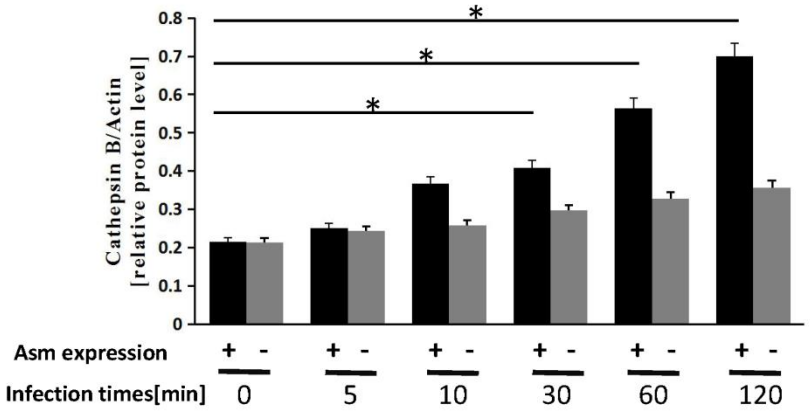


FIG 4.3 J

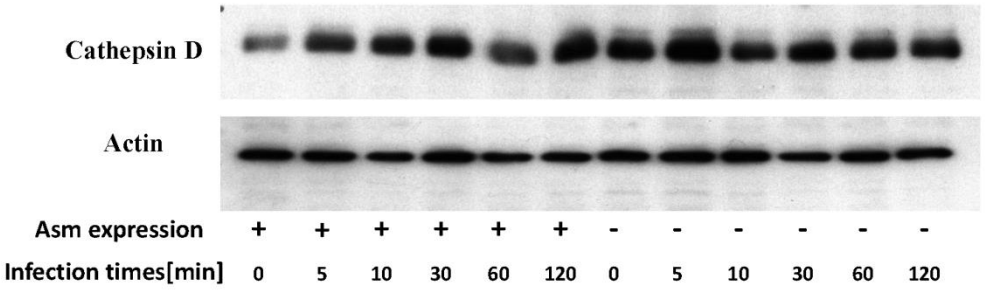


FIG 4.3 K

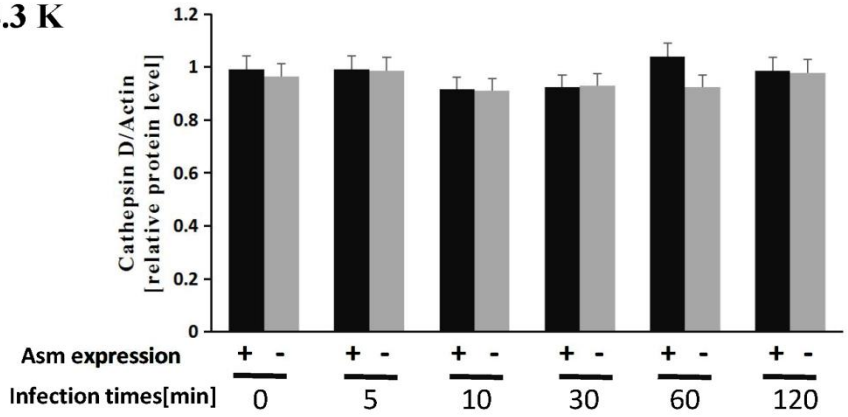


FIG 4.3 L

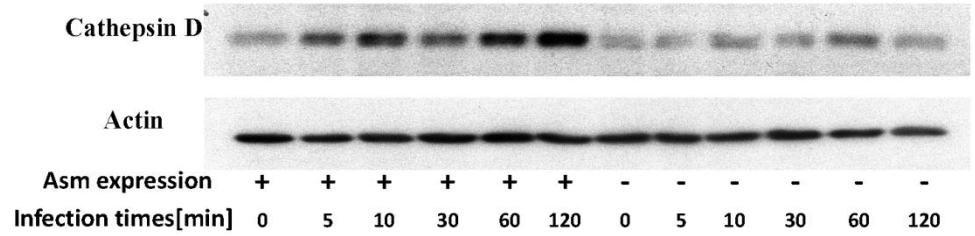


FIG 4.3 M

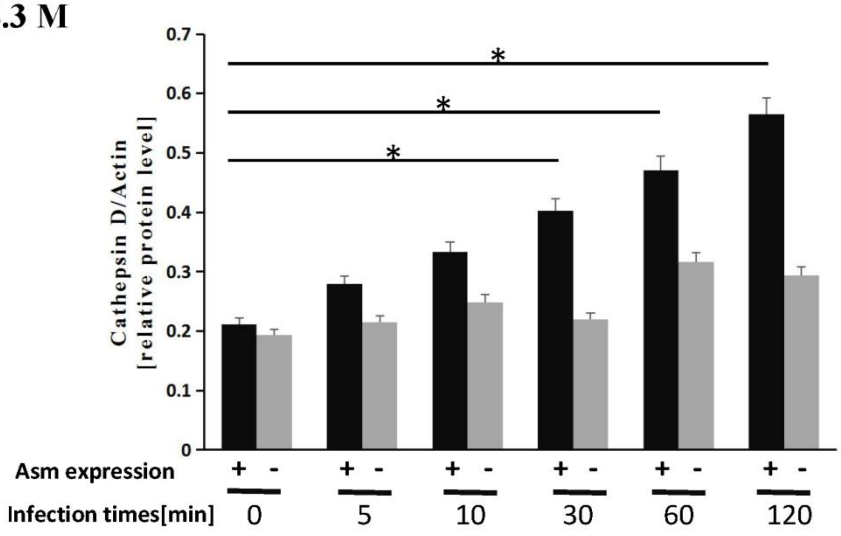


Figure 4.3 *S. aureus* α-toxin triggers a release of cathepsin B and D from lysosomes into the cytoplasm, which is prevented by acid sphingomyelinase deficiency.

(A, C) Confocal microscopy of α-toxin treated or -untreated wildtype and Asm deficient (Asm-/-) macrophages that were stained with anti-cathepsin B or anti-cathepsin D in combination with anti-Lamp-1 antibodies reveals a partial release of cathepsin B and D into the cytoplasm from lysosomes after treatment. The release is prevented by acid sphingomyelinase-deficiency (B, D). (E, G, I, K) Western blot studies demonstrate a similar expression of cathepsin B (E, G) and cathepsin D (I, K) in wildtype and acid sphingomyelinase-deficient (Asm-/-) macrophages. Separation of the cytoplasmic from lysosomal cell fractions reveals that treatment with *S. aureus* α-toxin induces a release of cathepsin B (G) and cathepsin D (K) into the cytoplasm of wildtype macrophages. Deficiency of the acid sphingomyelinase prevents the lysosomal release of cathepsins after α-toxin treatment (G, K). Actin blots served to demonstrate equal loading in all lanes. Panel F, H, J, K shows the quantification of the Cathepsin B or D by ImageJ. Results are given as mean ± SD, n=3, * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test. Please note that panel E and I were from the same lysates and therefore, the actin blots are the same. Shown are representative confocal microscopy studies or blots, respectively, from each 4 (A, B, C, D) or 3 (E, G, I, K) independent studies.

4.4 Pharmacological inhibition of the acid sphingomyelinase prevents the release of cathepsin B and D from lysosomes into the cytoplasm after *S. aureus* α-toxin treatment.

Amitriptyline has been shown to mediate a functional inhibition of the acid sphingomyelinase (Hurwitz R et al 1994, Becker KA et al 2009, Kornhuber J et al 2008, Gulbins E et al 2013). We therefore tested whether treatment of WT macrophages with amitriptyline blocks the release of cathepsin B and D. The results were consistent with the data obtained in acid sphingomyelinase-deficient cells and showed that amitriptyline inhibits the release of cathepsin B and D from lysosomes into the cytoplasm after treatment with α-toxin (Fig. 4). The residual release of cathepsin B and D into the cytoplasm in acid sphingomyelinase-deficient cells after treatment with α-toxin was further reduced by pre-treatment with amitriptyline.

FIG 4.4 A

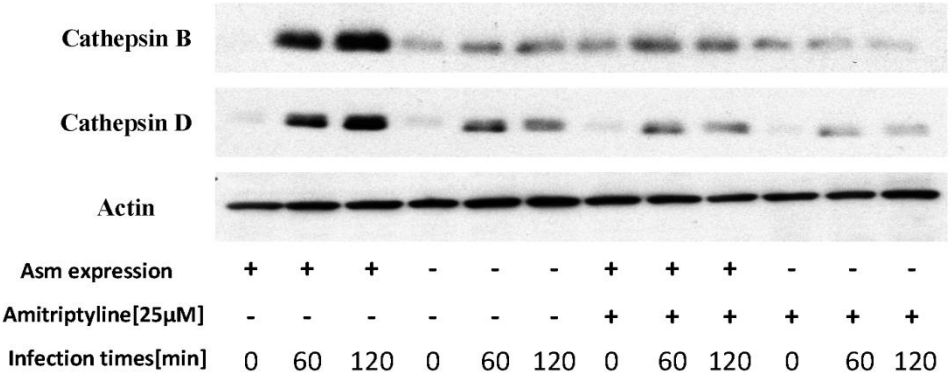


FIG 4.4 B

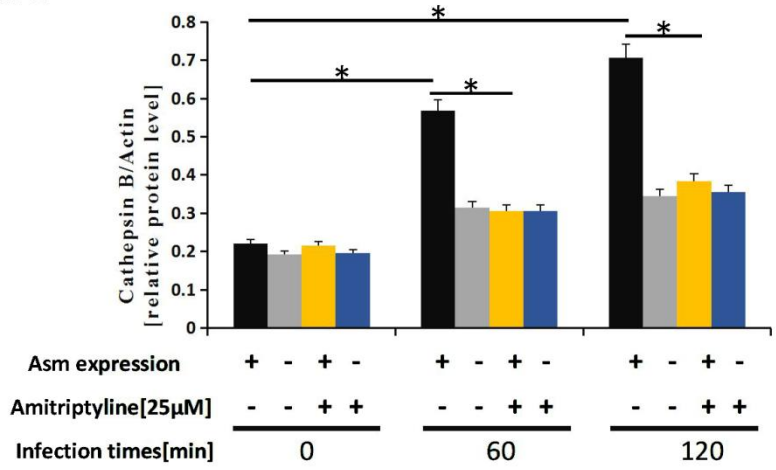


FIG 4.4 C

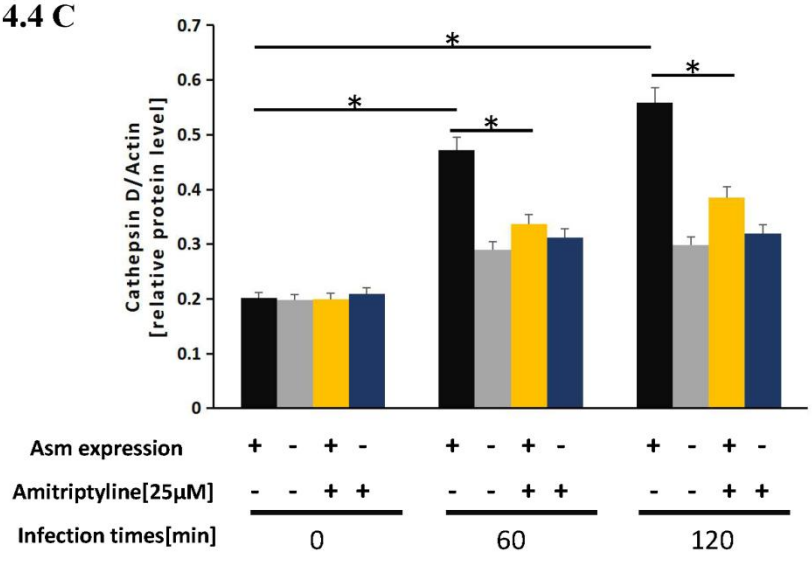


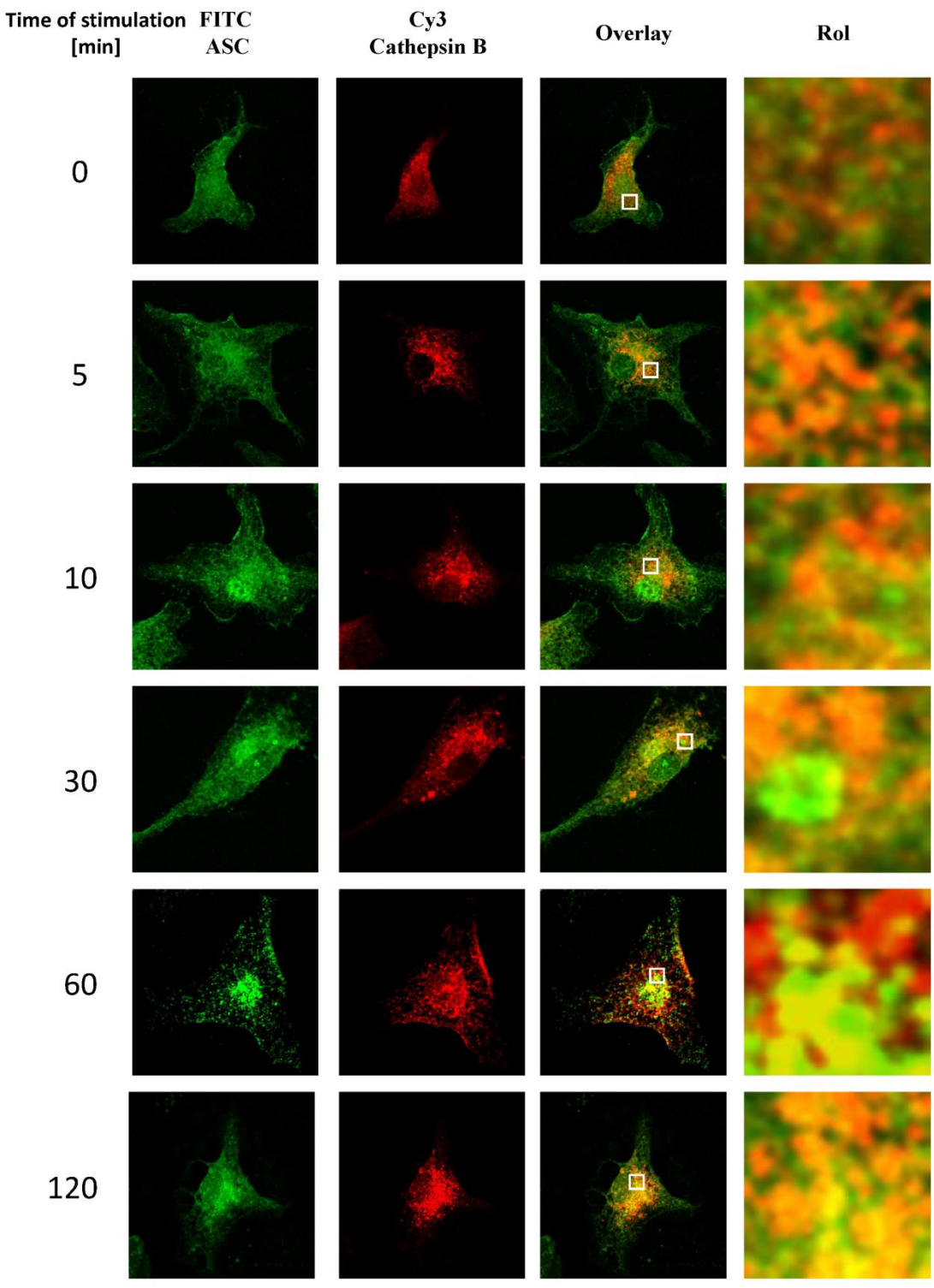
Figure 4.4 Pharmacological inhibition of the acid sphingomyelinase prevents the release of cathepsin B and D from lysosomes into the cytoplasm after *S. aureus* α-toxin treatment.

Cells were pre-treated with amitriptyline, a functional inhibitor of the acid sphingomyelinase, and the release of cathepsin B and cathepsin D from lysosomes into the cytoplasm after treatment with *S. aureus* α-toxin was determined by western blotting of cell fractions. Amitriptyline prevented the *S. aureus* α-toxin-induced release of cathepsins into the cytoplasm and even further reduced the residual release of cathepsins into the cytoplasm in cells lacking the acid sphingomyelinase ($Asm^{-/-}$). Actin blots served to demonstrate equal loading in all lanes. Displayed are representative results from each 3 independent studies. Panel B, C shows the quantification of the Cathepsin B or D by ImageJ. Results are given as mean ± SD, n=3, * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test.

4.5 Cytoplasmic cathepsin B associates with inflammasome proteins.

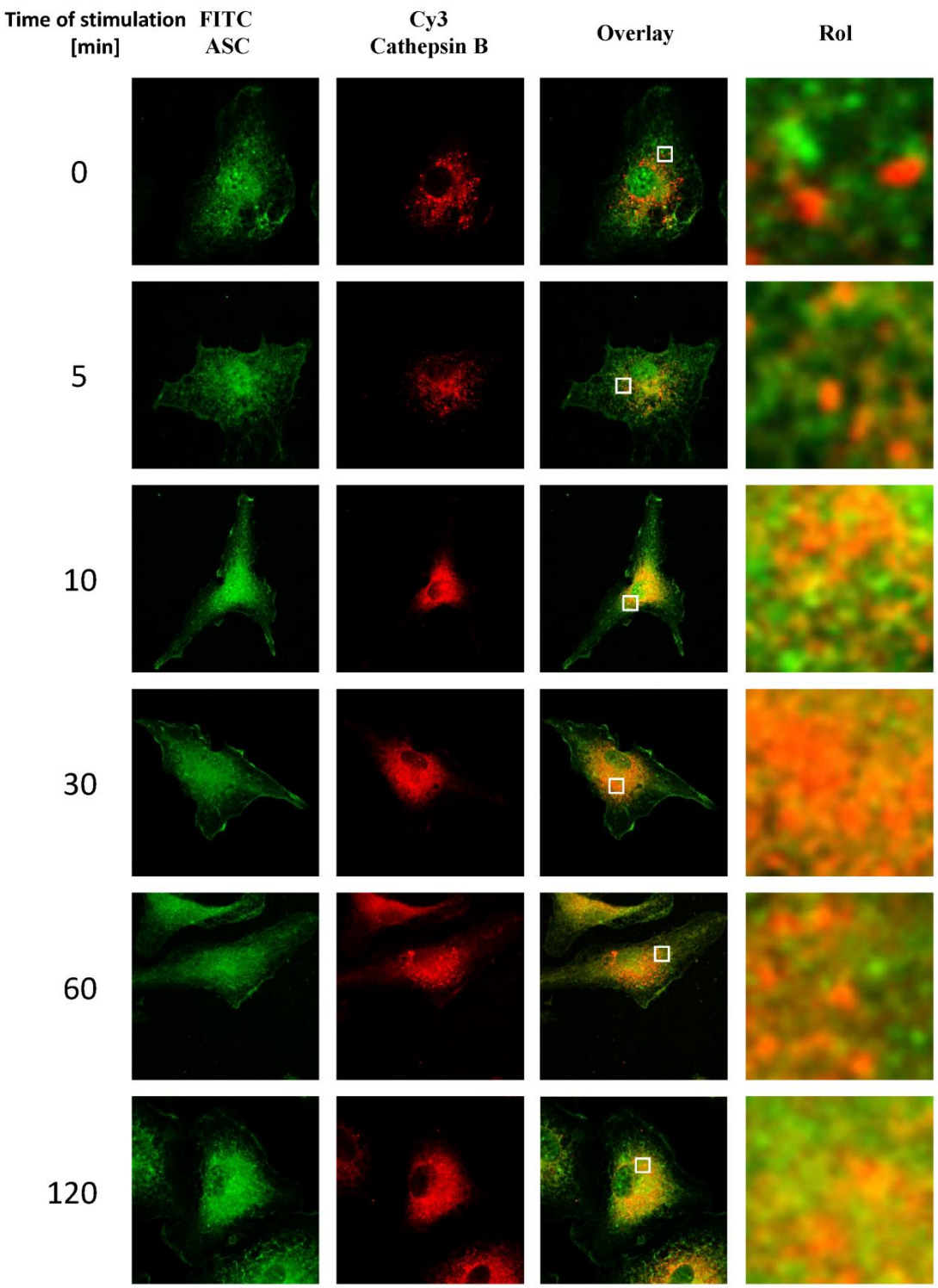
Next, we tested whether activation of the acid sphingomyelinase/ceramide system in lysosomes is linked to activation of the inflammasome and whether such a potential link is mediated by cathepsin B and/or D. To this end, we tested whether cathepsin B and D co-localize with Asc, a central regulator of the inflammasome, after treatment of macrophages with α-toxin. Confocal microscopy studies indicated a co-localization of cathepsin B with Asc (Fig. 5A) after treatment of WT macrophages with α-toxin, while much less cathepsin B co-localized with Asc in acid sphingomyelinase-deficient cells(Fig. 5B) . In contrast, confocal microscopy revealed that most of the cathepsin D did not co-localize with Asc (Fig. 5C, D). However, confocal microscopy studies certainly do not allow to determine whether two proteins really associate. We therefore performed co-immunoprecipitation experiments to test whether α-toxin induces a true association of Nlrc4, a downstream target of Asc and known to form a complex with Asc in the active inflammasome multiprotein complex, with cathepsin B. These studies demonstrate that Nlrc4 co-precipitated with cathepsin B after treatment of wildtype macrophages with α-toxin, while almost no association of Nlrc4 with cathepsin B was detected in acid sphingomyelinase-deficient macrophages treated with α-toxin (Fig. 5B). Likewise, pretreatment with amitriptyline reduced the association of Nlrc4 with cathepsin B after stimulation of wildtype macrophages with α-toxin (Fig. 5E). We did not detect a significant effect of α-toxin on the association of Nlrc4 with cathepsin D in co-immunoprecipitation experiments (not shown).

FIG 4.5 A



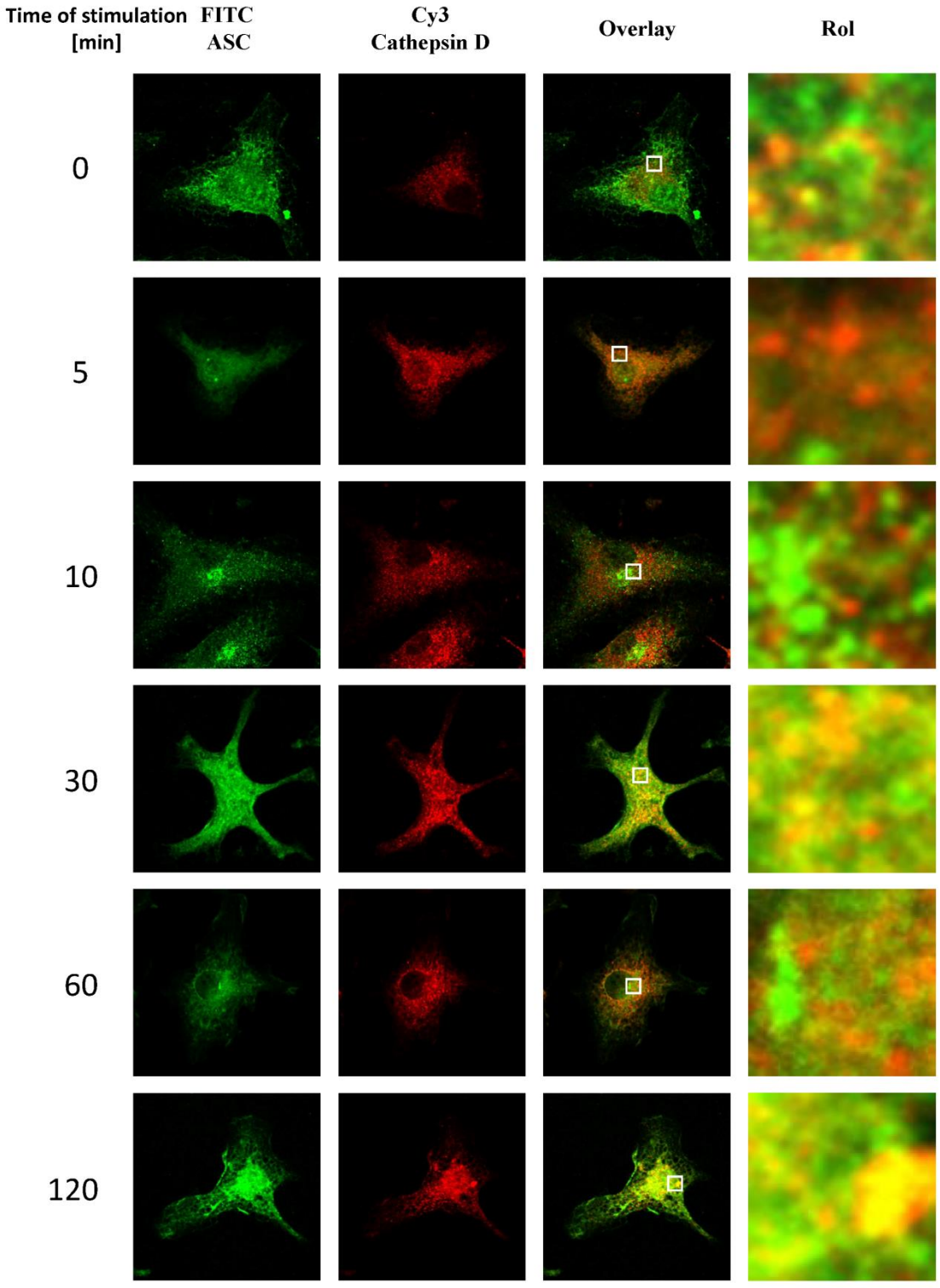
Wildtype

FIG 4.5 B


Time of stimulation [min]
FITC ASC
Cy3 Cathepsin B
Overlay
Rol
0
5
10
30
60
120

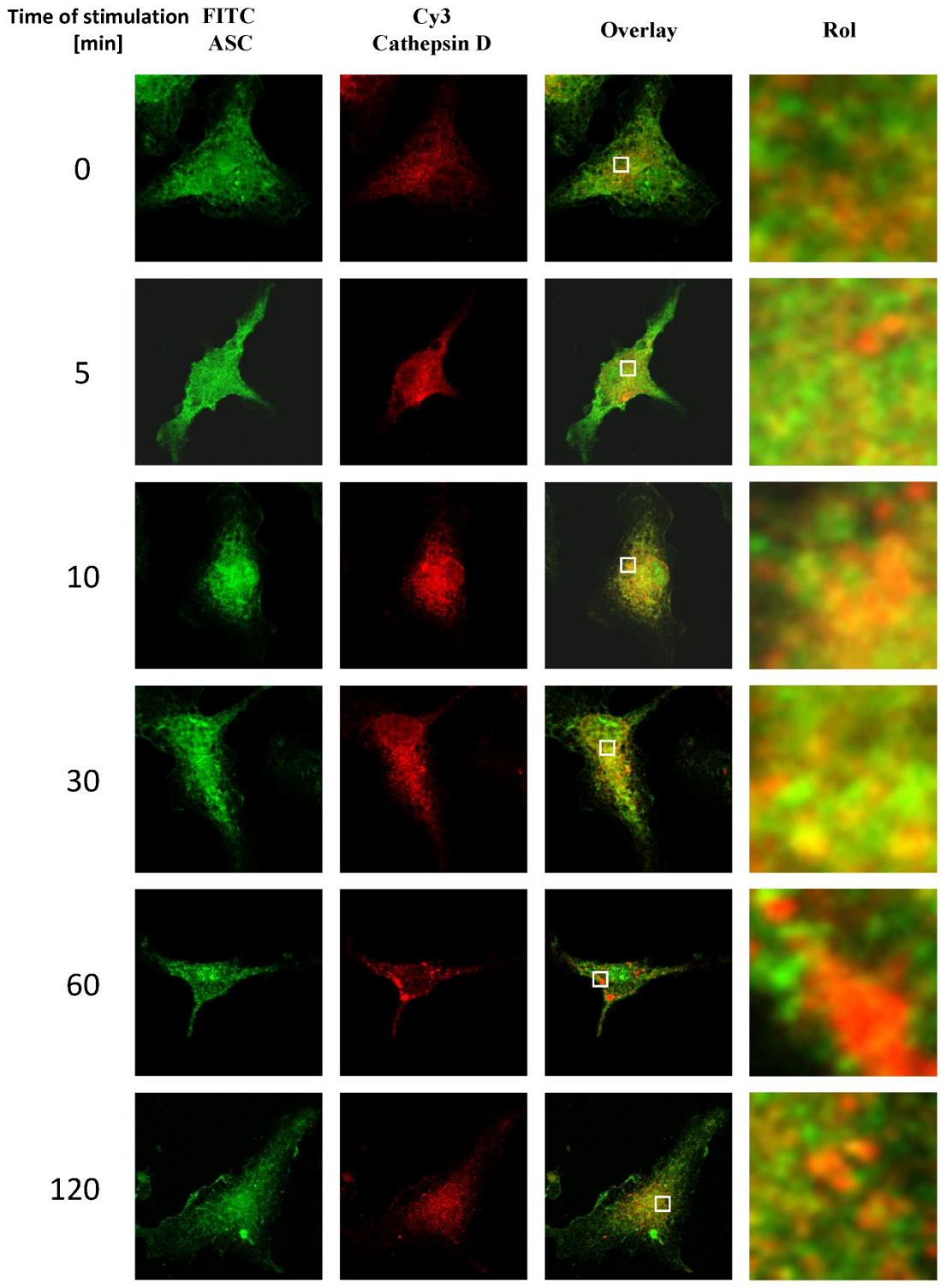
Asm$^{-/-}$

FIG 4.5 C



Wildtype

FIG 4.5 D


Time of stimulation [min]
FITC ASC
Cy3 Cathepsin D
Overlay
Rol
0
5
10
30
60
120

Asm$^{-/-}$

FIG 4.5 E

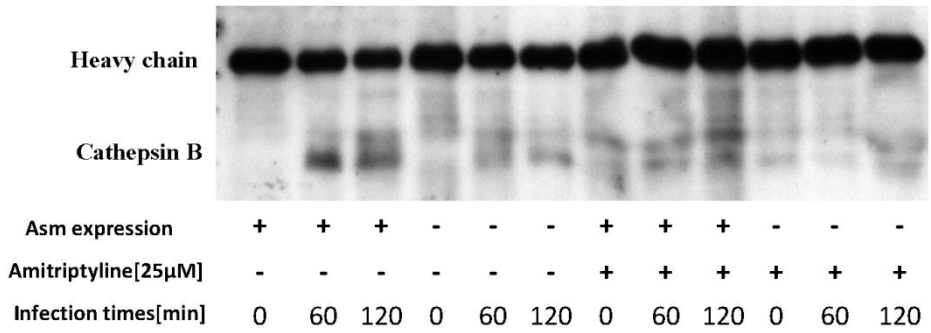


Figure 4.5 Cytoplasmic cathepsin B associates with inflammasome proteins

Confocal microscopy (A, B, C, D) and co-immunoprecipitation studies (E) reveal an association of cathepsin B, but not cathepsin D, with the inflammasome proteins Asc (A, B, C, D) and Nlrc4 (E) after treatment of macrophages with *S. aureus* α-toxin, which is reduced by genetic deficiency or pharmacological inhibition of the acid sphingo-myelinase. In the co-immunoprecipitation experiments we immunoprecipitated Nlrc4 and blotted for cathepsin B (E). Shown are representative results from each 4 (A, B, C, D) or 3 (E) independent studies.

4.6 Genetic deficiency or pharmacological inhibition of the acid sphingomyelinase reduces formation and release of Interleukin 1β and TNF-α in macrophages after *S. aureus* α-toxin treatment.

To determine the significance of the ceramide-mediated release of cathepsin B from lysosomes and its association with Nlrc4, we determined the formation of IL-1β and TNF-α in WT, acid sphingomyelinase-deficient and amitriptyline-treated WT macrophages after treatment with α-toxin (Fig. 6A and B). In addition, we measured the release of IL-1β and TNF-α in the cell culture supernatants (Fig. 6C and D). These studies revealed a marked, time-dependent formation of IL-1β and TNF-α in WT macrophages after stimulation with α-toxin, which was reduced in acid sphingomyelinase-deficient macrophages or WT macrophages treated with amitriptyline (Fig. 6A and B). Amitriptyline had no effect on the residual formation of IL-1β and TNF-α in acid sphingomyelinase-deficient macrophages after α-toxin stimulation (Fig. 6A and B), indicating that the inhibitory effect of amitriptyline on the release of these cytokines is entirely mediated by the acid sphingomyelinase. Treatment with the cathepsin B inhibitor further reduced IL-1β formation in acid sphingomyelinase-deficient cells (Fig. 6A) suggesting that a small part of cathepsin B is

released from lysosomes independent of acid sphingomyelinase expression.IL-1β and TNF-α were released from WT macrophages after stimulation with α-toxin (Fig. 6C and D). Treatment with amitriptyline or genetic deficiency prevented the release of IL-1β completely and reduced the release of TNF-α (Fig. 6 C and D).

To test the role of cathepsin B for the release of IL-1β after α-toxin stimulation, we treated WT and acid sphingomyelinase-deficient macrophages with the cathepsin B inhibitor CA-074Me (50 M). These studies revealed that the cathepsin B inhibitor reduced the formation and the release of IL-1β from WT macrophages after α-toxin stimulation and further reduced the residual formation and release of IL-1β from acid sphingomyelinase-deficient cells after α-toxin treatment (Fig. 6 A and C). In contrast, the cathepsin B inhibitor CA-074Me was without effect on the formation and the release of TNF-α after α-toxin treatment of macrophages (Fig. 6 B and D).

Fig. 6A

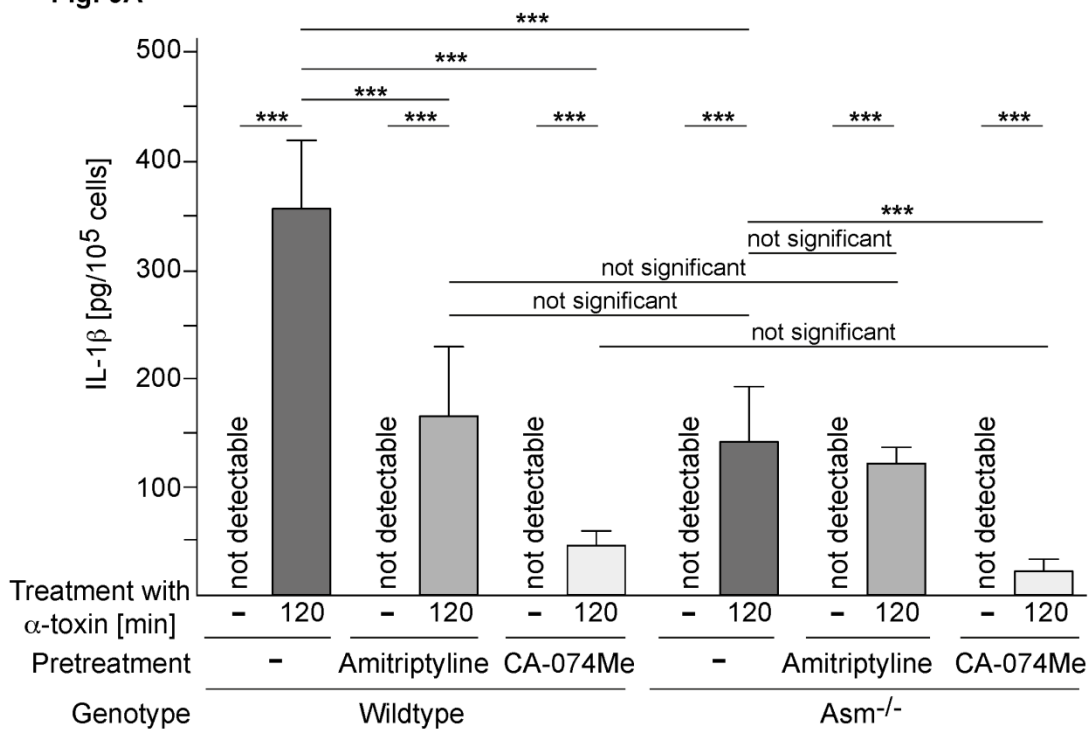


Fig. 6B

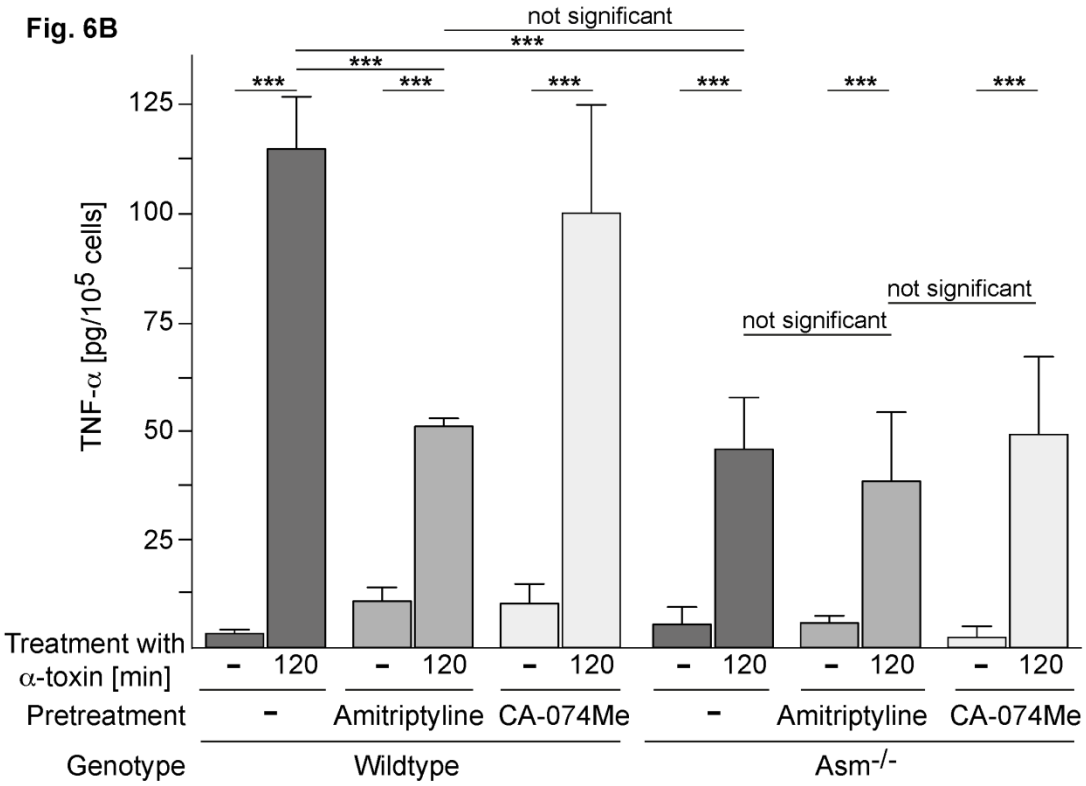
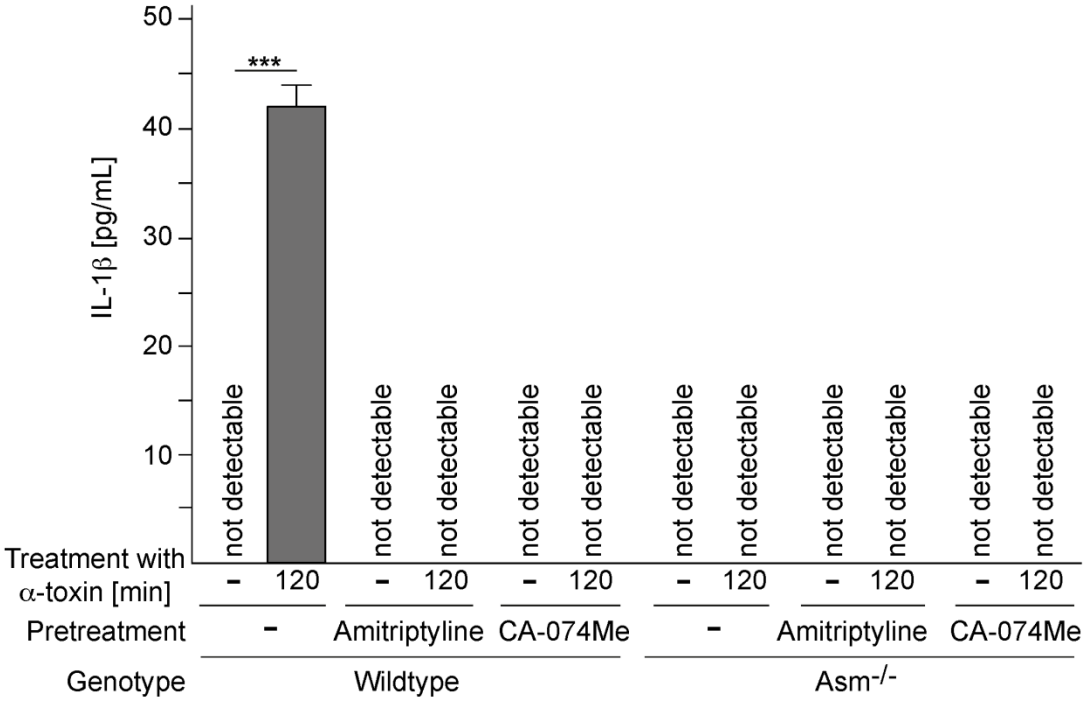

not significant
***
***
***
***
***
***
***
not significant
not significant
TNF-α [pg/10^5 cells]
125
100
75
50
25
Treatment with α-toxin [min]
- 120 - 120 - 120 - 120 - 120 - 120
Pretreatment
- Amitriptyline CA-074Me - Amitriptyline CA-074Me
Genotype
Wildtype
Asm-/-

Fig. 6C


***
IL-1β [pg/mL]
50
40
30
20
10
not detectable
Treatment with α-toxin [min]
- 120 - 120 - 120 - 120 - 120 - 120
Pretreatment
- Amitriptyline CA-074Me - Amitriptyline CA-074Me
Genotype
Wildtype
Asm-/-

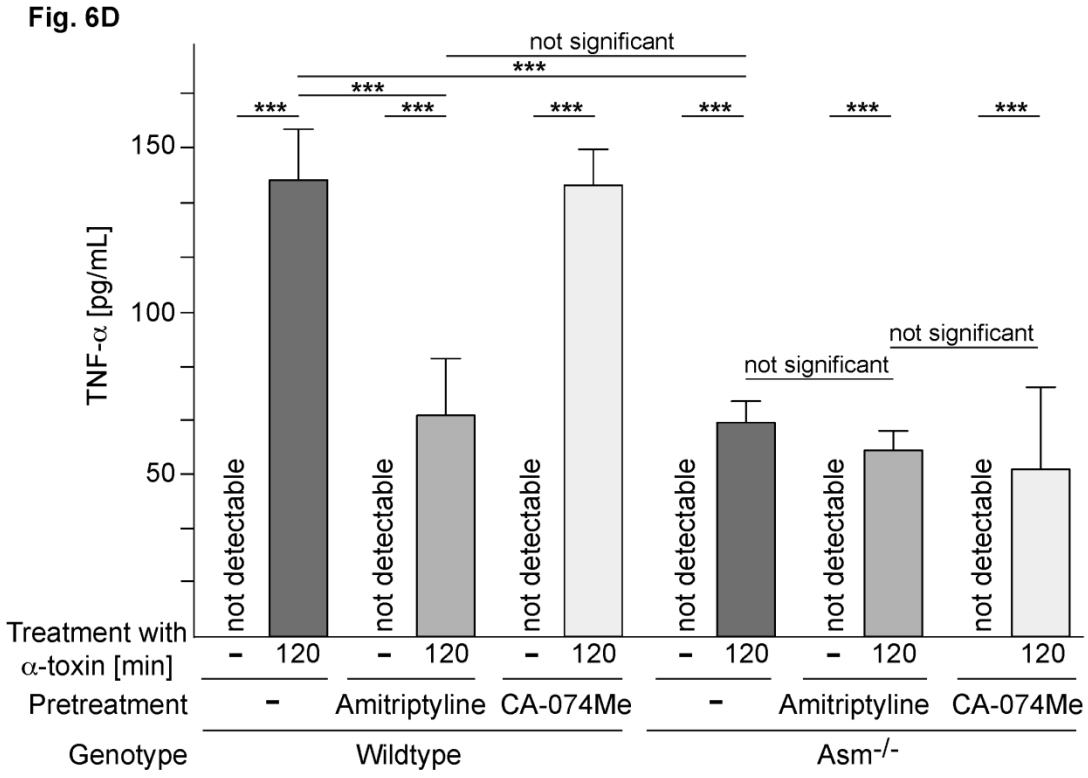


Figure 4.6 Genetic deficiency or pharmacological inhibition of the acid sphingomyelinase reduces formation and release of Interleukin 1β and TNF-α in macrophages after *S. aureus* α-toxin treatment

Treatment of wildtype macrophages with *S. aureus* α-toxin results in formation of Interleukin 1 (IL-1β) (A) and TNF-α (B) as determined by ELISA from the cell pellets, which is reduced by genetic deficiency of the acid sphingomyelinase or pharmacological inhibition of the acid sphingomyelinase using amitriptyline. Pharmacological inhibition of cathepsin B using CA-074Me blocks the formation of IL-1β, but is without effect on TNF-α-formation. (C) Treatment of macrophages with α-toxin results in a release of IL-1β into the supernatant, which is completely abrogated by genetic deficiency of the acid sphingomyelinase, treatment with amitriptyline or the cathepsin B inhibitor CA-074Me. (D) TNF-α is released from wildtype macrophages into the cell culture supernatant after treatment with α-toxin, which is reduced by treatment with pharmacological or genetic deficiency of the acid sphingomyelinase, but unaffacted by the cathepsin B inhibitor CA-074Me. Displayed are the mean ± SD from 3 independent studies, *p<0.05, ANOVA.

5 Disscussion

In the present study we identified a novel mechanism how *S. aureus* α-toxin induces inflammation. We demonstrate that α-toxin cause's activation of the acid sphingomyelinase and a formation of ceramides within lysosomes that results in the release of cathepsin B and D from lysosomes into the cytoplasm. We focused on the initial 2 hrs of the response of macrophages to α-toxin, since we assumed that effects of toxins on macrophages result in a rapid response.

In fact, our data show a rapid release of cathepsin B into the cytoplasm with a subsequent activation of the inflammasome. However, these data do not exclude that α-toxin also induces a long-term activation of the acid sphingomyelinase/ceramide system, which may result in a continuous activation of the inflammasome. Further, a large portion of the lysosomal cathepsin B has been released from lysosomes and the inflammasome has been activated already 2 hrs after α-toxin treatment suggesting that this time period of treatment is sufficient to induce most of the changes investigated in the present manuscript. Cytoplasmic cathepsin B physically associates with Asc and Nlrc4, key components of the inflammasome (Abais JM et al., 2015), and thereby activates the inflammasome as determined by a formation and release of IL-1β. Moreover, we demonstrate that α-toxin triggers the formation of TNF-α, at least partly, via acid sphingomyelinase, but independent of cathepsin B. Genetic deficiency or pharmacological inhibition of the acid sphingomyelinase prevented all of these events indicating the key role of the acid sphingomyelinase for the induction of pro-inflammatory cytokines in macrophages by *S.aureus* α-toxin. Lysosomal activation of the acid sphingomyelinase/ceramide system by bacterial toxins might be a novel mechanism how these toxins induce formation of pro-inflammatory cytokines.

5.1 *S. aureus* α-toxin activates the acid sphingomyelinase/ceramide system

Until now, it is unknown how *S. aureus* α-toxin activates the acid sphingomyelinase. Bubeck Wardenburg and Inoshima had proved that *S. aureus* α-toxin binds to its receptor ADAM10 at the plasma membrane (Bubeck Wardenburg J et al., 2007; Wilke GA et al., 2010; Inoshima I et al., 2011; Inoshima N et al., 2012) , which are best known for their role in shedding the extracellular domain of transmembrane proteins (Maeve Mullooly et al., 2015) and is also a pre-requisite for the oligomerization and membrane integration of the toxin (Powers

ME et al., 2012). Binding of α-toxin to ADAM10 or the wounding upon integration of the toxin into the membrane might trigger translocation of secretory lysosomes that contain the acid sphingomyelinase, to the plasma membrane bringing the acid sphingomyelinase in contact with the toxin. The acid sphingomyelinase may then generate ceramides in the plasma membrane and internalization of ceramide-enriched membrane domains together with the acid sphingomyelinase may finally result in an increase of ceramides within lysosomes. However, it seems to be more likely that the cell internalizes the toxin immediately after membrane incorporation to prevent cell damage and targets the toxin to lysosomes for degradation. Within lysosomes the cell responds to the toxin with an activation of the acid sphingomyelinase, formation of ceramides, the release of cathepsins, an activation of the inflammasome and the release of pro-inflammatory cytokines. The toxin may generate reactive oxygen species within the lysosome that have been previously shown to stimulate the enzyme activity of the acid sphingomyelinase (Dumitru CA and Gulbins E, 2006; Zhang Y et al., 2008; Li X et al., 2012; Henry B et al., 2013). Alternatively, activation of the acid sphingomyelinase by α-toxin might be mediated by a direct contact of the acid sphingomyelinase with α-toxin and a conformational change of the acid sphingomyelinase (Gulbins E et al., 1997). In addition, the toxin may activate proteases that induce a limited cleavage and thereby activation of the acid sphingomyelinase, similar to the activation of the acid sphingomyelinase by caspases in multivesicular bodies (Edelmann B et al., 2013).

5.2 Ceramide formation within lysosome.

Acid sphingomyelinase-induced ceramide release has been shown by many studies to induce apoptosis in response to various stimuli (Claudia A et al., 2009). Ceramides have been shown to induce a lysosomal release and activation of cathepsin D (Heinrich M et al., 1999; Heinrich M et al., 2004), and Lysosomal ceramide was reported to trigger the Cathepsin D-mediated apoptotic pathway (Heinrich M et al., 2004; Edelmann B et al., 2011). Cathepsin D has been linked to the induction of endoplasmatic reticulum stress (Uchiyama Y, 2001), induction of apoptosis (Deiss LP et al., 1996), suppression of autophagy (Liu F et al., 2016) and degradation of XIAP (Taniguchi M et al., 2015). The lysosomal cysteine protease cathepsin B plays an important role in physiological protein turnover and processing, making cathepsin B an important contributor to diverse processes including autophagy, antigen presentation, and activating cleavage-induced signaling cascades (Katunuma N et al., 1994; Foghsgaard L et al., 2001; Ha SD et al., 2010). M Taniguchi research suggested that with stimulated such as IL-2 can activated ASM–ceramide pathway in lysosomes and lysosomal

ceramides caused the release of Cathepsin B (M Taniguchi et al., 2015).

It might be possible that ceramide molecules bind to cathepsins and thereby facilitate the translocation of the proteins via the lysosomal membrane (Heinrich M et al., 1999; Heinrich M et al., 2004). Such a direct interaction of ceramides with cathepsin B/D has been previously shown (Heinrich M et al., 2004; M Taniguchi et al., 2015). In addition, the acid sphingomyelinase has been shown to be required for the fusion of phagosomes with lysosomes(Utermöhlen O et al., 2008), such as the research of *L. monocytogenes* show that the transfer of lysosomal contents into phagosomes containing *L. monocytogenes* are shown to depend on the activity of ASMase and ASMase regulates the efficient delivery of lysosomal hydrolases such as cathepsins D, B and L to phagosomes (Michael S et al., 2008), thus, targeting of *S. aureus* α-toxin to lysosomes might also require the acid sphingomyelinase and the formation of ceramides. The toxin may then permeabilize the lysosomal membrane to allow release of cathepsins. Obviously, these events are not exclusive and the acid sphingomyelinase/ceramide system might have multiple functions in the cellular response to *S. aureus* α-toxin.

Ceramide may not be the only lipids that trigger permeabilization of the lysosomal membrane after activation of the acid spingomyelinase. It has been shown that sphingosine generation within lysosomes also mediates lysosomal permeability and compromises lysosomal integrity (Ullio C et al., 2012; Höglinger D et al., 2015). It is certainly possible that *S. aureus* α-toxin does not only activate the acid sphingomyelinase, but also the acid ceramidase, and thereby triggers the formation of ceramide and sphingosine. Both lipids might contribute to an increased permeability of lysosomes and the release of cathepsins from the lysosome. An activation of the acid ceramidase with a subsequent formation of sphingosine from lysosomal ceramides, would also explain the finding that the release of cathepsins from lysosomes is not completely blocked in acid sphingomyelinase-deficient macrophages, because ceramides can also be generated within lysosomes by hydrolysis from other sphingolipids than sphingomyelin. Since amitriptyline does not only block the acid sphingomyelinase, but also the acid ceramidase (Elojeimy S et al., 2006), this scenario would also explain why this inhibitor further reduces the release of cathepsins in acid sphingomyelinase-deficient cells.

5.3 cathepsin B and D releasefrom lysosomes into the cytoplasmvia the acid sphingomyelinase

Previous studies have shown that the Asm-ceramide system is involved in infection with several pathogenic bacteria, such as Neisseria gonorrhoeae, N. meningitides, Pseudomonas aeruginosa, *S. aureus*, and Salmonella species (Hauck et al., 2000; McCollister et al., 2007; Peng et al., 2015; Simonis et al., 2014; Zhang et al., 2008). Amitriptyline has been shown to mediate a functional inhibition of the acid sphingomyelinase (ASMase) (Hurwitz R et al., 1994, Becker KA et al., 2009, Kornhuber J et al., 2008, Gulbins E et al., 2013), which is a specific mechanism of ceramide generation, is required for the activation of key pathways that regulate steatosis, fibrosis and lipotoxicity, including endoplasmic reticulum stress, autophagy and lysosomal membrane permeabilization (Carmen Garcia-Ruiz et al., 2015).More over the research from Michael Heinrich suggest that acid sphingomyelinase-derived ceramide targets endolysosomal Cathepsin(Michael HeinrichMarc et al., 2000) .

Previous studies by Colombini et al. have shown that ceramide molecules form short-lived pores in mitochondrial membranes (Siskind LJ et al., 2000), these pores are large enough to permit translocation of polypetides (Colombini M, 2010). Similar ceramide channels were recently shown in lysosomes (Yamanea M et al., 2017) and they may permit the translocation of cathepsin B from the lysosome into the cytoplasm after *S. aureus* α-toxin treatment. It will be very interesting to determine different ceramide species in lysosomes after treatment with *S. aureus* α-toxin and to measure whether the amounts and species of different ceramides are sufficient to mediate lysosomal ceramide channels.

In addition, ceramide accumulation in organelle membranes might also result in a very brief disruption of the bilayer, ceramide in one of the membrane monolayers causes a surface area mismatch between both monolayers, which leads to vesicle collapse with a random "translocation" of proteins that attach to this specific area of the lysosomal inner membrane leaflet to the cytoplasm (Artetxe I et al., 2017). MeiYang et al have shown that after being taken up by macrophages, carbon nanohorns was localized in the lysosomes and induce lysosomal membrane permeabilization followed by release of lysosomal proteases, such as cathepsins. The released cathepsins affected the mitochondria and caused ROS generation (MeiYang et al., 2014).

5.4 The activate of inflammasome via cathepsin B

The inflammasomes are important platforms that account for recognition and restriction of the infection by pathogenic microbes.It has been previously shown that bacteria activate the inflammasome via cathepsins (Lee HM et al., 2013; Duncan JA et al., 2009; Chen Y et al., 2015). For instance, it has been previously shown that infection of endothelial cells with *Lactobacillus casei* results in increased lysosomal permeability and a release of cathepsin B into the cytoplasm, finally mediating stimulation of the Nlrp3 inflammasome and arteritis (Chen Y et al., 2015). Interestingly, free fatty acids such as palmitate also triggered a cathepsin B-mediated activation of the inflammasome (Wang L et al., 2016) and it remains to be determined whether treatment with *S. aureus* α-toxin also mediates an intracellular release of free fatty acids contributing to increased lysosomal permeability. However, only a few studies report that bacterial toxins trigger an activation of the inflammasome via cathepsin B (Gupta R et al., 2014; Ali SR et al., 2011) and none of these studies has linked the acid sphingomyelinase/ceramide pathway to the inflammasome. Thus, it was shown that hemolysin from group B Streptococcus induces lysosomal leakage and inflammasome activation (Gupta R et al., 2014). Likewise, anthrax lethal toxin stimulates the inflammasome, which is prevented by inhibition of cathepsin B (Ali SR et al., 2011). Detailed insights into the mechanisms how these toxins activate the inflammasome require definition.

Our studies show a direct interaction of cathepsin B with Nlrc4 and Asc. Inhibition of cathepsin B (CA-074Me, a potent cathepsin B inhibitor, protects macrophages from cell death and prevents the activation of caspase-1) prevents activation of the inflammasome and the release of IL-1β upon *S.aureus* α-toxin treatment. This suggests that limited cleavage of components of the inflammasome by cathepsin B activates the inflammasome and mediates the release of pro-inflammatory cytokines. In contrast to cathepsin B, we did not detect an *S.aureus* α-toxin-triggered association of cathepsin D with components of the inflammasome. Further, inhibition of cathepsin B was sufficient to prevent activation of the inflammasome by *S. aureus* α-toxin. Thus, the release of cathepsin D does not seem to result primarily in activation of the inflammasome and the cellular consequences mediated by the release of cathepsin D from lysosomes into the cytoplasm upon treatment of macrophages with *S. aureus* α-toxin require further studies.

The formation of TNF-α was also dependent on expression of the acid sphingomyelinase and reduced in cells lacking the acid sphingomyelinase or treated with amitriptyline. However,

the formation of TNF-α after α-toxin-treatment was independent of cathepsin B indicating that the acid sphingomyelinase couples to several independent pathways regulating inflammation.

Macrophage response to pathogens by using difference receptors to stimulate phagocytosis and cytokine secretion.Our studies further show that genetic deficiency of the acid sphingomyelinase or treatment of WT macrophages with amitriptyline reduces the formation of IL-1β by approximately 50%, while the release of IL-1β from macrophages is completely prevented by genetic or pharmacological blockade of the acid sphingomyelinase. This indicates a dual role of the acid sphingomyelinase, which is important for intracellular formation of IL-1β and absolutely essential for the release of IL-1β from macrophages into the cell culture supernatant.

6 Summery

In summary, our studies indicate a novel signaling pathway how *S. aureus* α-toxin mediates activation of the inflammasome in macrophages: We demonstrate that the toxin activates the acid sphingomyelinase and triggers a formation of ceramides in lysosomes of bone marrow-derived macrophages. Activation of the acid sphingomyelinase/ceramide pathway results in release of cathepsin B from lysosomes that associates with Nlrc4 and Asc and mediates an activation of the inflammasome finally resulting in formation and release of IL-1β. Activation of the acid sphingomyelinase by α-toxin also triggers formation and release of TNF-α, which is independent of cathepsin B. These data link the lysosomal acid sphingomyelinase/ceramide system to the regulation of cytokines that are central for the regulation of inflammation in many human diseases.

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8 Publications

1. **Jie Ma**, Erich Gulbins, Michael J. Edwards, Charles C. Caldwell, Martin Fraunholz, Katrin Anne Becker. *Staphylococcus aureus* α-toxin induces inflammatory cytokines via lysosomal acid sphingomyelinase and ceramides. Cellular Physiology and Biochemistry 2017 43(6):2170-2184
2. Li Cao, Wu Yuqing, **Ma Jie**, et al. *Staphylococcus aureus* Survives in Cystic Fibrosis Macrophages, Forming a Reservoir for Chronic Pneumonia[J]. Infection and Immunity, 2017, 85(5):00883-16.

9 Abbreviations

ASM	AcidSphingomyelinase
ADAM10	A-disintegrin and metalloprotease 10
LRs	lipid rafts
Lamp-1	Lysosome-associated membrane protein 1
BMDMs	Bone marrow derived macrophages
Ami	Amitriptyline
FITC	Fluorescein isothiocyanate
FITC	Fluorescein isothicyanate
Cy3	Cyanine Dyes
IL	Interleukin
Smpd1	Sphingomyelin phoohodiesterase 1
PFA	paraformaldehyde
NLRP3	nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 OR Nod-like receptor protein 3
BLRC4	NLR family CARD domain containing 4
TNF-α	Tumor necrosis factor alpha
RIPA	radioimmunoprecipitation assay
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	sphingomyelin
SK	sphingosine kinases
SMases	sphingomylinases
PAMPs	Pathogen-associated molecular patterns
DAMPs	Damage-associated molecular patterns

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Curriculum vitae

The biography is not included in the online version for reasons of data protection

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