Acid sphingomyelinase inhibition protects mice from lung edema and lethal Staphylococcus aureus sepsis

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ABBREVIATIONS

ADAM10	A-disintegrin and metalloprotease 10
AIDS	acquired immune deficiency syndrome
AJ	adherens junction
ALI	acute lung injury
Ami	Amitriptyline
APC	activated protein C
ARDS	acute respiratory distress syndrome
Asm	Acid sphingomyelinase
BAL	bronchoalveolar lavage
CA-MRSA	community acquired MRSA
CFUs	colony-forming units
DAG	diacylglycerol
DAMP	danger-associated molecular pattern
E. coli	Escherichia coli
EDVD	endothelium-dependent vasodilation
ESR	Electron Spin Resonance
GM-CSF	granulocyte-macrophage colony stimulating factor
HA-MRSA	hospital associated MRSA
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
IL	interleukin
LPS	lipopolysaccharide
LRs	lipid rafts
Methi	Methicillin
MLST	Multilocus sequence typing
MMPs	matrix metalloproteinases
MRSA	methicillin-resistant S. aureus

MSOF	multisystem organ failure
MSSA	methicillin-sensitive S. aureus
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NLR	NOD-like receptors
NMR	nuclear magnetic resonance
Nox	NADPH oxidases
P. aeruginosa	Pseudomonas aeruginosa
PAF	platelet-activating factor
PAMPs	pathogen-associated molecular patterns
PDTC	pyrrolidine dithiocarbamate
PFGE	Pulsed-field gel electrophoresis
PFT	Pore-forming cytotoxins
PMN	polymorphonuclear
PRRs	pattern-recognition receptors
ROS	Reactive oxygen species
RP-HPLC	reversed phase HPLC
S. aureus	Staphylococcus aureus
SCCmec	staphylococcal chromosomal cassette mec
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
SSTI	Skin and soft tissue infection
ST	sequence type
TJ	tight junction
TLC	Thin Layer Chromatography
TLRs	Toll-like receptors
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultraviolet
Vanco	Vancomycin

1. INTRODUCTION

1.1. *Staphylococcus aureus* (*S. aureus*)

1.1.1. Overview of *S. aureus*

S. aureus was discovered in the 1880s (Ogston 1880, Ogston 1881, Ogston 1882a, Ogston 1882b) and is a faculative pathogenic Gram-positive bacterium. Currently, *S. aureus* is one of the most virulent and common pathogens inside and outside hospitals and infections with *S. aureus* are associated with considerable morbidity and mortality (Laupland et al 2013, Tom et al 2014, van Hal et al 2012).

Humans and other mammals are major reservoirs for *S. aureus*. Three out of ten people in the United States are asymptomatically colonized with *S. aureus* on their skin or mucous membranes, most commonly in the anterior nares (Gorwitz et al 2008). Some people are only intermittent carriers, and some carry the bacteria for longer periods. Nasal colonization with *S. aureus* increases the risk of developing serious infection by the same strain (von Eiff et al 2001, Williams et al 1959), as when the bacterium is introduced into deeper tissues by cuts in the skin, in-dwelling catheters, aspiration, or surgery. Moreover, immune-deficient patients with advanced surgery, malignant diseases, prolonged stays in intensive care units and long-term care facilities and old patients are increasing since the beginning of the 21st century, which is another reason for *S. aureus* infections.

Skin and soft tissue infection (SSTI) is the most common clinical manifestation of *S. aureus* disease (Hayward et al 2008, Lautz et al 2011), recent studies describe an increase in the incidence of SSTI in Australia and New Zealand over the past decade (O'Sullivan et al 2011, Vaska et al 2012, Williamson et al 2013, Williamson et al 2014). In addition to *S. aureus* SSTI, *S. aureus* also causes a spectrum of invasive infections, including osteomyelitis, necrotizing pneumonia, joint infections, endocarditis, sepsis, and death. In particular, *S. aureus* bacteremia is associated with considerable morbidity and mortality, with reported

global incidence rates varying between 14 and 41 per 100,000 population (El Atrouni et al 2009, Laupland et al 2013), although it should be noted that differences in case ascertainment and study methodology limit comparisons between regions.

1.1.2. Evolution of *S. aureus*

In the pre-antibiotic era, prior to the introduction of penicillin for the treatment of *S. aureus* infections, the mortality rate of individuals with an *S. aureus* infection was about 80% (Skinner et al 1941). In 1929, Alexander Fleming reported his observations of the bactericidal effects of a fungal contaminant that produced penicillin, which can kill *S. aureus* on culture plates and is non-toxic to animals in enormous doses (Fleming 1929). Subsequently, with mass production of penicillin, the death rates from bacterial pneumonia and meningitis in World War II dramatically dropped, comparing to World War I (Neushul 1993).

Alexander Fleming noted that the growth of E. coli and a number of other bacteria belonging to the colityphoid group was not inhibited by penicillin (Fleming 1929). Further work has been done to find the cause of the resistance of these organisms to the action of penicillin. In 1940, an enzyme, which was called "penicillinase" and capable of hydrolyzing the active β-lactam ring in penicillin, was described in *Escherichia coli* (*E. coli*) (Abraham 1940). Soon thereafter, in 1944, penicillinase was extracted from penicillin resistant *Staphylococci* (Kirby 1944). Later on, more and more penicillin-resistant *S. aureus* strains were observed in the hospital and community. Currently, 90 to 95% of all *S. aureus* strains are resistant to penicillin, with the plasmid encoded penicillinase readily transferable via transduction or conjugation.

In 1961, 2 years after the introduction of methicillin, a penicillinase-resistant penicillin, the first strain of methicillin-resistant *S. aureus* (MRSA) was reported in a United Kingdom hospital due to the acquisition of the mecA gene (Eriksen 1961). This gene encodes for a penicillin-binding protein (PBP2a) which is expressed in the bacterial cell wall and which has a low affinity for β-lactam antibiotics. Thus, this group of antibiotics can't disrupt the synthesis of the peptidoglycan layer of bacteria and is ineffective against bacteria expressing

this gene. As a consequence, *S. aureus* will survive. There is evidence that strains of methicillin-sensitive *S. aureus* (MSSA) became methicillin resistant through the acquisition of the SCCmec element, probably from coagulase-negative staphylococcal strains, and that this has occurred on multiple occasions (Robinson et al 2004). The gene encoding meticillin resistance, mecA, is part of a larger genetic element known as the staphylococcal chromosomal cassette mec (SCCmec) (Ito et al 2001). The SCCmec element contains the mecA gene, chromosomal cassette recombinase (ccr) genes, mec regulatory genes and a junkyard region which contains non-essential components of SCCmec (Deurenberg et al 2008).

In recent years MRSA is now reported in about 60 to 70% of all *S. aureus* isolates found worldwide. There are now almost 100,000 cases of life threatening MRSA infections in the U.S. each year, evidences from the Centers for Disease Control and Prevention suggest with about 19,000 related deaths, more than the number of deaths from acquired immune deficiency syndrome (AIDS), which is induced by human immunodeficiency virus (HIV) (Klevens et al 2007).

Glycopeptides, such as vancomycin, are the treatment of choice for infections due to MRSA. Unfortunately, up to now, three vancomycin-resistant MRSA isolates have been reported from the US since 2002 (Boyce et al 2005, Courvalin 2006, Weigel et al 2007). By far, the biggest threat in *S. aureus* is the spread of vancomycin-resistant MRSA isolates. Lack of previous or additional reports of vancomycin-resistant MRSA isolates might be because of lack of detection (several of those isolates had vancomycin intermediate susceptibility) or to lack of stability of the plasmid-mediated vancomycin-resistant determinants in *S. aureus* (Perichon et al 2004). However, a recent report indicates that a single plasmid transfer from vancomycin-resistant enterococci to MRSA may be sufficient for expression of resistance (Weigel et al 2007).

Linezolid, quinupristin-dalfopristin, tigecycline, ceftopibrole and daptomycin are all available therapeutic options for treating vancomycin-resistant MRSA infections since they are all

active *in vitro* against those isolates. However, the clinical efficacy of the best antibiotic combinations remains to be determined using animal models of infection since we (fortunately) have not faced outbreaks with those isolates to this day.

1.1.3. Typing methods for S. aureus

For the first three decades after their appearance, MRSA strains typically have remained hospital associated MRSA (HA-MRSA). In addition, since the 1990s, in an unexpected epidemiological 'move', MRSA strains also began to appear in the community among people who had none of the usual risk factors for such infections. Such community acquired MRSA (CA-MRSA), characterized by the presence of the toxin Panton-Valentine leukocidin, spread worldwide, first in the community, but later on also in healthcare facilities. At this moment, the distinction between CA-MRSA and HA-MRSA is beginning to fade (Lowy 1998, Lowy 2003).

A thorough knowledge of the spread and the molecular evolution of MRSA is required to effective develop strategies to control the dissemination of MRSA. Therefore, several typing methods have been developed during the last decades. MRSA strains can be typed using both phenotypic and molecular methods. There are many phenotypic typing methods, including the use of colonial characteristics, biochemical reactions, antibiotic susceptibility pattern, susceptibility to various phages and toxin production. The most important molecular typing methods in current use comprise pulsed-field gel electrophoresis, multilocus sequence typing, SCCmec typing and spa locus typing (Aires de Sousa et al 2004).

Pulsed-field gel electrophoresis (PFGE). As one of the earlier molecular methods, PFGE remains the most popular technique to differentiate MRSA isolates. In PFGE for *S. aureus*, the chromosomal DNA is digested with the restriction enzyme SmaI, and the obtained DNA fragments are separated by agarose gel electrophoresis in an electric field with an alternating voltage gradient. The resulting banding patterns are analyzed using a special software package (Tenover et al 1995). The advantage of this method is that it provides great discrimination between strains and is useful in the investigation of outbreaks by allowing differentiation of

unrelated strains. Disadvantages associated with the method relate principally to difficulties with inter-laboratory comparison of results. Thus reliable comparison of strains between regions and internationally is not always possible.

Multilocus sequence typing (MLST). MLST is based on the sequence analysis of fragments of seven *S. aureus* housekeeping genes, i.e. arcC, aroE, glpF, gmk, pta, tpi and yqiL, each approximately 500-bp in length (Enright et al 2000). The DNA sequences are compared to those of previously identified alleles at each locus on the MLST online database (http://www.mlst.net). Each allele is given a number, and a string of seven numbers then represents the allelic profile designated sequence type (ST) of an isolate. The MLST scheme for *S. aureus* was developed in 2000 and the details of more than 1500 isolates are available at the *S. aureus* MLST website http://saureus.mlst.net (Enright 2006). The advantage of MLST is that the geographic source and clinical information on each isolate can be stored with its sequence online, making it useful for international and local surveillance purposes. Disadvantages associated with MLST are the economic and time-consuming costs of performing seven PCRs and 14 DNA-sequencing reactions per isolate.

SCCmec typing. Seven major SCCmec types and their subtypes, which range in size from 20 to 67 kb, are defined by combinations of different classes of mec and ccr genes (Chongtrakool et al 2006). The SCCmec gene cluster evolves rapidly and becomes another target for sequencing methods to differentiate MRSA strains. Unfortunately, a major disadvantage of such a method is that it is not feasible for routine applications, since the relative large number of PCR reactions that are needed to determine the structure of SCCmec are time consuming (Kondo et al 2007).

Spa locus typing. Typing of a single locus zone in the polymorphic region X of *S. aureus* protein A (spa) has also become increasingly popular during recent years (Frenay et al 1996, Moodley et al 2006). The diversity of the spa gene, consisting mainly of a number of repeats of 24 bp in length, is attributed to point mutations, as well as to deletions and duplications of the repeats (Kahl et al 2005, Shopsin et al 1999). Spa typing is less expensive and

time-consuming than MLST and can be used to study both the molecular evolution as well as inter-hospital comparisons since it requires sequencing of one locus.

In summary, although a lot of knowledge on the spread of MRSA clones has been gained in last decades, there are still a number of questions unanswered. Further investigations, addressing both basic research and performing epidemiological studies, are needed to understand completely the molecular evolution of *S. aureus*.

1.2. Sepsis

1.2.1. Overview of sepsis

Sepsis is a very heterogeneous clinical syndrome broadly defined as the systemic host response to an infection. Indeed, sepsis can be initiated by any microorganism, whether it is bacterial, fungal, viral, parasitic, or by microbial products and toxins, and is then propagated by a complex network of inflammatory mediators and cellular dysfunction.

In 1989, Roger Bone and colleagues proposed the term “sepsis syndrome” for the first time to define patients who have severe sepsis, by establishing a set of clinical parameters (Bone et al 1989). Sepsis syndrome was defined as hypothermia (less than 96 ^{o}F [35.5 ^{o}C]) or hyperthermia (greater than 101 ^{o}F [38.3 ^{o}C]); tachycardia (greater than 90 beat/min); tachypnea (greater than 20 breath/min); clinical evidence of an infection site; and the presence of at least one end-organ demonstrating inadequate perfusion or dysfunction expressed as poor or altered cerebral function, hypoxemia (PaO_2 less than 100 mbar on room air), elevated plasma lactate, or oliguria (urine output less than 30 mL/h or 0.5 mL/kg body weight/h without corrective therapy) (Bone et al 1989). If untreated, the patient may develop systemic inflammatory response syndrome (SIRS), septic shock and multisystem organ failure (MSOF), which are the deadly forms of the disease.

The most common sites of bacterial infection are the lungs, abdominal cavity, the skin, the urinary tract and primary infections of the blood stream. A microbiological diagnosis is made

in about half the cases; Gram-negative bacteria account for about 60% of cases, Gram-positive for the remainder (Alberti et al 2002, Angus et al 2001).

Sepsis remains a significant problem in medical management, with an annual worldwide incidence of approximately 18 million cases with an associated 30-40% mortality rate (Blanco et al 2008, Karlsson et al 2007). In the U.S. alone, approximately 750,000 patients annually are diagnosed with sepsis, with a mortality rate ranging from 30% to 50% (Angus et al 2001). Most patients with sepsis are admitted to intensive care units and are on mechanical ventilation. The U.S. Center for Disease Control and Prevention indicates that sepsis is one of the top ten leading causes of death in the U.S., and estimates the annual costs for the treatment of patients with sepsis are estimated to exceed $17 billion (Angus et al 2001). Sepsis may be responsible for a majority of the mortality associated with significant public health concerns such as malaria, tuberculosis, HIV/AIDS and others. What's more, the incidence of sepsis increases annually probably mainly due to an increase in the number of immunocompromised patients, increase in antibiotic resistance and the aging population.

Unfortunately, very few new treatments have been introduced over the past several decades although many people are working in the field. Current management of septic patients is predominantly non-specific, relying on a range of interventions such as intravenous fluids and medications, antibiotics, mechanical ventilation, nutritional support and corticosteroid therapy. Therefore, there is an urgent need for new effective treatments for sepsis.

1.2.2. Sepsis pathogenesis

Sepsis develops when the host response to a pathogen or a microbial toxin is accelerated to an inappropriate degree. The immune system relies on a process of molecular pattern recognition to determine the appropriate immunologic response to a foreign protein. These bacterial motifs, which are recognized by the innate immune system, have been named pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (Janeway et al 1998). In Gram-negative bacteria, lipopolysaccharide (LPS; known also as endotoxin) correlates with the ability to activate host cell membranes. In Gram-

positive bacteria, identified structural components in the bacteria cell wall account for their biological activity since there is no endotoxin in these type of bacteria (Majcherczyk et al 1999, Morath et al 2001). However, Gram-positive bacteria can also produce potent exotoxins, which exhibit the properties of super antigens and cause massive T-cell activation and release of pro-inflammatory lymphokines, suggesting a plausible role for these toxins as a cause of the profound shock that is seen in patients with toxic shock (Lavoie et al 1999).

PAMP's as well as other danger-associated molecular pattern (DAMP) molecules will bind to their receptors in the host cell membrane and begin a process of intracellular signaling and cellular activation: On one hand, the mechanisms involve widespread fibrin deposition causing microvascular occlusion, the development of tissue exudates further compromising adequate oxygenation, and disorders of microvascular homeostasis resulting from the elaboration of vasoactive substances such as platelet-activating factor (PAF), histamine and prostanoids (Anderson et al 1991). On the other hand, cellular infiltrates, particularly neutrophils, damage tissue directly by releasing lysosomal enzymes and superoxide-derived free radicals. TNF-α and other cytokines increase the expression of the inducible nitric oxide synthase and increased production of nitric oxide causes further vascular instability (Azevedo et al 2006) and may also contribute to the direct myocardial depression that occurs in sepsis (Landry et al 2001). Microvascular occlusion and vascular instability results in coagulopathy, fever, vasodilation and capillary leak, ultimately sepsis and multiple organ failure (Cohen 2002) (**Figure 1.2.2.1.**).

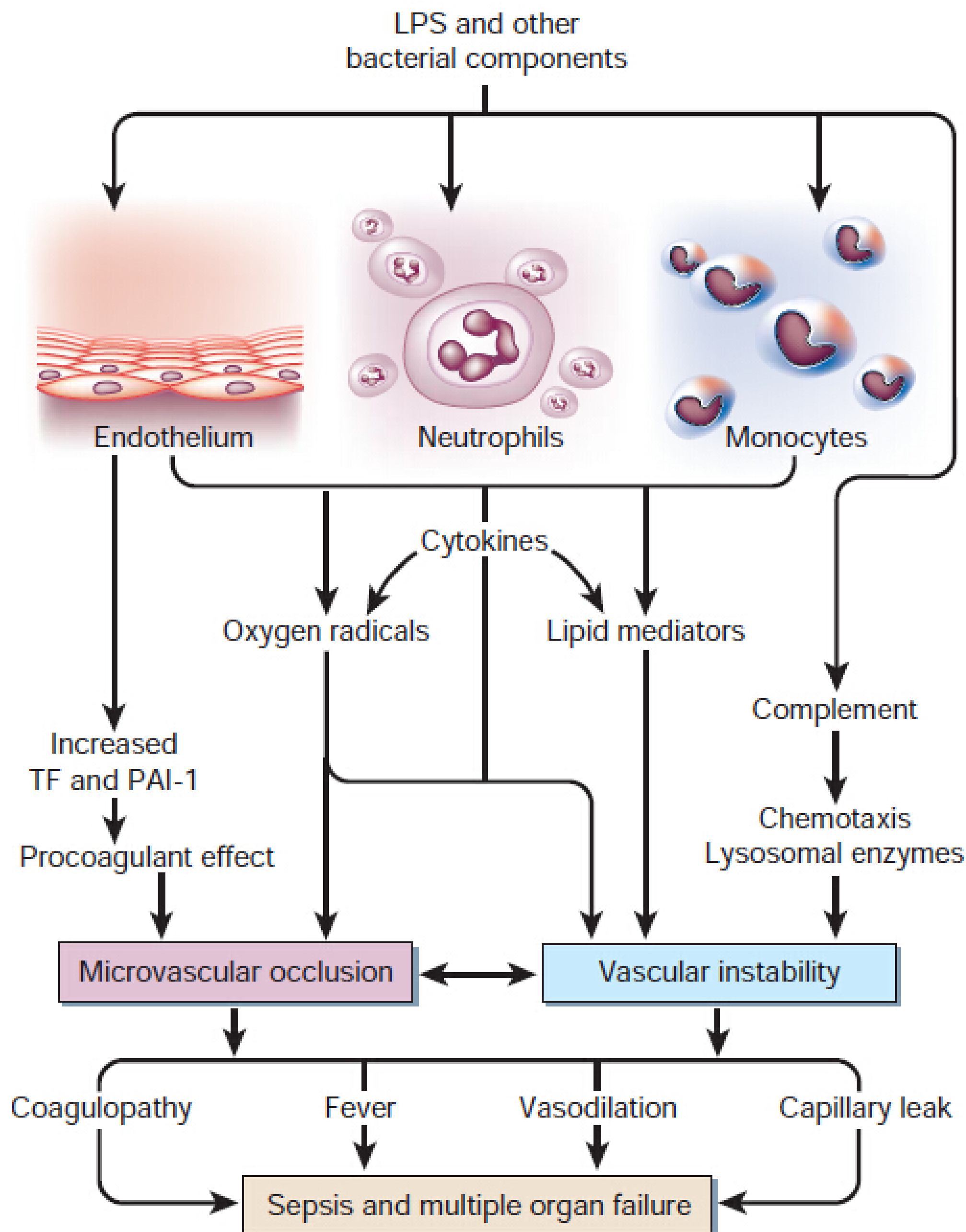

Figure 1.2.2.1. Pathogenetic networks in septic shock

Lipopolysaccharide (LPS) and other microbial components simultaneously activate multiple parallel cascades that contribute to the pathophysiology of adult respiratory distress syndrome (ARDS) and septic shock. The combination of poor myocardial contractility, impaired peripheral vascular tone and microvascular occlusion results in tissue hypoperfusion and inadequate oxygenation, and finally multiple organ failure (Cohen 2002).

During a very long time, the prevailing concept of the pathogenesis of sepsis was that mortality is the consequence of an uncontrolled hyper-inflammatory response of the host. The disappointing results of nearly 40 years of anti-inflammatory strategies and the development

of animal models that more closely mimic clinical sepsis have led to the reconsideration of the pathophysiology of sepsis. Sepsis is now considered a misbalance between pro-inflammatory reactions (designed to kill invading pathogens but at the same time responsible for tissue damage) and anti-inflammatory responses (designed to limit excessive inflammation, but at the same time making the host more vulnerable for secondary infections) (Anas et al 2010) (**Figure 1.2.2.2.**).

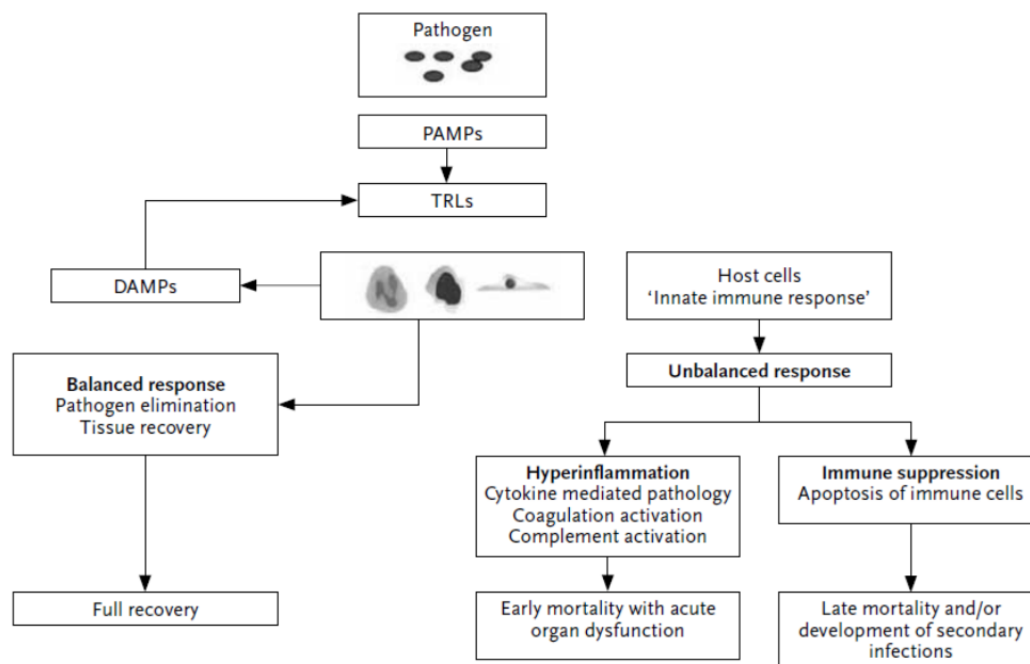

Figure 1.2.2.2. Important components of the host response to sepsis

The interaction between pathogens and the host is mediated initially via an interaction between PAMPs (pathogen associated molecular pathogens) and TLRs (Toll-like receptors). The resulting innate response of immune cells can result in a balanced reaction leading to pathogen elimination and tissue recovery or an unbalanced reaction that on the one hand can lead to exaggerated inflammation and tissue injury and on the other hand to immune suppression caused by immune cell apoptosis (Anas et al 2010).

In addition, new players have been described in the field of vascular dysfunction, such as platelet-derived microparticles, which are associated with apoptosis of vascular cells and cardiac failure (Azevedo et al 2007). However, the correlation of these pathways to outcome is so far poorly understood.

1.2.3. Mechanisms of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)

Currently, the incidence of ALI and ARDS in the USA has been reported to be 79 and 59 per 100,000 people per year, respectively (Bernard et al 1994, Herridge et al 2005, Peek et al 2006, Rubenfeld et al 2005, Wheeler et al 2007). Based on the growth of the population, the incidence will likely double in the next 25 years (Rubenfeld et al 2005).

The highest incidence of ALI is seen during sepsis, with approximately 25% of all ARDS cases stemming from severe sepsis (Brun-Buisson et al 2004) and 42.8% of sepsis was pneumonia (Blanco et al 2008). Moreover, ALI/ARDS are associated with a lethality of approximately 40%, accounting for around 75,000 deaths per year in the USA (Rubenfeld et al 2005, Wheeler et al 2007).

ALI/ARDS was first described in 1967 (Ashbaugh et al 1967) and a large body of research has been ongoing in order to understand ALI/ARDS and to improve the clinical outcomes of this entity. ALI/ARDS is a clinical syndrome, characterized by the acute onset of severe hypoxemia with diffuse bilateral infiltrates in the chest radiograph and without evidence of left atrial hypertension. The difference between both entities is the degree of hypoxemia. In ALI, the ratio of arterial oxygen tension (PaO_2) to the fraction of inspired oxygen (FiO_2) is $\leqslant$ 300, while in ARDS it is $\leqslant$200 (Bernard et al 1994).

The pathophysiology of ALI/ARDS is not completely understood. Initially, a direct pulmonary or indirect extrapulmonary insult is believed to cause a proliferation of inflammatory mediators that promote neutrophil accumulation in the microcirculation of the lung. These neutrophils activate and migrate in large numbers across the vascular endothelial and alveolar epithelial surfaces, releasing proteases, cytokines, and reactive oxygen species. This migration and mediator release lead to pathologic vascular permeability, gaps in the alveolar epithelial barrier, and necrosis of type I and II alveolar cells. This, in turn, leads to the pulmonary edema, hyaline membrane formation, and loss of surfactant that decrease pulmonary compliance and make air exchange difficult. Subsequent infiltration of fibroblasts

can result in collagen deposition, fibrosis, and further progression of the disease (Matthay et al 2003, Matthay et al 2005, Ware et al 2000).

Increased permeability of microvascular barriers, resulting in extravascular accumulation of protein-rich edema fluid, is a cardinal feature of acute inflammation and a central pathophysiologic mechanism in ALI and ARDS (**Figure 1.2.3.)** (Bachofen et al 1977, Matthay et al 2003, Ware et al 2000).

Neutrophils are proposed to have an important role in mediating ALI. When recruited to a site of infection/inflammation, they exert a variety of beneficial functions (phagocytosis, production of reactive oxygen species and nitric oxide species, and degranulation of lytic enzymes) that, when well regulated, enable clearance of the invading pathogen. However, it is also hypothesized that the recruitment of activated PMNs may be potentially harmful when these same functions are dysregulated and directed at otherwise normal host tissue, culminating in injury and organ damage.

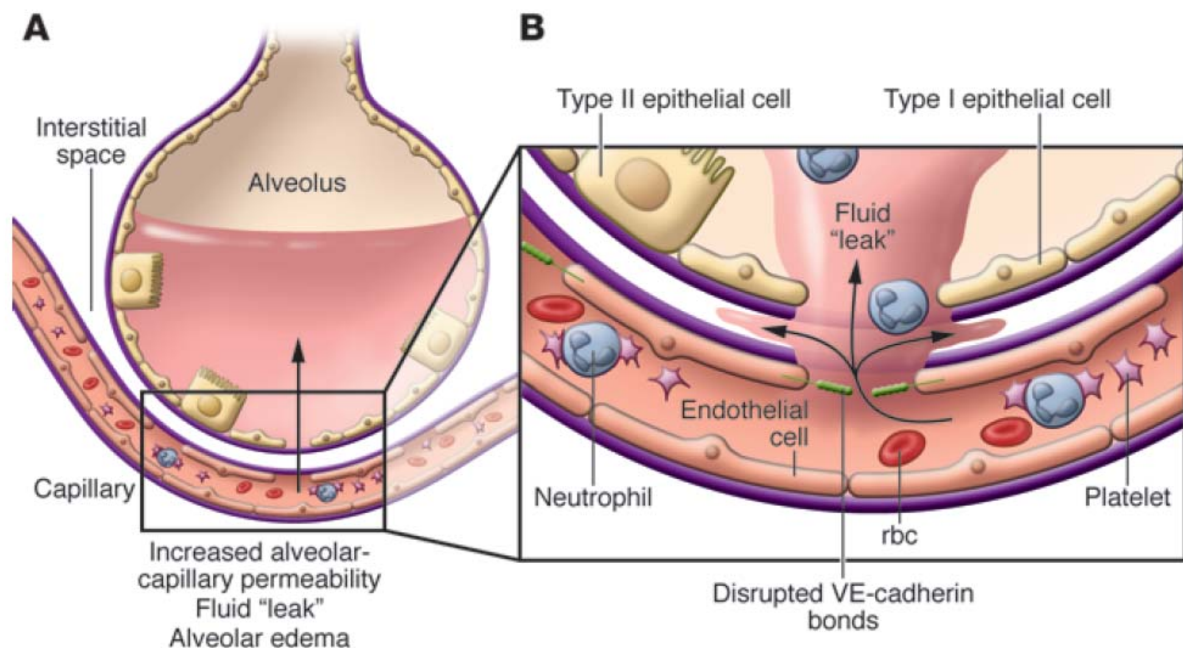

Figure 1.2.3. Mechanism of lung edema

(A) Disrupted alveolar barrier function, resulting in increased permeability to water, proteins, and other solutes, is a hallmark of clinical and experimental ALI. Intra-alveolar accumulation of neutrophils, other leukocytes, erythrocytes and inflammatory mediators is also associated with altered endothelial and epithelial barrier function. (B) Disruption of VE-cadherin bonds is a central mechanism of altered endothelial barrier function in experimental ALI and in models of sepsis and systemic vascular destabilization. Disruption of VE-cadherin bonds also facilitates transendothelial migration of leukocytes and, in some studies, is associated with accumulation of leukocytes and platelets in microvessels (Matthay 2003).

1.2.4. ALI/ARDS therapy

Despite the importance of ALI/ARDS healthcare issue, very few new treatments have been introduced over the past three decades, and the mortality and morbidity rates of ALI/ARDS -related conditions remains high. Current management of ALI/ARDS patients is predominantly non-specific, relying on a range of interventions such as intravenous fluids and medications, antibiotics, mechanical ventilation, nutritional support, corticosteroid therapy, and prevention of stress ulcers and venous thromboembolism (Adhikari et al 2004, Briel et al 2010, Dellinger et al 2008, Geerts et al 2008, Lin et al 2010, National Heart et al 2006, Peter et al 2008, Petrucci et al 2013).

Despite the large amount of research elucidating the molecular mechanisms underlying ALI/ ARDS, the investigation of pharmacologic therapies has led almost exclusively to negative results, often in contrast to very promising results in animal studies. Although some animal studies support the potential efficacy of anti-inflammatory therapies for decreasing lung injury, clinical trials have not demonstrated a convincing reduction in mortality using granulocyte-macrophage colony stimulating factor (GM-CSF) or glucocorticoids, antioxidants, or anticytokine therapies that were tested in patients with sepsis (Bernard et al 1987, Bernard et al 1997, Meduri et al 1998, Meduri et al 2007, Paine et al 2012, Steinberg et al 2006). Although pulmonary hypertension and lung vascular injury are important features of ALI and ARDS, vasodilator therapies including prostaglandin E1 and nitric oxide have not reduced mortality (Abraham et al 1999, Taylor et al 2004). Treatment to accelerate the resolution of pulmonary edema with aerosolized or intravenous β-adrenergic agonists also failed to improve survival (Gao Smith et al 2012). Nutritional supplement with ω-3 fatty acid may be

harmful (Rice et al 2011).

Newer approaches, such as targeting the coagulation system, have been considered. Recombinant human activated protein C (APC) was found to reduce 28-day mortality in patients with severe sepsis (Bernard et al 2001). However, follow-up studies among patients with severe sepsis and a low risk of death and in children with severe sepsis were negative (Abraham et al 2005, Nadel et al 2007). Furthermore, a recent trial of APC has provided evidence that this anticoagulant and anti-inflammatory agent does not have efficacy for patients with severe sepsis, the most lethal cause of ALI and ARDS (Ranieri et al 2012). Strategies and rationale for anticoagulants for ALI and ARDS will now need to be reevaluated.

Explanations for these outcomes are likely multifactorial, the lack of efficacy of many of these agents does raise the question of whether or not these treatments may perform better in more homogeneous cohorts of patients. Therefore, further research is needed to recommend any of these agents. Without therapeutic product currently approved for treatment of ALI/ARDS, there is clearly an urgent need for new effective and affordable treatments.

1.3. Ceramide-enriched membrane platforms

1.3.1. Lipid interactions and domain formation

The biological membranes of eukaryotic cells are comprised mainly of sphingolipids, (glycerol-) phospholipids and cholesterol. Sphingolipids are characterized by a 1, 3-dihydroxy-2-aminoalkane backbone, also named sphingoid base (Hakomori 1983). Sphingosine, the most prevalent backbone of mammalian sphingolipids refers to (2S, 3R, 4E)-2-amino-4-octadecene-1, 3-diol. Sphingoid bases vary in length of the alkyl chain and position and number of the double bonds. Ceramide is generated from sphingosine by attachment of a fatty acid via an amide ester bond. The fatty acids in the ceramide moiety also vary in chain length and saturation. Thus, ceramide is composed of D-erythro-sphingosine and a fatty acid usually containing 2 – 32 carbon atoms in the acyl chain that are connected

via an amide ester bond. Ceramides are further modified by attachments of headgroups to form sphingomyelin, gangliosides, sulfatides, globosides or cerebrosides, for example, forming sphingomyelin by reaction with phosphorylcholine (Barenholz et al 1980, Kolesnick et al 2000). The most prevalent membrane sphingolipid is sphingomyelin, which consists of a very hydrophobic ceramide moiety (a D-erythro-sphingosine bound to a fatty acid by an amide ester) and a hydrophilic phosphorylcholine headgroup (Hakomori 1983). The acyl chain of the fatty acid may contain 2 to 32 carbon atoms.

In 1972, Singer and Nicolson described the fluid mosaic model of the cell membrane; this model suggested a random distribution of lipids and proteins in the cell membrane, which was in a liquid-disordered phase. This model predicted free movement of proteins in the lipid bilayer, which was based on biophysical experiments that determined the melting temperatures of lipids (Singer et al 1972). However, biophysical studies in the last 15 years revised this model and suggested the formation of small domains in the cell membrane that exist in a liquid-ordered phase and thus form distinct domains in the cell membrane (Brown et al 1998, Simons et al 1997).

Sphingomyelin is the most prevalent cellular sphingolipid and synthesized on the luminal side of the Golgi apparatus or the plasma membrane. Thus, it localizes predominantly to anti-cytoplasmic leaflets of the cell membrane and intracellular vesicles (Emmelot et al 1975, Futerman et al 1990, Jeckel et al 1990). This distribution pattern of sphingomyelin, which results in lipid bilayer asymmetry, is critical for the genesis of distinct membrane domains and, as discussed below, signal transduction. Sphingomyelin consists of a highly hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup (Barenholz et al 1980, Kolesnick et al 2000). Sphingolipids interact with each other via hydrophilic interactions between the sphingolipid headgroups (Brown et al 1998, Kolesnick et al 2000, Simons et al 1997). In addition, sphingolipids, and in particular the predominant sphingolipid sphingomyelin, interact with cholesterol via hydrogen bonds with the hydroxy group in the cholesterol molecule and via hydrophobic van der Waal interactions between the ceramide moiety and the sterol ring system. These binding forces result in a relatively tight interaction

between these lipids and in the spontaneous formation of domains that are in the liquid-ordered phase or even of gel-like domains with higher melting temperatures than other phospholipids in the cell membrane (Brown et al 1998, Kolesnick et al 2000, Simons et al 1997). These distinct sphingolipid- and cholesterol-enriched membrane domains were named rafts (Simons et al 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). Extraction of cholesterol from rafts by interference with the cholesterol metabolism employing drugs such as filipin, nystatin or methyl-beta-cyclodextrin (Keller et al 1998) destroys rafts supporting the critical role of cholesterol for the integrity of at least some rafts.

However, at present only indirect evidence exists to support the presence of rafts in cells under in vivo conditions, for instance at physiological temperature. The concept of rafts is still somewhat controversial (Munro 2003), since the use of detergents employed in most studies characterizing rafts may change the membrane and only cause the formation of rafts (Munro 2003). Recently, Brugger and co-workers suggested the lipid composition of HIV particles released from living cells (Brugger et al 2006). This study demonstrated a high concentration of sphingolipids and cholesterol in the viral coat suggesting that viral budding occurs in distinct domains of the cell membrane that are enriched with these lipids arguing that rafts exist *in vivo*.

1.3.2. Ceramide synthesis and metabolism

Diverse stress stimuli, such as cytokines, environmental stress and chemotherapeutic or anti-cancer drugs (Hannun et al 2002, Senchenkov et al 2001, Spiegel et al 2002) (**Figure 1.3.2.A.**) induce ceramide formation, and therefore ceramide is involved in the regulation of cell growth, differentiation, cell cycle arrest and apoptosis.

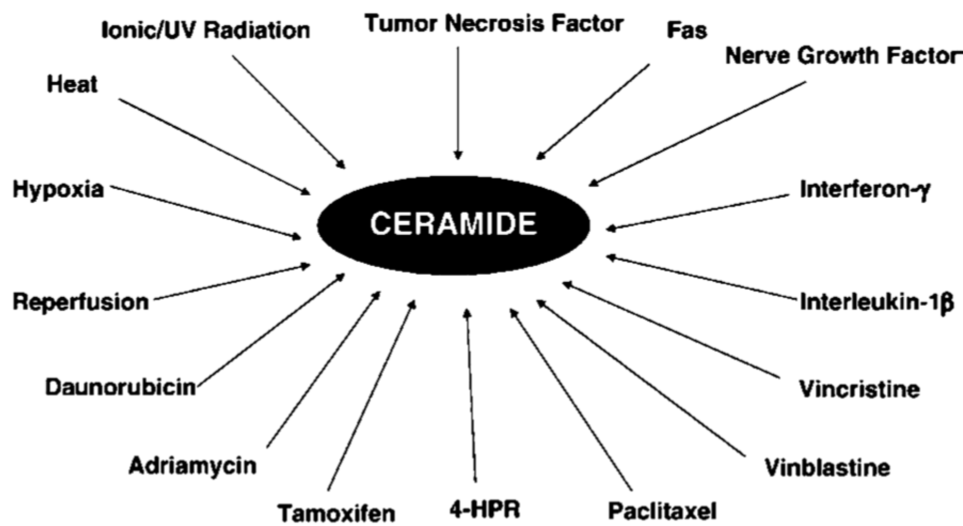

Figure 1.3.2.A. Ceramide formation in response to diverse stress stimuli

Ceramide formation is induced in response to environmental stress, ionic/ultraviolet radiation, heat, hypoxia, reperfusion, cytokines and growth factors, tumor necrosis factor, interferon-gamma and interleukin-1-beta as well as chemotherapeutic agents/anticancer drugs (Pandey et al 2007).

The hydrolysis of sphingomyelin is catalyzed by the activity of acid, neutral, or alkaline sphingomyelinase and results in the generation of ceramide (Gulbins et al 2003, Hannun et al 2008, Quintern et al 1989). Further, ceramide is generated in membranes by a de novo pathway involving the enzyme ceramide synthase (Kolesnick et al 2000) (**Figure 1.3.2.B.**). 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). Ceramide can also be converted into other sphingolipids, such as ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate.

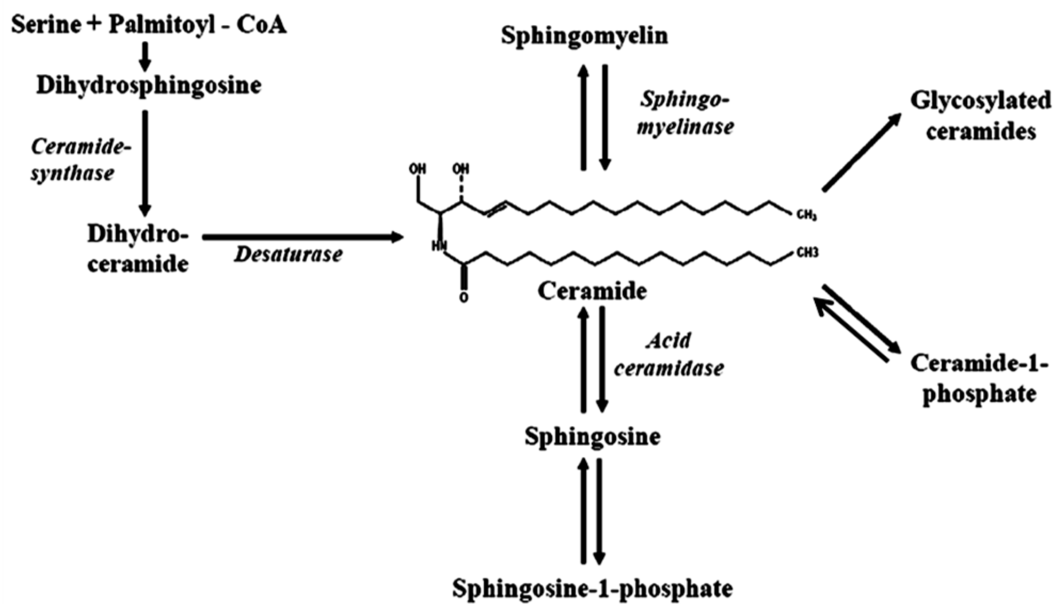

Figure 1.3.2.B. Ceramide synthesis and metabolism

Ceramide can be generated from the sphingomyelin via sphingomyelinase pathway (sphingomyelin metabolism), degradation of glycosylated sphingolipids or via the *de novo* synthesis pathway where cells synthesize ceramide from serine and palmitoyl-CoA (*de novo* synthesis). Ceramide can be further converted into other sphingolipids such as ceramide-1-phosphate, glycosylated ceramide, sphingosine and sphingosine-1-phosphate (Becker et al 2010a).

1.3.3. Acid sphingomyelinase (Asm) and ceramide-enriched membrane platforms

Acid sphingomyelinase (Asm) is the first and best-characterized sphingomyelinase and previous study already showed the enzyme critically involved in many forms of cell activation reviewed for instance by Gulbins et al. (Gulbins et al 2003). There are two forms of Asm, a lysosomal Asm and a secretory Asm that are both derived from the same gene, but differ in their glycosylation pattern and are also differentially processed at the NH2-terminus (Schissel et al 1998a). Most of Asm seems to reside in classical lysosomes, where it mediates the catabolism of sphingomyelin. Asm-deficiency leads to the accumulation of sphingomyelin and a lysosomal storage disorder named Niemann-Pick disease type A and B. Many studies suggested that the pool of Asm insecretory lysosomes seems to participate in signal transduction events (Bao et al 2010, Grassmé et al 2001a, Herz et al 2009, Schissel et al 1996, Schissel et al 1998b). Appropriate cellular activation triggers the fusion of secretory

lysosomes and the cell membrane, and this fusion results in the exposure of Asm on the outer leaflet of the cell membrane (Bao et al 2010, Herz et al 2009).

Usually, Asm hydrolyzes sphingomyelin to ceramide, preferentially at an acidic pH. However, because other lipids crucially influence the Michaelis-Menten constant (Km) of the enzyme to its substrate, the enzyme seems to be able to hydrolyze sphingomyelin also under almost neutral PH conditions (Schissel et al 1996, Schissel et al 1998a). Since Asm is predominantly present in the outer leaflet of the cell membrane (Calderon et al 1997, Grassmé et al 2001a, Grassmé et al 2001b), the hydrolysis of sphingomyelin results in ceramide-enriched membrane domains that are primarily in the outer leaflet of the cell membrane, or in general in anti-cytoplasmic leaflets of cellular membranes.

Changes in the glycosylation pattern of Asm result in the expression of a secretory form of Asm that is released upon stimulation, for instance via interleukin-1 receptors (Schissel et al 1996, Schissel et al 1998b). Further, several studies demonstrated that stimuli such as CD95, DR5 and CD40 or infection with some pathogenic bacteria and viruses mobilize intracellular vesicles/secretory lysosomes, a process that results in exposure of Asm on the outer leaflet of the cell membrane (Cremesti et al 2001, Dumitru et al 2006, Grassmé et al 2001a, Grassmé et al 2002b, Grassmé et al 2003a, Grassmé et al 2003b, Grassmé et al 2005).

Lysosomal Asm and secretory Asm hydrolyze sphingomyelin from the plasma membrane and generate ceramide within cell membranes. The release of ceramide within the cell membrane alters the biophysical characteristics of membranes, because they spontaneously self-associate to small, highly hydrophobic, and ordered ceramide-enriched membrane microdomains (Holopainen et al 1998, Kolesnick et al 2000, Nurminen et al 2002). These microdomains spontaneously fuse to larger ceramide-enriched membrane domains, also named membrane platforms, that can reach a width of up to 5 µm (Gulbins et al 2003). Ceramide-enriched membrane platforms seem to be very hydrophobic and stable, since ceramide molecules are highly packed and ordered. Furthermore, the presence of ceramide excludes cholesterol molecules from membrane domains, at least in artificial membranes, suggesting that ceramide

also changes the composition of rafts (Megha et al 2004, Megha et al 2006) with an increased concentration of ceramide and a decreased concentration of cholesterol, respectively.

1.3.4. Visualization of ceramide-enriched membrane domains

The formation of ceramide-enriched membrane platforms in the plasma membrane upon generation of ceramide might be critical for the signaling function of ceramide, which were demonstrated by several methods *in vivo* and *in vitro*.

First, activation of several receptors, such as CD95, DR5, CD40, and the platelet-activating factor receptor, but also some bacterial and viral infections or stress stimuli, trigger the surface exposure of Asm and the formation of ceramide-enriched membrane platforms by fluorescence and electron microscopy (Cremesti et al 2001, Dumitru et al 2006, Grassmé et al 2001a, Grassmé et al 2002b, Grassmé et al 2003a, Grassmé et al 2003b, Grassmé et al 2005) (**Figure 1.3.4.**). Secondly, studies on phosphatidylcholine/sphingomyelin-composed unilamellar vesicle that were treated with sphingomyelinase immobilized onto a microbead confirmed the formation of ceramide-enriched membrane macrodomains (Holopainen et al 1998, Nurminen et al 2002). Furthermore, *in vitro* studies also indicated the formation of distinct membrane domains by magnetic resonance spectroscopy and atomic force microscopy studies, which revealed laminar phase separation of long chain ceramides in glycerol-phospholipid/cholesterol bilayers and formation of stable, distinct domains that correspond with a transition of fluid phospholipid bilayers into a gel-like phase (Huang et al 1999, ten Grotenhuis et al 1996, Veiga et al 1999). Finally, Ira and Johnston used a combination of atomic force microscopy and total internal reflection fluorescence to directly visualize clustering of small membrane domains into larger domains in artificial membranes composed of 1, 2-dioleoyl-sn-glycero-3-phosphocholine/sphingomyelin/cholesterol mixtures upon treatment with Bacillus cereus sphingomyelinase to generate ceramide or incubation with C_{16}-ceramide. The studies revealed that enzymatic hydrolysis of sphingomyelin to ceramide in model membranes resulted in very rapid reorganization of the membrane, clustering of domains and the formation of larger distinct domains that presumably correspond to ceramide-enriched membrane domains. The addition of C_{16}-ceramide also

resulted in the formation of larger domains in these bilayers, albeit with different kinetics and less impact on membrane organization (Ira et al 2008).

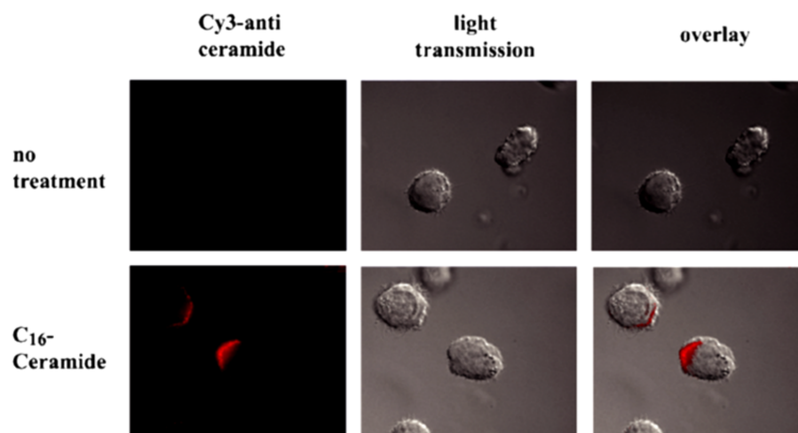

Figure 1.3.4. Ceramide forms ceramide-enriched membrane platforms in the cell membrane

Jurkat T cells (0.5 x·10^6 cells/sample) were centrifuged for 5 min at 1400 rpm, resuspended in 90 µl PBS. Cells were rested for 8 min at 37 ℃, shaked at 400 rpm, and treated with 1 µM C_{16} ceramide for 5 min. Cells were fixed in 1% PFA for 20 min, washed once, unspecific binding sites were blocked with 2% fetal calf serum (FCS) and stained with anti-ceramide antibody clone 15B4 (1:20 dilution). Cells were incubated on ice for 45 min, washed once and incubated for 45 min on ice with 100 µl of a Cy3 F (ab) 2 fragment of a donkey anti-mouse IgM (1:300). Cells were washed once and analyzed on a fluorescence microscope (Grassmé et al 2007).

1.3.5. Function of ceramide-enriched membrane platforms

Ceramide-enriched membrane domains may have several biological functions. First, the composition and fluidity of ceramide-enriched membrane domains differ from the surrounding areas in biological membranes, which are perfect structures to sort proteins in cells and to provide a mean for the spatial re-organization of receptors and/or intracellular signaling molecules within these membrane domains. Trapping and clustering of activated receptors in ceramide-enriched membrane domains was demonstrated for CD95, DR5, or CD40 (Dumitru et al 2006, Grassmé et al 2001a, Grassmé et al 2002a, Grassmé et al 2002b), but many more receptors may use the mechanism of clustering to reach a very high density in circumscribed areas of the cell membrane. The reorganization of receptors within ceramide-enriched membrane domains results in a very high receptor density within a relatively small area of the cell membrane, which is required for the transmission of signals

via many receptor molecules. Receptor aggregation and trapping in ceramide-enriched membrane domains may limit lateral diffusion and, thus, stabilize the interaction of a ligand with its receptor, in particular if the ligand is also trapped in distinct membrane domains. The interaction of ligand-bound receptors with the very hydrophobic ceramide-enriched membrane platform and/or individual ceramide molecules may in addition stabilize conformational changes that may occur upon activation of a receptor by its ligand. Furthermore, ceramide-enriched membrane domains may sort intracellular signalling molecules, for instance via farnesyl or geranyl-moieties, which may result in the spatial association of activated receptors with signalling molecules that transmit the signal from the receptor into the cell, while at the same time inhibitory molecules are excluded from this area of the cells. Thus, in general, ceramide-enriched membrane platforms may primarily function to re-organize receptor and signaling molecules in and at the cell membrane to facilitate and amplify signaling processes via a specific receptor.

Second, in addition to its function in membrane platforms, ceramide was also shown to directly interact and regulate several molecules including cathepsin D (Heinrich et al 1999), phospholipase A2 (Huwiler et al 2001), kinase suppressor of Ras (identical to ceramide-activated protein kinase) (Zhang et al 1997), ceramide-activated protein serine-threonine phosphatases(CAPP) (Dobrowsky et al 1993), protein kinase C isoforms (Muller et al 1995) and c-Raf-1 (Yao et al 1995). A direct binding of ceramide was demonstrated for cathepsin D (Heinrich et al 1999), phospholipase A2 (Huwiler et al 2001) and CAPP (Chalfant et al 2004, Dobrowsky et al 1993), although at present the details and the specificity of ceramide-protein interactions are still unknown except for cathepsin D. Cathepsin D binds ceramide within a short domain of the cathepsin D molecule, which triggers the autocatalytic cleavage of cathepsin D to its active form and promotes, via still unknown mechanisms, the translocation of active cathepsin D from lysosomes into the cytoplasm where cathepsin D triggers cell death via Bid, Bax and Bak (Heinrich et al 1999, Schneider-Brachert et al 2004). Ceramide might facilitate the transport of cathepsin D over the double membrane by forming a hydrophobic coat around the protein, the formation of ceramide channels (Siskind et al 2000) or by a very short disruption of the membrane bilayer.

Third, ceramide has been shown to regulate several ion channels. Ceramide inhibits the potassium channel Kv1.3 and the calcium release activated calcium channel in lymphocytes (Bock et al 2003, Gulbins et al 1997, Lepple-Wienhues et al 1999, Szabo et al 1996). This is also consistent with the finding that Kv1.3 localizes to ceramide-enriched membrane domains after stimulation via CD95 (Bock et al 2003, Lepple-Wienhues et al 1999). However, these studies do not exclude that ceramide also affects ion channels by a direct interference or by the change of the lipid composition in ceramide-enriched membrane platforms. The calcium release activated calcium channels that are central in the regulation of cellular Ca^{2+} concentrations and, thus, involved in multiple cellular pathways (Lee et al 2004), are also inactivated upon stimulation of cells via CD95 (Hannun et al 2000) or the TNF-receptor (Zemann et al 2007) or upon treatment with synthetic ceramides, C_2, C_6 and C_{16}-ceramides, respectively. Genetic deficiency of the Asm abrogated the inhibition of the calcium release activated calcium channels by CD95 and TNF-receptor stimulation. Finally, ceramide-molecules seem to form channels or pores, at least in the outer mitochondrial membrane (Siskind et al 2000, Siskind et al 2006). These channels might be important for the induction of apoptosis, although it is unknown whether ceramide pores are also formed *in vivo*. The regulation of ion channels by ceramide is a poorly investigated field, although its potential for many physiological and pathophysiological processes appears immense.

In summary, ceramide-enriched membrane domains serve the temporal and spatial organization of signaling molecules to regulate multiple cell functions. However, the mechanisms responsible for the action of ceramide on these downstream targets are not fully understood.

1.3.6. Asm/ceramide in bacterial infections

Asm/ceramide in internalization of bacteria. Numerous studies, using either genetic deficiency of pharmacological inhibition of Asm, demonstrated that the activation of Asm and the concomitant release of ceramide upon the infection of human epithelial and myeloid cells with *Neisseria gonorrhoeae* (Grassmé et al 1997, Hauck et al 2000), the infection of

endothelial cells with *S. aureus (Esen et al 2001)*, the infection of pulmonary epithelial cells and fibroblasts with *Pseudomonas aeruginosa* (*P. aeruginosa*) (Grassmé et al 2003b), the infection of immature dendritic cells with *Escherichia coli* (Falcone et al 2004), the infection of macrophages with *Listeria monocytogenes* (Utermohlen et al 2003) or *Salmonella typhimurium (McCollister et al 2007)*, and the infection of mononuclear cells with *Mycobateria avium (Utermohlen et al 2008)*, resulted in uptake of the bacteria (**Figure 1.3.6.A.**). The bacteria very rapidly activate the enzyme, induce a rapid surface translocation of the Asm and trigger the release of ceramide and, thus, the formation of ceramide-enriched membrane platforms, which seem to be central for the uptake of pathogens.

***Asm/ceramide in bacterial killing*.** Infection of macrophages with *Listeria monocytogenes* (Utermohlen et al 2008) or *Salmonella typhimurium* (McCollister et al 2007) results in intracellular killing of the bacteria. In wild-type macrophages, the phagosome rapidly fuses with the lysosome to form a phago-lysosome and to kill and digest the bacteria. In contrast, Asm-deficient macrophages were unable to kill the bacteria. Studies with *Listeria monocytogenes* also demonstrated that Asm deficient macrophages impaired the maturation and fusion of intracellular phagosomes with lysosomes and led to development of sepsis and greatly increased mortality of Asm deficient mice (Schramm et al 2008, Utermohlen et al 2003). Moreover, Yang and colleagues showed that the Asm is also required to produce ROS upon infection, which also kills the pathogens. Deficiency of the Asm prevents ROS release and reduces bacterial killing (Zhang et al 2008) (**Figure 1.3.6.B.**).

***Asm/ceramide in cell death induced by bacterial infection*.** In addition to mediating the internalization of pathogens and fusion of intracellular vesicles, Asm and ceramide have been shown to be also crucial for the induction of cell death on infection of endothelial cells with *S. aureus* (Esen et al 2001), on infection of immature dendritic cells with *E. coli* (Falcone et al 2004), on infection of pulmonary epithelial cells and fibroblasts with *P. aeruginosa* (Grassmé et al 2003b, Kannan et al 2008). The molecular mechanisms by which Asm and ceramide are involved in induction of cell death are still not well-known. Below is a summary of possible mechanisms: a. Ceramide-enriched membrane platforms cluster CD95 and induce cell death

on infection of epithelial cells with *P. aeruginosa* (Becker et al 2012, Grassmé et al 2000). b. Ceramide-enriched membrane platforms are required to internalize *P. aeruginosa*, induce apoptosis and regulate the cytokine response in infected cells. Impaired bacterial killing in Asm-deficient mice induced overwhelming inflammation and cell death (Grassmé et al 2003b, Kannan et al 2006) (**Figure 1.3.6.C.**).

Asm/ceramide in cytokine release induced by bacterial infection. The release of cytokines, which is moderately increased in the lungs of wild-type mice upon infection, is uncontrolled and exaggerated in Asm-deficient mice infected with *P. aeruginosa*, resulting in a cytokine storm and the death of the mice (Grassmé et al 2003b). This finding is confirmed by Kannan and co-workers' work (Kannan et al 2006). However, the mechanisms that mediate the activation of Asm and cytokines release by *P. aeruginosa* require definition.

Asm/ceramide in host survival. Our group revealed that Asm/ceramide are critical for the internalization of *P. aeruginosa* into epithelial cells and fibroblasts, the induction of death of infected cells, and controlled release of cytokines critical, finally facilitating the mice survival *P. aeruginosa* infection (Grassmé et al 2003b). Schramm and colleagues ascertained the role of Asm/ceramide in *Listeria monocytogenes* infection by demonstrating that Asm-deficient macrophages showed impaired phagolysosome fusion and maturation correlated with severe sepsis and increased mortality of Asm-deficient mice (Schramm et al 2008). Moreover, recent studies showed that Asm-deficienct mice controled the bacteria in small granulomas to protect the mice from uncontrolled inflammation upon *Mycobacteria avium* infection, but not wild-type mice (Utermohlen et al 2008).

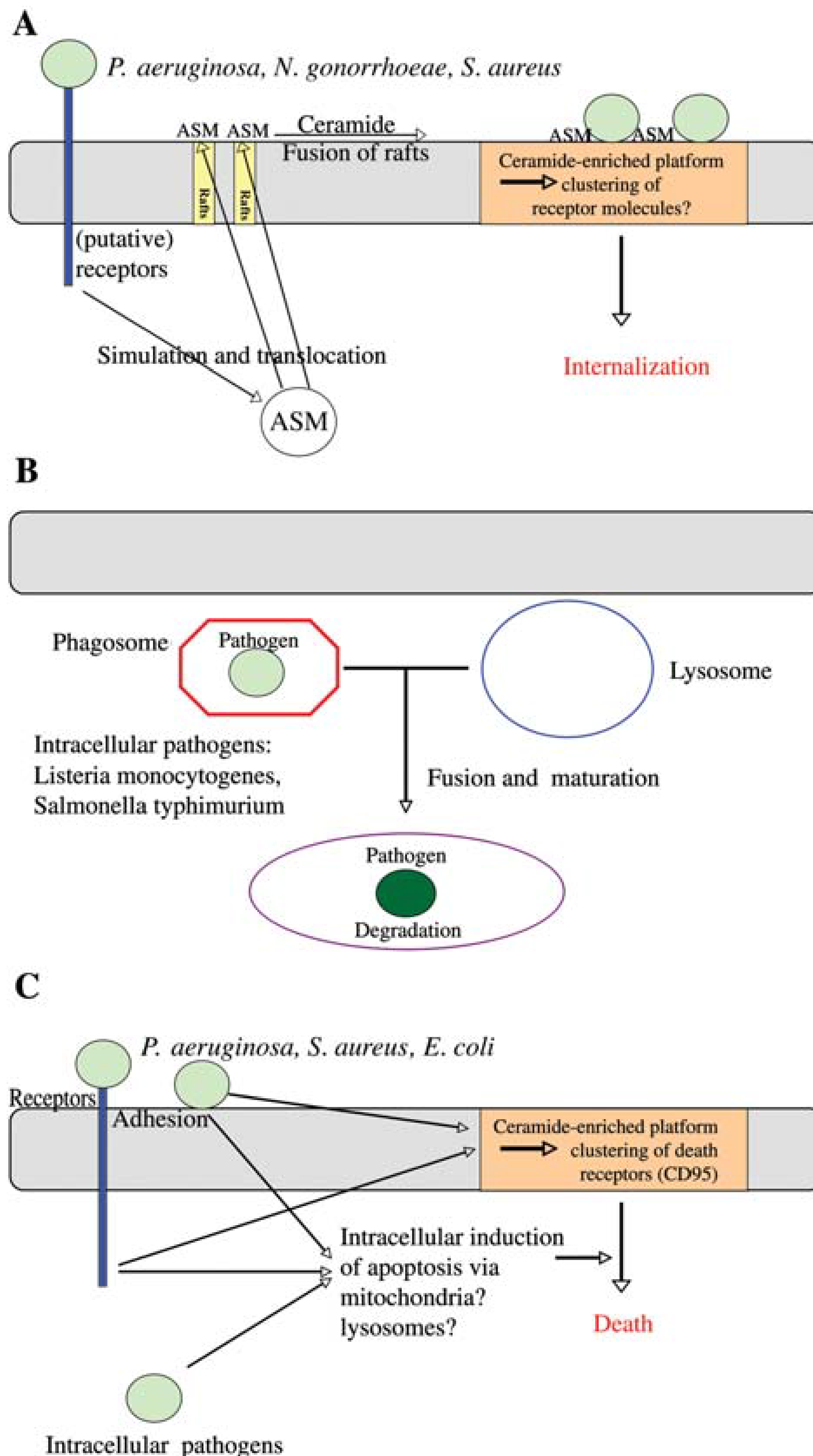

Figure 1.3.6. Functions of Asm and ceramide during bacterial infections

Asm functions in the outer leaflet of the cell membrane to mediate internalization of pathogens (A) and in lysosomes to mediate fusion of phagosomes with lysosomes (B). Asm also seems to be

involved in maturation of phagolysosomes (B). Finally, surface and intracellular ceramide generated by Asm activity is important for induction of cell death upon infection with some pathogens (C) (Grassmé et al 2008).

1.4. Redox signaling

1.4.1. Overview of Reactive oxygen species (ROS)

ROS are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis (Devasagayam et al 2004). Various ROS (**Figure 1.4.1.**), including $O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$, and $ONOO^{\cdot-}$, participate in cell signaling under certain physiological or pathological conditions. The most important of these ROS is $O_2^{\cdot-}$, which is unstable and short-lived because it has an unpaired electron, and it is highly reactive with a variety of cellular molecules, including proteins and DNA. $O_2^{\cdot-}$ is reduced to H_2O_2 by superoxide dismutase (SOD), an enzyme which catalyzes the dismutation of superoxide radicals ($O_2^{\cdot-} + O_2^{\cdot-} + 2H+$ -- $O_2 + H_2O_2$) (McCord et al 1969). Both $O_2^{\cdot-}$ and H_2O_2 can diffuse from their sites of generation to other cellular locations. H_2O_2 is further reduced to generate the highly reactive $^{\cdot}OH$ through the Haber-Weiss or Fenton reaction under pathological conditions. In contrast to $O_2^{\cdot-}$ and H_2O_2, $^{\cdot}OH$ is highly reactive and, therefore, causes primarily local damage. In addition, $O_2^{\cdot-}$ can also interact with NO to form another reactive oxygen free radical, $ONOO^{\cdot-}$. Under physiological conditions, $O_2^{\cdot-}$ preferably produces H_2O_2 via the dismutation reaction. However, when excess $O_2^{\cdot-}$ is produced, a substantial amount of $O_2^{\cdot-}$ reacts with NO to produce $ONOO^{\cdot-}$ (**Figure 1.4.1.**).

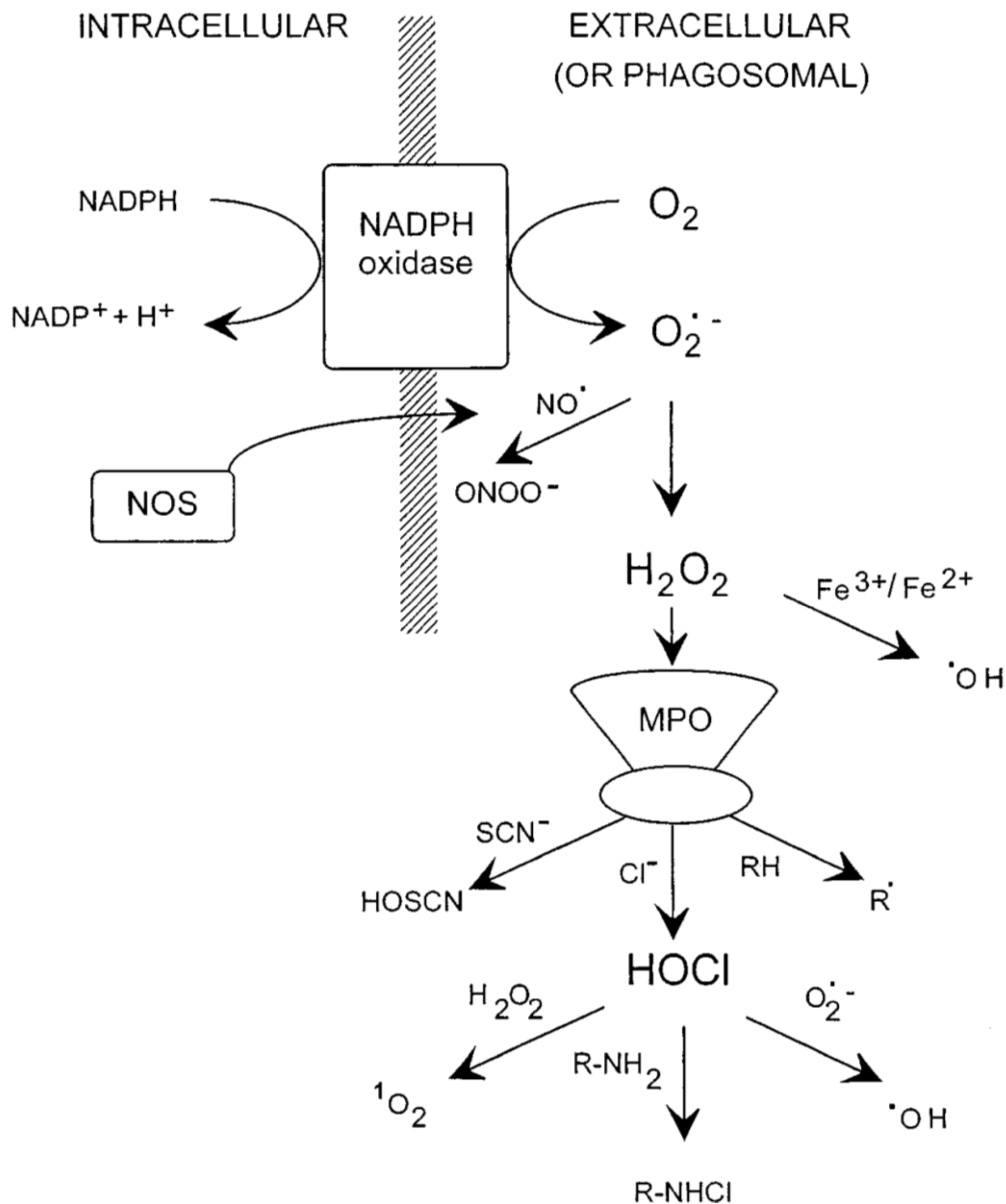

Figure 1.4.1. Possible oxidant generating reactions with stimulated neutrophils. NOS, nitric oxide synthase; MPO, myeloperoxidase (Hampton et al 1998)

1.4.2. ROS production and regulation

$O_2^{\cdot-}$ is the progenitor of other ROS. In mammalian cells, many pathways are involved in the production of $O_2^{\cdot-}$, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, mitochondrial respiration chain, and NO synthase-uncoupling. NADPH oxidase has been detected in nearly every tissue, and in many cells, such as those in phagocytes and vascular cells, it is the primary source of ROS. Recent studies suggest that

NADPH oxidase localizes to specific subcellular compartments, including lamellipodial focal complexes and focal adhesions, membrane ruffles, caveolae and lipid rafts, endosomes, sarcoplasmic reticulum, and the nucleus. NO synthases normally localize in caveolae and function as homodimers to synthesize NO. When exposed to oxidative or nitrosative stress, NOS becomes structurally unstable (“uncoupling state”) and exhibits NADPH oxidase activity resulting in $O_2^{\cdot-}$ formation. Given that ROS are short-lived and diffusible, the localization of ROS signals in specific subcellular compartments suggests that mammalian cells contain temporally and spatially organized redox signaling pathways that regulate various cellular functions.

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies 1997).

ROS formation and redox signaling are well known to play a major role in physiology as well as in a variety of pathologies. For instance, in the heart, cardiomyocyte differentiation, and excitation-contraction coupling are under tight redox control (Burgoyne et al 2012, Steinberg 2013). On the other hand, cardiac pathologies, such as ischemia/reperfusion injury, heart failure, and arrhythmias can be prevented or blocked by inhibiting specific processes that result in ROS generation in several experimental models (Anderson et al 2014, Kaludercic et al 2014, Takimoto et al 2007, Youn et al 2013). Thus, it appears that pro-oxidant generation and antioxidant defense need to be tightly regulated (Chance et al 1979). Indeed, disruption of redox signaling and control, and imbalance in favor of pro-oxidant species is defined oxidative stress, term first coined in 1985 (Jones 2006, Sies et al 1985). Conversely from pathological modifications (Chance et al 1979, Powers et al 2008), it appears that

physiological redox signaling is characterized by reversible oxido-reductive modifications, confined both spatially and temporally in subcellular compartments and microdomains.

1.5. Endothelial Barrier

1.5.1. Overview of Endothelial Barrier

The vascular endothelium lining the inner surface of blood vessels serves as the first interface for circulating blood components to interact with cells of the vascular wall and surrounding extravascular tissues. In addition to regulating blood delivery and perfusion, a major function of vascular endothelia, especially those in exchange microvessels (capillaries and postcapillary venules), is to provide a semipermeable barrier that controls blood-tissue exchange of fluids, nutrients, and metabolic wastes while preventing pathogens or harmful materials in the circulation from entering into tissues.

Blood fluid, solutes, and even circulating cells can cross the endothelium via two routes: through the cell body (transcellular), or between the cells (paracellular, or intercellular)(Mehta et al 2006) (**Figure 1.5.1.**). The transcellular pathway, which contributes very little to the leakage of events in pathophysiological conditions, includes vesicle-mediated endocytosis, vacuole-vesicular organelles (VVOs) and regulated water channels (aquaporins). On the other hand, the paracellular pathway, which is responsible for the majority of leakage of blood fluid and proteins across the microvascular endothelium under pathophysiological conditions, is mainly mediated by junctional proteins (**Figure 1.5.1.**).

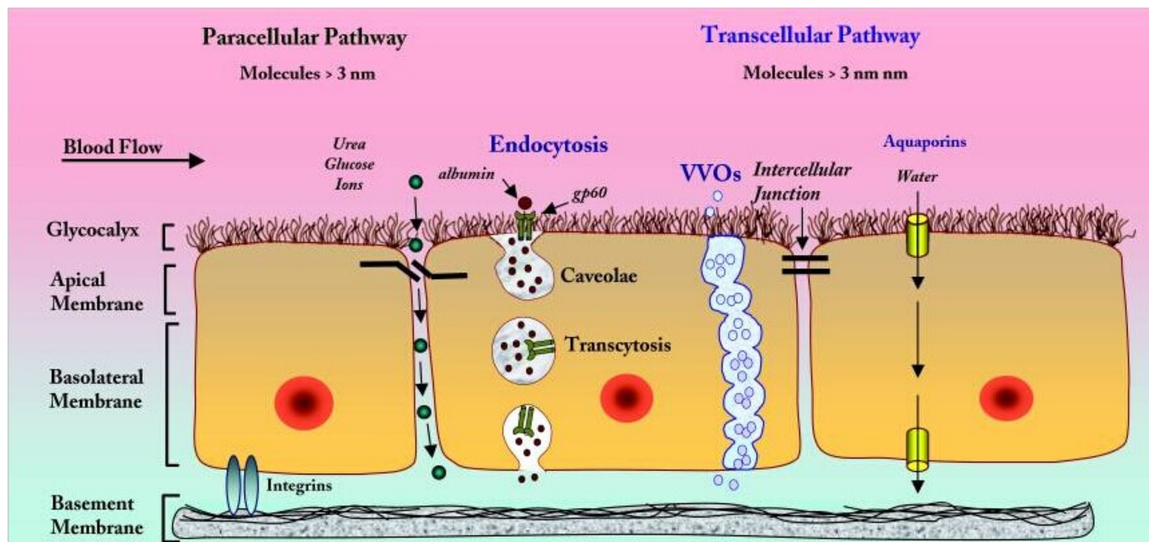

Figure 1.5.1. Transcellular and paracellular permeability pathways across the microvascular endothelium

Barrier function of the microvasculature is provided by closely apposed endothelial cells of themicrovessel walls. The thin layer of endothelium is attached to the microvascular basement membrane via endothelial membrane-bound integrins. Endothelial cells are joined together by intercellular junction proteins that allow the selective passage of solutes and fluids across the endothelium. Intercellular junctions can become more porous, or even form large-sized gaps under pathophysiological conditions. The glycocalyx forms a selective filter across the endothelial luminal surface, forming an additional permeability barrier. Solutes can also traverse the cell interior via receptor-mediated vesicle endocytosis originating at caveolae, or via vacuole-vesicular organelles (VVOs) that can fuse with trafficking vesicles and form open transcellular pores. Transcellular water transport can occur in parallel with other fluxes, through regulated water channels (aquaporins) in the endothelial cell membrane (Yuan et al 2010).

Two types of intercellular junctions have been characterized as the cell–cell adhesive barrier structures in the microvascular endothelium: the adherens junction (AJ) and tight junction (TJ) (Komarova et al 2010, Mehta et al 2006). The former has been identified in nearly all types of vascular beds, especially in the peripheral microvasculature. Vascular endothelial (VE)–cadherin is believed to be the most important protein in forming the molecular basis, as well as regulating the function of AJs. Intracellularly, VE–cadherin is connected to the actin cytoskeleton via a family of catenins (α-, β-, γ-, and p120-catenins) (Mehta et al 2006). Thus, the stability of the VE–cadherin–catenin–cytoskeleton complex is essential to the maintenance of endothelial barrier function (Alcaide et al 2008, Sallee et al 2006, Vincent et al 2004).

Endothelial tight junctions are composed of interactions of tight junction proteins: occludin, claudins, and JAM-A (Abbott et al 2010, Hawkins et al 2005, Mehta et al 2006), which are connected to the actin cytoskeleton via zona occludens proteins (ZO-1, ZO-2) and α-catenin. ZO proteins play both structural and signaling roles in tight junctions (Hawkins et al 2005, Shen et al 2009).

During host defense against infection or tissue injury, endothelial barrier dysfunction occurs as a consequence as well as cause of inflammatory responses. Endothelial barrier dysfunction is characterized by leakage of fluid, proteins, or small molecules, measured as excessive flux of these molecules across the endothelium (termed hyper-permeability), and clinically manifests as accumulation of plasma-like, protein-rich fluid in the extravascular space leading to tissue swelling (termed edema) (Yuan et al 2010).

1.1.1. Leukocytes and Endothelial Barriers

Endothelial hyper-permeability occurs following trauma, pathogen infection, or chronic disease states, which is a generalized response to inflammation (Kumar et al 2009, Lush et al 2000). A hallmark of inflammation is extravasation of leukocytes from the blood to the tissue across the microvascular endothelium (Cavanagh et al 1998, Lewis et al 1986, Nathan 2006). Leukocytes, which are white blood cells circulating in the blood, include lymphocytes (T-cells, B-cells, and natural killer cells), monocytes, and polymorphonuclear (PMN) granulocytes (neutrophils, eosinophils, and basophils) (Moser et al 2010).

Neutrophil extravasation is a multi-stage process: rolling, activation, adhesion, and transmigration, requiring complex interactions of PMNs or other leukocytes with the microvascular endothelium (**Figure 1.5.2.**) (Butcher 1991, Kubes 2002). Leukocyte trans-endothelial migration occurs in response to endothelial hyper-permeability caused by bacterial invasion or tissue inflammatory injury. In the presence of a compromised microvascular endothelial barrier, leukocytes can become immobilized by firm adhesion to the micro-vessel luminal surface and cross the endothelium into the tissues step by step.

Generally, PMNs are the first leukocyte cell type to arrive at the site of barrier dysfunction (Nathan 2006). After and/or during transmigration across the micro-vessel wall, PMNs will become activated and undergo a respiratory burst, characterized by release of granule secretions of numerous compounds (Lewis et al 1986), which can attack and liquify tissue surrounding the compromised vasculature (pus formation) (Nathan 2006). PMNs also secrete chemokines or induce endothelial expression of chemokines (Middleton et al 1997) to attract other leukocytes (macrophages, monocytes, and immune cells) to the site of inflammation. Hence, leukocyte activation and migration across the endothelium are both cause and consequence of endothelial hyper-permeability and barrier dysfunction (Nathan 2006).

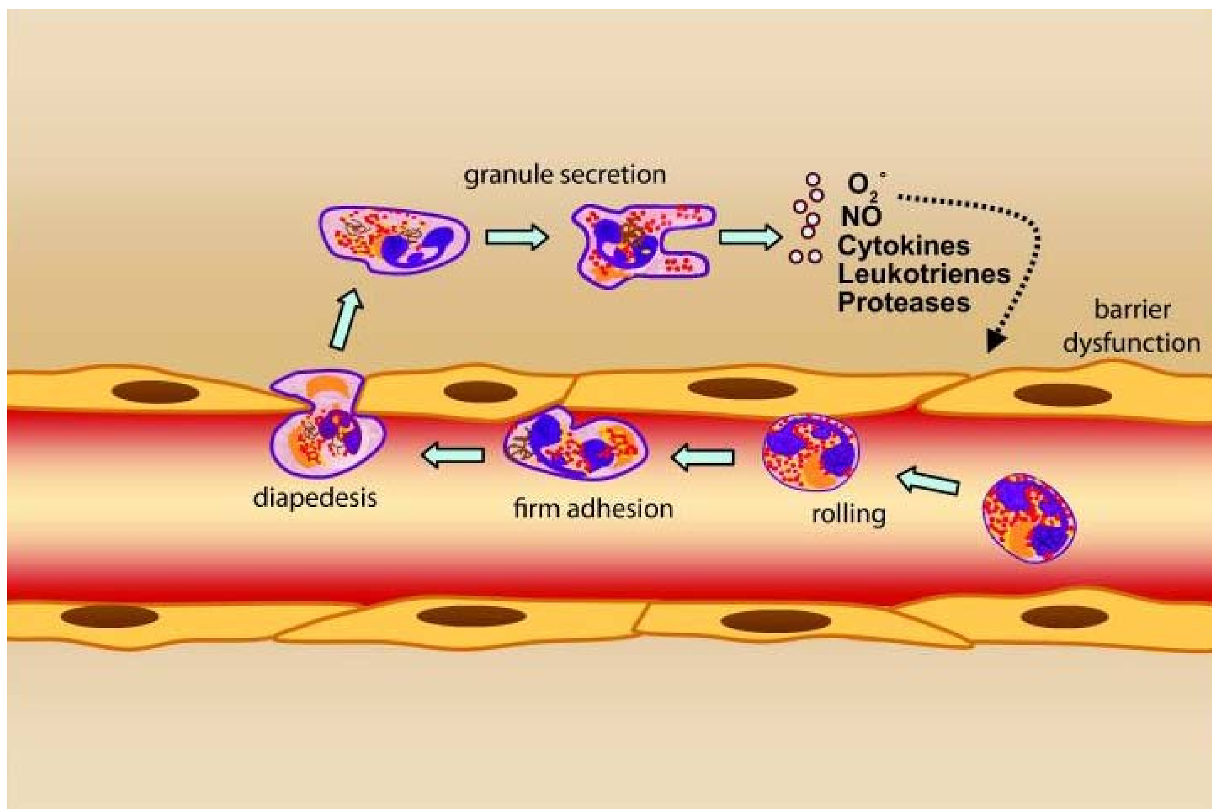

Figure 1.5.2. The stages of neutrophil extravasation

Neutrophil transmigration is a sequential process of (1) rolling along the microvessel wall, (2) firm adhesion to the endothelium (via interactions with cell surface adhesion molecules), (3) diapedesis (transmigration) coordinated by interactions between cell surface glycoproteins, followed by migration into the extravascular space, and (4) neutrophil activation, characterized by granule secretions of

hyper-permeability-inducing agents (oxygen free radicals ($O_2^{\cdot -}$), NO, cytokines, and arachidonic acid metabolites: leukotriences and prostaglandins), further contributing to barrier dysfunction (Yuan et al 2010).

1.6. Aims of the study

Sepsis is a devastating and complex syndrome and continues to be a major cause of morbidity and mortality among critically ill patients at the surgical intensive care unit setting in the United States (Angus et al 2001, Dombrovskiy et al 2007, Kung et al 2008, Martin et al 2003, Melamed et al 2009, Russell 2006). Antibiotics alone are often insufficient to cure patients with *S. aureus*–induced sepsis. Although treatment with effective doses of bactericidal antibiotics indeed prevents the bacterial burden, antibiotics often fail to prevent fatal lung edema after septic infection with *S.*
aureus.

Our group has demonstrated that Asm/ceramide system activates several receptors, mediates entry of several microorganisms into cells and participates in signal transduction events. Particularly, we discovered *S. aureus* infection activates the Asm/ceramide system, and finally induces apoptosis of human endothelial cells (Esen et al 2001). However, it is unknown whether the Asm/ceramide system also plays an important role in endothelial cells injury and lethal lung edema induced by systemic *S. aureus* infection. The endothelium is a highly dynamic cell layer that is involved in a multitude of physiological functions, thus, it is very important to emphasize the potential value of the endothelium as a target for lethal lung edema therapy in sepsis.

The present thesis first defines the role of Asm in regulating lung edema induced by systemic *S. aureus* infections. Because ceramide is an important signaling molecule that regulates redox signaling (Zhang et al 2007), the present study further investigated whether lung edema induced by *S. aureus* infections is prevented by Asm deficiency. The importance of Asm for lung injury was tested in Asm-deficient mice.

Furthermore, the mechanism of Asm activation inducing lung edema was addressed. It was investigated whether *S. aureus* infections induces activation of Asm, subsequent ceramide release and activation of ROS, leading to degradation of tight junctions, neutrophils trafficking and lung edema.

The last part of the study focused on the clinical significance of the Asm/ceramic system in a *S. aureus* sepsis. It was tested whether treatment of already septic mice with Asm inhibitor amitriptyline prevents the development of lung edema and whether the combination of amitriptyline and antibiotic prevents sepsis.

Our results will increase the understanding of the signaling mechanism that Asm deficiency protects lung edema induced by *S. aureus* infections. Moreover, the study will announce a novel approach to treat severe systemic and often lethal infections and to prevent lung injury in patients with incipient sepsis.

2. MATERIALS

2.1. Chemicals

Aqua ad Injectabilia DeltaSelect GmbH, Dreieich

Acetic acid ($C_2H_4O_2$) Merck, Darmstadt

Acetone Sigma-Aldrich Chemie GmbH, Steinheim

Adenosine Tri-Phosphate (ATP) Sigma-Aldrich Chemie GmbH, Steinheim

Amitriptyline Sigma-Aldrich Chemie GmbH, Steinheim

β-mercaptoethanol Sigma-Aldrich Chemie GmbH, Steinheim

Bromphenol blue Sigma-Aldrich Chemie GmbH, Steinheim

C_{16}-Ceramide Biomol, PA, USA

Calcium chloride ($CaCl_2$) Sigma-Aldrich Chemie GmbH, Steinheim

Cardiolipin Sigma-Aldrich Chemie GmbH, Steinheim

Chloroform ($CHCl_3$) Ridel-de Haen, Seelze

CDP-STAR with Nitro-Block II enhancer PerkinElmer, Boston, USA

DABCO (1,4-Diazabicyclo(2,2,2)octane) Sigma-Aldrich Chemie GmbH, Steinheim

Deoxycholic acid ($C_{24}H_{40}O_4$) Sigma-Aldrich Chemie GmbH, Steinheim

Dithiothreitol (DTT) Carl-Roth GmbH & Co, Karlsruhe

Dimethylsulfoxid (DMSO) Sigma-Aldrich Chemie GmbH, Steinheim

Eosin (gelblich) E.Merk, Darmstadt

Ethidium bromide Sigma-Aldrich Chemie GmbH, Steinheim

Ethanol (C_2H_5OH) Sigma-Aldrich Chemie GmbH, Steinheim

Ethylenediamine Tetraacetic Acid (EDTA) Serva Electrophoresis GmbH, Heidelberg

Evan's Blue Dye Sigma-Aldrich Chemie GmbH, Steinheim

Eukitt® quick-hardening mounting medium Sigma-Aldrich Chemie GmbH, Riedstrasse

Formamide Sigma-Aldrich Chemie GmbH, Steinheim

Glucose	Sigma-Aldrich Chemie GmbH, Steinheim
Glycerol	Fluka Chemie GmbH, Buchs
H_2DCFDA	Molecular Probes, Eugene, OR
HEPES	Carl-Roth GmbH & Co, Karlsruhe
Hydrochloric acid (HCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Hematoxylin	Carl-Roth GmbH & Co, Karlsruhe
Imidazole ($C_3H_4N_2$)	Sigma-Aldrich Chemie GmbH, Steinheim
Isopropanol	Sigma-Aldrich Chemie GmbH, Steinheim
Ketamine	Ceva Tiergesundheit GmbH, Duesseldorf
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium sulphate ($MgSO_4$)	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol (CH_3OH)	Fluka Chemie GmbH, Buchs
Methicillin	Sigma-Aldrich Chemie GmbH, Steinheim
Mowiol	Kuraray Specialities Europe GmbH, Frankfurt
N-acetylcysteine	Sigma-Aldrich Chemie GmbH, Steinheim
N-octylglucopyranoside	Sigma-Aldrich Chemie GmbH, Steinheim
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim
Paraplast plus Tissue Embedding Medium	Leica Microsystems GmbH, Netherlands
Pepsin	Invitrogen, Frederick, USA
Phosphatase inhibitor	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium dihydrogenphosphate (KH_2PO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Protease inhibitor	Carl-Roth GmbH & Co, Karlsruhe
RPMI-1640	Gibco/Invitrogen, Karlsruhe, Germany
Saponin	Serva Electrophoresis GmbH, Heidelberg
Sodium acetate (CH_3COONa)	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
Surgipath Paraplast	Leica Microsystems GmbH, Netherlands
Taq Polymerase	Invitrogen, Karlsruhe, Germany
Tiron	Fluka Chemie GmbH, Buchs
Tryptic soy broth (TSB)	BD Biosciences, Heidelberg, Germany
Tris-HCl and Tris-Base	Carl-Roth GmbH & Co, Karlsruhe
Triton X-100	Sigma-Aldrich Chemie GmbH, Steinheim
Tween-20	Sigma-Aldrich Chemie GmbH, Steinheim
Vancomycin	Sigma-Aldrich Chemie GmbH, Steinheim
Xylazin	Ceva Tiergesundheit GmbH, Duesseldorf
Xylene	Applichem GmbH, Darmstadt, Germany

2.2. Tissue culture materials

Cell dissociation buffer enzyme-free	Gibco/Invitrogen, Karlsruhe
DMEM (EAGLE)	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
Tissue culture flasks 75 cm^2	TPP, Trasadingen, Switzerland
Tissue culture flasks 25 cm^2	TPP, Trasadingen, Switzerland
Tissue culture test plates	TPP, Trasadingen, Switzerland
Trypsin	Gibco/Invitrogen, Karlsruhe

2.3. Antibodies

Alkaline phosphatase-coupled secondary antibodies	Santa Cruz Biotechnology, CA, USA
Anti-E-cadherin(H-108) rabbit IgG	Santa Cruz Biotechnology, CA, USA
Anti- Ly-6G and Ly-6C (GR1) rat IgG	BD Biosciences, Heidelberg, Germany
Anti-Occludin rabbit IgG	Invitrogen, CA, USA
Anti-ZO1 rabbit IgG	Invitrogen, CA, USA
Anti-ZO2 (H-110) rabbit IgG	Santa Cruz Biotechnology, CA, USA
Cy3-donkey-anti-rabbit IgG	Jackson Immunoresearch, West Grove, PA, USA
Cy3-donkey anti-rat IgG	Jackson Immunoresearch, West Grove, PA, USA
FITC-labled Isolectin B4	Vector Laboratories, CA, USA

2.4. PCR primers

Asm-PA 1-2 5´-CGA GAC TGT TGC CAG ACA TC-3´	Hermann GbR, Freiburg
Asm-PA 2-2 5´-GGC TAC CCG TGA TAT TGC TG-3´	Hermann GbR, Freiburg
Asm-PS-2 5´-AGC CGT GTC CTC TTC CTT AC-3´	Hermann GbR, Freiburg
Myco P1 5´-GTG CCA GCA GCC GCG GTA ATA C-3´	Hölle & Hüttner AG, Germany
Myco P4 5´-TAC CTT GTT ACG ACT TCA CCC CA-3´	Hölle & Hüttner AG, Germany

2.5. Cell lines

EOMA CRL-2586	established murine endothelial cell line

The cell line was tested monthly by PCR to exclude mycoplasma contamination.

2.6. Animals

Asm-deficient mice were kindly provided by Dr. R. Kolesnick (Memorial Sloan-Kettering Cancer Center, NY, USA) and backcrossed for more than 10 generations on a C57BL/6 background. Syngenic wild-type littermates from the same heterozygous breeding were used as control.

The mice used in the present study show the earliest clinical manifestation of Niemann-Pick disease type A at approximately 12 weeks of age; therefore, all the Asm-deficient mice used in our experiments were younger than 10 weeks of age, before any biochemical, histological or clinical manifestations of Niemann-Pick disease type A were apparent. This excluded that the effects observed in the Asm-deficient cells were due to altered cellular processes but instead, were dependent on the lack of Asm. Wild-type and Asm-deficient mice were propagated in the Animal Facility of the Uniklinikum Essen. The genotype was verified by PCR analysis.

Mice were housed in pathogen-free conditions under diurnal lighting alternated with a dark phase between 18:00–6:00, allowed daily food "Zuchthaltungsfutter Maus-Ratte 10 H 10" (Eggersmann) and water ad libitum. All mice were repeatedly tested for the presence of pathogens and were free of any pathogens according to the criteria of the Federation of Laboratory Animal Science Associations.

2.7. Radioactive substances

[^{32}P] gamma-ATP	Hartmann Analytic, Braunschweig
[^{14}C] Sphingomyelin	Perkin Elmer, Boston, MA, USA

2.8. Other materials

Coverslips 12 mm diameter	Carl-Roth GmbH & Co, Karlsruhe
Cryo 1C Freezing container	Nalgene, USA
Cryovials	Carl-Roth GmbH & Co, Karlsruhe
Cuvettes 10 x 4 x 45 mm	Sarstedt, Nümbrecht, Germany
Leica Confocal software (Leica)	Leica Microsystems, Germany
Microscopy glass slides 76 x 26 mm	Engelbrecht, Edermunde, Germany
Minisart syringe filters	Vivascience AG, Hannover, Germany
Neubauer chamber 0.1 mm	Marienfeld, Germany
Parafilm	Peckiney, Chicago, IL, USA
PCR Tubes	Sarstedt, Nümbrecht, Germany
Polyethylene vials 20 ml	Packard, USA
Silica G60 TLC plates	Merck, Darmstadt
sn-1,2-Diacylglycerol (DAG) Biotrak Assay Reagents System	Amersham Biosciences, Freiburg
Steritop Vacuum-driven disposable top filters	Millipore, Billerica, MA, USA
Tryptic soy agar plates with 5% sheep blood	BD Biosciences, Heidelberg, Germany
Whatman filter paper	Whatman, Maidstone, UK
X-Ray films	Amersham Biosciences, Buckinghamshire, UK

2.9. Special laboratory equipment

Fluorescence Microplate Reader	BMG Labtech, Offenburg, Germany
Leica TCS SP confocal microscope equipped with a 100× oil objective	Leica Microsystems, Wetzlar, Germany
Microtome	Techno-Med, GmbH, Bielefelt, Germany

Paraffin-Embedding-System	Techno-Med, GmbH, Bielefelt, Germany
Portable Datalogging Spectrophotometer	Bachofer, Reutlingen, Germany
Sonorex bath sonicator	Bandelin electronic, Berlin, Germany
SpeedVac (Vacuum Concentrator)	Bachofer, Reutlingen, Germany
TriCarb Liquid scintillation	Perkin Elmer, USA

2.10. Buffer and Solutions

Anesthesia cocktail	10% Ketamin 2 ml
	2% Xylazin 0.5 ml
	ddH_2O 10 ml
ASM lysis buffer	0.1% Triton X-100
	50 mM sodium acetate pH 5.0
Complete DMEM (Gibco) medium	500 ml DMEM (Gibco)
	10% FCS
	10 mM HEPES, pH 7.4
	2 mM L-Glutamine
	1 mM Sodium pyruvate
	100 µM non-essential amino acids
	100 units/ml Penicillin
	100 µg/ml Streptomycin
DAG-assay Buffered Saline Solution	135 mM NaCl
	1.5 mM $CaCl_2$
	0.5 mM $MgCl_2$
	5.6 mM Glucose
	10 mM HEPES, pH 7.2
DAG-assay detergent solution	7.5% N-octylglucopyranoside
	5 mM cardiolipin
	1 mM DETAPAC
DAG-kinase diluent	1 mM DETAPAC, pH 6.6

	0.01 M imidazole/HCl
DAG-kinase reaction buffer	100 mM imidazole/HCl pH 6.6
	100 mM NaCl
	25 mM $MgCl_2$
	2 mM EDTA
	2.8 mM DTT
	5 µM ATP
	10 µCi [^{32}P] gamma-ATP
Freezing medium	1 ml DMSO
	2 ml FCS
	7 ml complete DMEM
HEPES buffer	132 mM NaCl
	20 mM Hepes pH 7.4
	5 mM KCl
	1 mM $CaCl_2$
	0.7 mM $MgCl_2$
	0.8 mM $MgSO_4$
HEPES/Saline	132 mM NaCl
	20 mM HEPES, pH 7.4
	5 mM KCl
	1 mM $CaCl_2$
	0.7 mM $MgCl_2$
	0.8 mM $MgSO_4$
Mowiol	6 g Glycerol
	2.4 g Mowiol
	6 ml ddH_2O
	12 ml 0.2 M Tris-Base, pH 8.5
	0.1% DABCO
PFA 2%	2.5 ml PFA stock solution

	7.5 ml PBS
PFA stock solution	8 g PFA
	100 ml PBS
SA medium	500 ml DMEM
	1 mM HEPES, pH 7.4
Trypsin	0.25% Trypsin
	5 mM Glucose
	1.3 mM EDTA

3. METHODS

3.1. Tissue culture techniques

3.1.1. Culture and passage of established cell lines

EOMA cells were maintained in complete DMEM medium (see Materials) at 37°C in a 10% CO_2 atmosphere. Because EOMA cells grow adherent, passage of cells was achieved by incubation with trypsin solution to dislodge the cells from the flask wall. Prior to that, the cultures were examined using a light microscope, to assess the degree of confluence. Medium, PBS and trypsin were pre-warmed at 37°C. The cell monolayer was washed with PBS for two times and trypsin was added. Detachment of cells was assessed by light microscope. The digestion was stopped by addition of medium and the cells were centrifuged at 1500 rpm for 5 minutes to pellet the cells. Cells were then re-suspended in medium, transferred to fresh flasks and kept incubated at 37°C.

3.1.2. Freezing and thawing of cells

The basic principle of successful cryo-preservation is a slow freeze and a quick thaw of cells. For the freezing step, DMSO was used to protect cells from ice crystal formation, which causes cell rupture. The freezing medium (see Materials) was prepared in advance and kept at 4°C. Cells were collected, counted with a Neubauer chamber and re-suspended at a concentration of $1x10^6$ cells/ml in freezing medium. The cell suspension was transferred in cryo-protective vials, which were placed at -80°C in a Cryo 1C Freezing Container overnight. For long-term storage, cells were placed in a liquid nitrogen storage vessel. To thaw the cells, the vials from liquid nitrogen storage were transferred to a water bath at 37°C. The vials were moved back and forth to ensure a quick thaw. After thawing, cells were washed with medium to remove the DMSO, re-suspended in fresh medium, transferred to culture flasks and incubated at 37°C.

3.2. Infection experiments

3.2.1. Preparation of *Staphylococus aureus* (*S. aureus*)

S. aureus was stored at -80°C and plated with plastic swaps on Trypticase Soy Agar plates with 5% sheep blood (Becton Dickinson #254053). The plates were incubated for 16 hrs at 37°C. Bacteria were then transferred into 40 ml 37°C pre-warmed Trypticase Soy Broth (TSB, Becton Dickinson #221093) in Erlenmeyer flasks at an optical density at 550 nm of 0.2/ml (equals to 1.85 x 10^8 colony forming units (CFU)/ml). The bacteria were incubated for 70 min at 37°C with shaking at 125 rpm and collected during the early logarithmic growth phase by centrifuging at 3000 rpm for 10 min. The bacterial pellet was washed twice in pre-warmed DMEM supplemented with 10 mM HEPES or phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCL, 7 mM CaCl2, 0.8 mM MgSO4, 1.4 mM KH2PO4, and 6.5 mM Na2HPO4) and then resuspended in DMEM supplemented with 10 mM HEPES for infection of EOMA cells or at 5×10^6 CFU per 100 µL in PBS for infection of mice. Cells or mice were then infected within the next 10 min.

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3.2.2. Infection cells with *S. aureus*

To evaluate the role of *S. aureus* on EOMA cells, EOMA cells were plated in 24 well plates with cover slip at 5x10^4 per well with 1 ml complete DMEM or 6 well plate at 8x10^5 per well with 4 ml complete DMEM, grown for 2 days. Prior to infection, the cells were washed with pre-warmed PBS for 2 times and maintained in buffered DMEM medium supplemented with 10 mM HEPES during infection and were inoculated with *S. aureus* at bacteria-to-host cell ratio of 200:1 or 10:1 (Multiplicity of infection (MOI) 200:1 or 10:1). Synchronous infection conditions and an enhanced bacterium-host cell interaction were achieved by a 2-min centrifugation (1000 rpm) of the bacteria onto the cells. The end of the centrifugation was defined as the starting point of infection. If required for the experiment, we added the anti-oxidant Tiron or N-acetylcysteine (NAC) or the Asm inhibitor amitriptyline 20 minutes prior to infection as follows.

3.2.3. Infection mice with *S. aureus*

The littermates of 8 weeks old Asm wild-type or Asm-deficient mice were infected intravenously with $5x10^6$ CFU *S. aureus*. Between the first and the last mouse infected, we allowed a time interval of maximally 10 min, to exclude a change in viable counts of the bacteria.

For pretreatment with inhibitors before infection, wt mice were injected intraperitoneally with 10 mg/kg amitriptyline, 100 mg/kg Tiron or 100 mg/kg NAC twice daily for 2.5 days. The last dose was given 1 h before infection. For treatment with amitriptyline post infection, wt mice were injected i.p. 1 h or 2 hrs after infection with 16 mg/kg amitriptyline. Antibiotics were also injected i.p. 1 h after infection with 100 mg/kg methicillin (Sigma) or 100 mg/kg vancomycin (Sigma). The injection of methicillin or vancomycin was repeated 9 hrs after infection. For mortality experiment, 10 mg/kg amitriptyline, 100 mg/kg methicillin or 100 mg/kg vancomycin were treated twice daily in indicated group until 11 days.

The mice were sacrificed at indicated infection times by cervical dislocation. The lungs were removed and used for CFU counting and histology as described below.

3.3. Determination colony-forming units (CFUs) of *S. aureus* in the liver and spleen

To quantify *S. aureus* colony forming units (CFUs) in mouse livers and spleens, the organs were removed after 12 hrs infection and homogenised in a loose Dounce homogenizor. The homogenates were lysed for 10 min in 5 mg/ml saponin (SERVA) at 37 °C to release intracellular bacteria. The samples were centrifuged at 3200 rpm for 10 min, resuspended in PBS, and plated on normal LB plates in duplicates. Bacterial CFUs were counted after the plates had been incubated overnight at 37 °C.

3.4. Immunocytochemistry

EOMA cells were grown on coverslips, infected or uninfected for indicated time and with designed treatment as above. They were fixed in 2% PFA/PBS for 10 min. For intracellular staining, cells were permeabilized with 0.1% Triton X-100/PBS for 5 min at room temperature. Cells were washed again with PBS and incubated for 60 min in PBS supplemented with 5% FCS for all antibodies to block nonspecific binding sites. Cells were washed and incubated for 45 min with either anti-ZO1 IgG, anti-ZO2 IgG, anti-E-cadherin IgG, or an anti-Occludin IgG(see Materials). Cells were then washed three times in PBS with 0.05% Tween-20 and incubated for an additional 45 min with Cy3-labeled donkey anti-rabbit antibodies. Cells were then washed again in PBS with 0.05% Tween-20. After a final PBS wash, cells were mounted on glass coverslips with moviol. Control experiments were performed with irrelevant rabbit antibodies and secondary antibodies. Control antibodies did not significantly bind to the cells. Cells were examined on a Leica TCS SP confocal microscope equipped with a 100x oil objective and images were analyzed using Leica Confocal software (Leica).

3.5. Histology

3.5.1. Preparation of the lung sample

Mice were sacrificed and the lungs were subsequently removed. The left lungs were fixed in 4% PFA for 38 hours, serially dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm.

3.5.2. Hematoxylin and eosin staining

Lung tissue (6-mm paraffin embedded sections) were dewaxed, rehydrated and stained for 2 min with hematoxylin and washed with water for 5 min prior to being stained with eosin for 1 min. After a final short wash with water the sections were mounted in Mowiol and evaluated using a Leica TCS-SP2 microscope.

3.5.3. Fluorescence staining for the lungs

The sections were then dewaxed, rehydrated and incubated in pepsin (see Materials) for 30 min at 37°C incubator. The sections were washed and incubated for 10 min in PBS supplemented with 5% FCS to block nonspecific binding sites. The sections were washed again and incubated overnight at 4 °C with either anti-ZO1 IgG, anti-ZO2 IgG, , anti-E-cadherin IgG, anti-Occludin IgG, or anti-Ly-6G and Ly-6C (GR1) or FITC-labeled anti-Lectin antibodies, respectively. The sections were washed in PBS with 0.05% Tween-20 and incubated for an additional 45 min with Cy3-labeled donkey anti-rabbit or anti-rat antibodies. The sections were then washed again in PBS with 0.05% Tween-20, once in PBS wash, and mounted in moviol. Control experiments were performed with irrelevant rabbit or goat antibodies and secondary antibodies. Control antibodies did not significantly bind to the lungs. The sections were examined on a Leica TCS SP confocal microscope equipped with a 40x oil objective and images were analyzed using Leica Confocal software (Leica).

3.6. Electron Spin Resonance Detection of Endothelial $O_2^{\cdot -}$

ROS production was measured by electron spin resonance (ESR), as we described previously (Abais et al 2014, Li et al 2013b). 2×10^5 endothelial Cells were infected with *S.aureu*s for the indicated time, the medium removed, the cells scraped into 20 mM HEPES (PH 7.5), 1 mM EDTA, and 255 mM sucrose and shock frozen in liquid nitrogen. Proteins were isolated and resuspended with modified Krebs-HEPES buffer containing deferoximine (100 μM, Sigma) and diethyldithiocarbamate (5 μM, Sigma). A spin trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen, Elzach, Germany) (1 mM final concentration), was then added to the mixture in the presence or absence of manganese-dependent superoxide dismutase (SOD, 200 U/mL; Sigma, St. Louis, MO). The mixture was loaded into glass capillaries and immediately kinetically analyzed for $O_2^{\cdot -}$ production for 10 min. The SOD-inhibited fraction of the signal was used to calibrate the system. The ESR settings were as follows: biofield, 3,350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; points of resolution, 4,096; receiver gain, 100; and kinetic time, 10 min. The ESR signal strength was

recorded in arbitrary units and the final results were expressed as the fold changes compared to the control as described (Xu et al 2013).

3.7. Asm activity assay

The activity of Asm was measured as the consumption of radioactive [^{14}C]-sphingomyelin to ceramide and [^{14}C]-phophorylcholine. To this end, $8x10^5$ EOMA cells were infected with *S. aureus* for indicated times, washed, lysed in 300 µl/sample ice-cold ASM-lysis buffer (see Materials). The cells were removed from the plate, transferred into eppendorf tubes and immediately sonicated three times (3x10 s). Since [14C]-sphingomyelin is insoluble in water, it was first dried by SpeedVac centrifugation and solubilized into micelles in ASM-lysis buffer, using a bath sonicator for 10 min. Cell lysates were incubated with 0.05 µCi per sample [^{14}C]-labeled sphingomyelin (2 GBq/mmol) for 30 min at 37°C on a thermomixer. Lipids were extracted by addition of 1 ml/sample of $CHCl_3$:CH_3OH (2:1, v/v), followed by vigorous vortexing for 30 sec and centrifugation at 14000 rpm for 5 min. An aliquot (300 µl) of the aqueous phase was applied for liquid scintillation counting. Hydrolysis of [^{14}C]-sphingomyelin by Asm results in release of [^{14}C]-choline chloride into the aqueous phase, whereas ceramide and unreacted [^{14}C]-sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]-choline chloride (pmol/10^5 cells/h) serves to determine the activity of the Asm.

3.8. Ceramide measurement by DAG kinase assay

3.8.1. Lipid extraction and enzymatic reaction

Cellular ceramide was measured by DAG kinase assay, in which ceramide is converted to a quantifiable product (ceramide-1-phosphate) by transfer of [^{32}P]-phosphate from [^{32}P]-gamma ATP to ceramide. To this end, cells were infected as above, first extracted in $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v). The resulting biphasic mixture is composed of a lower lipid-containing organic phase, and an upper aqueous phase. An aliquot of the lower organic phase was collected and dried by evaporation of the chloroform in a SpeedVac. The dried lipids were solubilized in 20 µl of DAG-assay detergent solution (see Materials) and

sonicated for 10 min in a bath sonicator, and 50 µl of DAG-kinase reaction buffer (see Materials), and 10 µl of diluted enzyme (dilution 1:1, v/v in DAG-kinase diluent) (see Materials) were added. The kinase reaction was performed for 30 min at room temperature on a thermomixer. The samples were re-extracted in 1 ml/sample $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v), 170 µl/sample DAG-assay Buffered Saline Solution (see Materials) and 30 µl of a 100 mM EDTA solution, followed by vortexing. The resulting upper phase was removed, and the lower organic phase was again concentrated by SpeedVac centrifugation. The dried lipids were dissolved in 20 µl/sample $CHCl_3$:CH_3OH (1:1, v/v).

3.8.2. Separation of lipids by Thin Layer Chromatography (TLC)

Lipids were separated on a Silica G60 TLC plate. A solvent system of $CHCl_3$:CH_3COCH_3:CH_3OH:CH_3COOH:H_2O (10:4:3:2:1, v/v/v v/v) was added to the TLC chamber, and was allowed to saturate the atmosphere for 1 h by using a sheet of Whatman filter paper. The silica plates were loaded with the solubilized lipids, placed into the TLC chamber and the solvent front was allowed to migrate to the top of the plate. The plate was then removed, air dried for 45 min and exposed to X-ray films for 24 hours. Ceramide-spots were identified by comigration with a C_{16}-ceramide standard, and incorporation of ^{32}P into ceramide was quantified by liquid scintillation counting. Comparison with a standard curve using C_{16}-ceramide permitted the determination of ceramide amounts.

3.9. Evans blue microvascular permeability analysis of lung edema

To assess vascular leakage, 4% Evans blue dye (20 mg/kg) was injected into the external jugular vein 30 min before the termination of the experiment as described (Moitra et al 2007). Evans blue dye has a very high binding affinity for serum albumin. When the vascular barrier in the lung is compromised, albumin-bound Evans blue moves into the lung parenchyma. The lungs were perfused free of blood with phosphate-buffered saline via right heart, removed, dried, weighed, and was homogenized in PBS (1 ml/100 µg tissue), incubated with 2 volumes of formamide to extract the dye (18 h, 60°C), and centrifuged at 5,000 × g for 30 minutes, and the optical density of the supernatant was determined at 620 nm and 740 nm with a

fluorescence microplate reader (BMG Labtech, Offenburg, Germany). The extravasated EB concentration in lung homogenate was calculated against a standard curve and was expressed as micrograms of Evans blue dye per gram of lung.

3.10. DNA techniques

3.10.1. DNA isolation

3.10.1.1 DNA isolation from mouse tails

For genotyping of wt and Asm-deficient mice, app. 1-2 mm of mouse tail was cut and placed into 80 µl Tissue Lysis Buffer (TLB) (see Materials). The samples were incubated at 56°C overnight and diluted with 800 µl autoclaved ddH_2O.

3.10.1.2 DNA isolation from cell lines

To test cultured cell lines for the presence of Mycoplasma, 5 $x10^5$ cells/sample were pelleted and re-suspended into 50 µl TLB. The samples were incubated at 56°C for 3 h and boiled at 95°C for 10 min. The volumes were then raised to 100 µl with autoclaved ddH_2O.

3.10.2. Polymerase Chain Reaction (PCR)

3.10.2.1 Asm PCR

For the detection of Asm by PCR, 1 µl of overnight tail digest (see **3.10.1.1.**) was added to 1.2 µl 10 x PCR Buffer, 2.5 mM $MgCl_2$, 1µl dNTP mix 5 units/ml Taq Polymerase and 0.1 µl each of primers Asm-PA1-2, Asm-PA2-2 an Asm-PS-2 in 0.2 ml PCR tubes. The temperature of the lid of the PCR machine was raised to 104°C and the temperature of the PCR block was raised to 96°C for 17 min, after which the following cycle was carried out 35 times:

Denaturation: 95°C for 1 min

Annealing: 58°C for 1 min

Elongation: 72°C for 1 min 45 sec

After the last cycle, the PCR block remained at 72°C for 5 min, after which the samples were placed at 4°C.

3.10.2.2 Mycoplasma PCR

For the identification of mycoplasma by PCR, 1 µl of cell digest (see 3.10.1.2.) was added to 2.5 µl 10 x PCR Buffer, 4.1 mM $MgCl_2$, 0.5 µl dNTP mix 1.25 units/ml Taq Polymerase and 0.25 each of primers P1 and P4 in 0.2 ml PCR tubes. The temperature of the lid of the PCR machine was raised to 104°C and the temperature of the PCR block was raised to 96°C for 17 min, after which the following cycle was carried out 25 times:

Denaturation: 95°C for 1 min

Annealing: 60°C for 1 min

Elongation: 72°C for 1 min 30 sec

After the last cycle, the PCR block remained at 72°C for 7 min, after which the samples were placed at 4°C.

3.10.3. Agarose gel electrophoresis

PCR products were analysed on 1% agarose gels. Agarose was poured in TBE buffer (see Materials) that contained 0.01 µg/ml ethidium bromide. The samples (15 µl) were loaded on the gel along with 0.1 µg/µl of a 100-bp-standard. The gel was run under 5 V/cm current. Visualization of the DNA fragments was performed under UV-light.

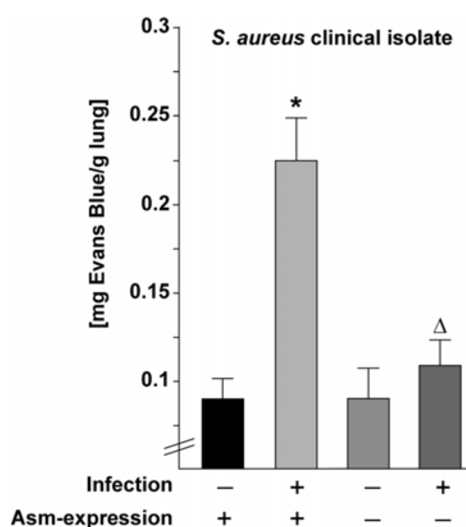
4. RESULTS

4.1. Asm deficiency prevent *S. aureus*-induced lung edema

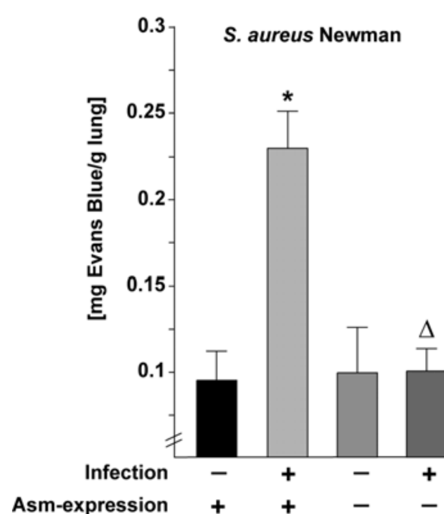
4.1.1. Asm deficiency mitigates pulmonary edema upon *S. aureus* infection

To investigate whether activation of Asm is required in the *in vivo* development of lung edema upon *S. aureus* infection, we systemically infected C57BL/6 wild type (wt) and Asm-deficient mice with *S. aureus* for various time periods. To date, a large amount of studies indicated that degradation of tight junctional proteins leads to microvascular leakage and finally pulmonary edema (Corada et al 1999, Jang et al 2011). To determine pulmonary edema, we injected Evans blue dye into the mice 30 min before sacrificing the mice to analyze lung edema. The studies revealed massive leakage of Evans blue dye into the lungs of wt mice but almost no leakage into the lungs of Asm-deficient mice upon systemic infection with a clinical *S. aureus* strain (**Figure 4.1.1.A-1.**) or the *S. aureus* Newman strain (**Figure 4.1.1.A-2.**). Moreover, hematoxylin and eosin (H&A) staining of the lungs demonstrated that the clinical *S. aureus* infection induced massive lung edema in a time-dependent manner in wt mice, a finding that was absent or much less pronounced in Asm-deficient mice (**Figure 4.1.1.B.**).

A-1



A-2



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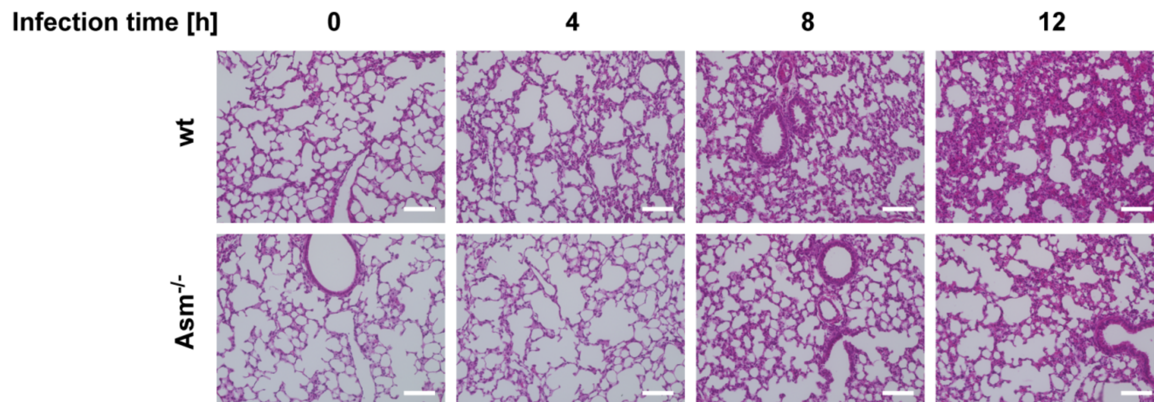


Figure 4.1.1. Effect of Asm deficiency on pulmonary edema upon *S. aureus* infection

(A) Wild-type (wt) and Asm-deficient mice were infected with a clinical *S. aureus* strain (A-1) or the *S. aureus* Newman strain (A-2) for the indicated time periods. Evans blue dye was injected 30 min before sacrificing the mice, flushing the lung via the right heart to remove intravascular Evans Blue and removal of the lungs. The amount of dye leaking into the lung tissue was quantified. Shown is the number (mean ± SD) of the concentration of Evans blue dye in the lungs from each 5 wildtype and Asm-deficient mice. *, significant differences between uninfected mice and infected mice; Δ, significant differences between infected wild-type mice and Asm-deficient mice (all $P<0.05$; t-test).

(B) Wild-type and Asm-deficient mice were infected with *S. aureus* for the indicated time periods. They were sacrificed and lung sections were stained with H&E and analyzed by light microscopy for the detection of lung edema. Scale bar is 100 µM (magnification, 63×). Representative images from three independent experiments are shown.

4.1.2. Asm deficiency prevents neutrophil trafficking to the lung

Neutrophils have a pivotal role in the defense against bacterial infections. However, overwhelming activation of neutrophils is known to elicit tissue damage and contribute to severe sepsis (Adams et al 2001, Guo et al 2002, Windsor et al 1993).

This led us to examine whether Asm expression is also required for pulmonary neutrophil trafficking during systemic *S. aureus* infection. To this end, we stained lung sections from infected wt and Asm-deficient mice with anti-GR1 antibodies, a neutrophil marker. Confocal fluorescence microscopy studies reveal that *S. aureus* infection induced excessive neutrophil trafficking to the lung in a time-dependent manner in wild type mice (**Figure 4.1.2.A.**). However, neutrophil trafficking into the lung tissue was reduced markedly in Asm-deficient mice after infection (**Figure 4.1.2.A.**). Quantitative analysis of neutrophils demonstrates that

Asm is an important regulator for *S. aureus*–induced neutrophil trafficking to the lung during *S. aureus* sepsis (**Figure 4.1.2.B.**).

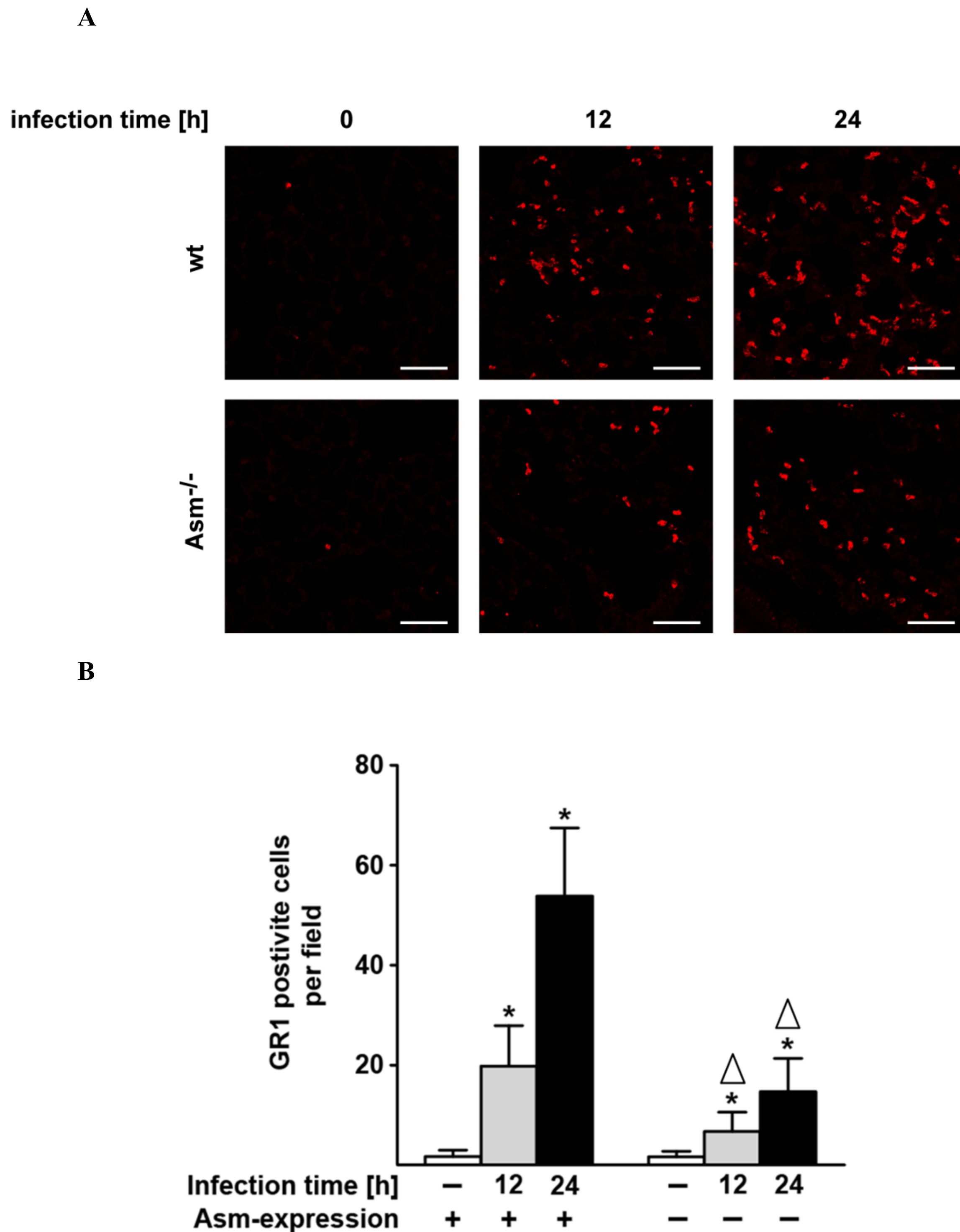


Figure 4.1.2. Effect of Asm deficiency on neutrophil trafficking induced by *S. aureus* infection

(A and B) For determination of neutrophil trafficking, wt and Asm-deficient mice were left uninfected or were infected with a clinical *S. aureus* strain for different time points. Lung sections were stained with Cy3-labeled anti-GR1 antibodies and analyzed by fluorescence microscopy. Scale bar is 50 µM. Shown are representative images from three independent experiments. Cells staining positive for GR1,

a neutrophil marker, were quantified by analysis of 50 fields per group. Shown is the number (mean ± SD) of GR1-positive cells per field of 63x magnification. *, significant differences between uninfected mice and infected mice; Δ, significant differences between infected wild-type mice and Asm-deficient mice (all $P<0.05$; t-test).

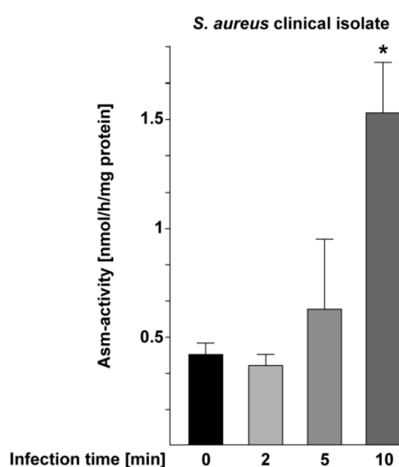
Taken together, these findings indicate that Asm plays a key role in the development of pulmonary edema induced by systemic infection with *S. aureus*. Asm deficiency prevents the development of lung injury during infection.

4.2. Infection of endothelial cells with S. aureus activates Asm and leads to the production of ROS in a positive feedback loop

4.2.1. *S. aureus* infection rapidly activates the Asm

To test whether systemic *S. aureus* infections activates Asm, we infected murine endothelial (EOMA) cells with a clinical *S. aureus* strain (MOI 200:1) and measured Asm activity. *S. aureus* infection induced a marked activation of the Asm (**Figure 4.2.1.A.**). Additionally, the well-characterized *S. aureus* sepsis strain Newman was tested to determine whether systemic *S. aureus* infections induced Asm activation is a general phenomenon. Following infection with *S. aureus* sepsis strain Newman (MOI 200:1), the kinetics of Asm activation showed a similar pattern in EOMA cells than after infection with the clinical *S. aureus* strain (**Figure 4.2.1.B.**).

A



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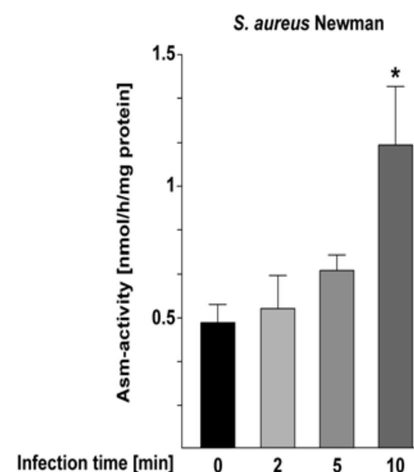


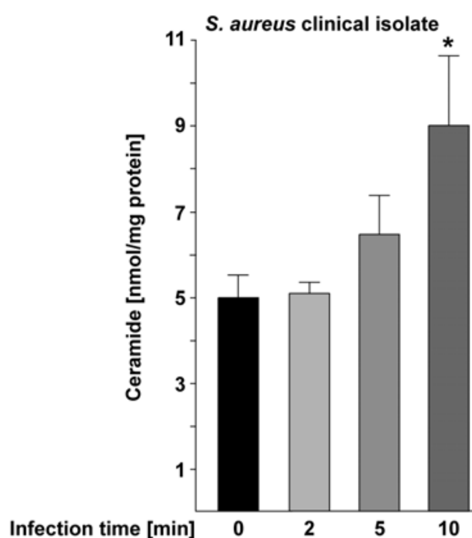
Figure 4.2.1. ***S. aureus*** **infection activates Asm**

Asm activity were measured in EOMA cells after infection with a clinical *S. aureus* strain (MOI 200:1) (A) or the *S. aureus* Newman strain (MOI 200:1) (B) for 0, 2, 5, or 10 min. Results show the mean ± SD of three independent experiments. *, significant differences compared to uninfected control mice (P<0.05, t-test).

4.2.2. *S. aureus* infection also induces a marked formation of the ceramide

As we know Asm is one of the most important sphingomyelinase which hydrolyzes sphingomyelin to ceramide and phosphorylcholine. To further define whether systemic *S. aureus* infections induces the formation of ceramide, which is the product of Asm activity, we infected EOMA cells with a clinical *S. aureus* strain or the *S. aureus* Newman stain (MOI 200:1) and measured ceramide production. The results showed a rapid release of ceramide upon these two *S. aureus* strains infection (**Figure 4.2.2.A. and B.**).

A



B

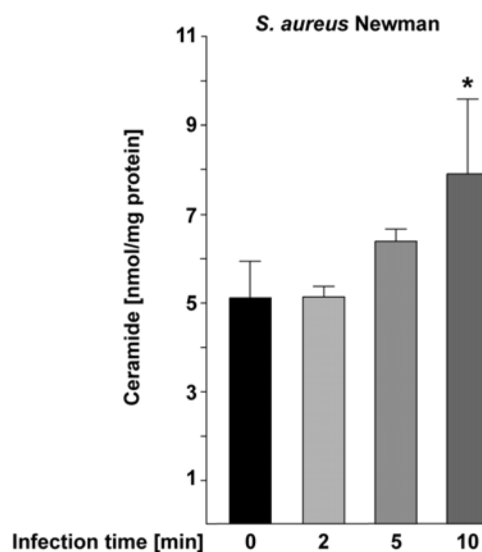


Figure 4.2.2. ***S. aureus*** **infection induced ceramide release**

Ceramide concentrations were measured in EOMA cells after infection with a clinical *S. aureus* strain (MOI 200:1) (A) or the *S. aureus* Newman strain (MOI 200:1) (B) for 0, 2, 5, or 10 min. Results show the mean ± SD of three independent experiments. *, significant differences compared to uninfected control mice (P<0.05, t-test).

4.2.3. *S. aureus* infection induces a rapid production of the ROS

Amitriptyline is a tricyclic antidepressant (TCA). It is the most widely used TCA and is efficacious for the treatment of depression (Barbui et al 2001, Garattini et al 1998). Several publications indicated that amitriptyline works as a functional Asm inhibitor and for instance reduces the pulmonary accumulation of ceramide in cystic fibrosis (Becker et al 2010b, Grassmé et al 1997, Kornhuber et al 2008, Kornhuber et al 2010, Kornhuber et al 2011, Teichgraber et al 2008).

ROS have been shown for a long time to play a critical role in host-pathogen interactions (Djaldetti et al 2002, Karupiah et al 2000, Moore et al 2012, Pai et al 2012, Wyllie et al 2011). Previous studies showed a critical role of the Asm in ROS production in macrophages and hepatocytes (Hatanaka et al 1998, Pai et al 2012, Reinehr et al 2005, Zhang et al 2007). To investigate whether *S. aureus* infection induces ROS production and whether *S. aureus*-induced ROS production depends on Asm, we infected EOMA cells with two different strains (MOI 200:1) and analyzed the production of oxygen radicals. The results showed that *S. aureus* infection induced a rapid production of ROS in EOMA cells (**Figure 4.2.3.A. and B.)**, which was prevented by pre-incubation with the functional Asm inhibitor amitriptyline (Ami) (**Figure 4.2.3.A. and B.)** indicating that the production of ROS after infection with *S. aureus* requires the activation of Asm.

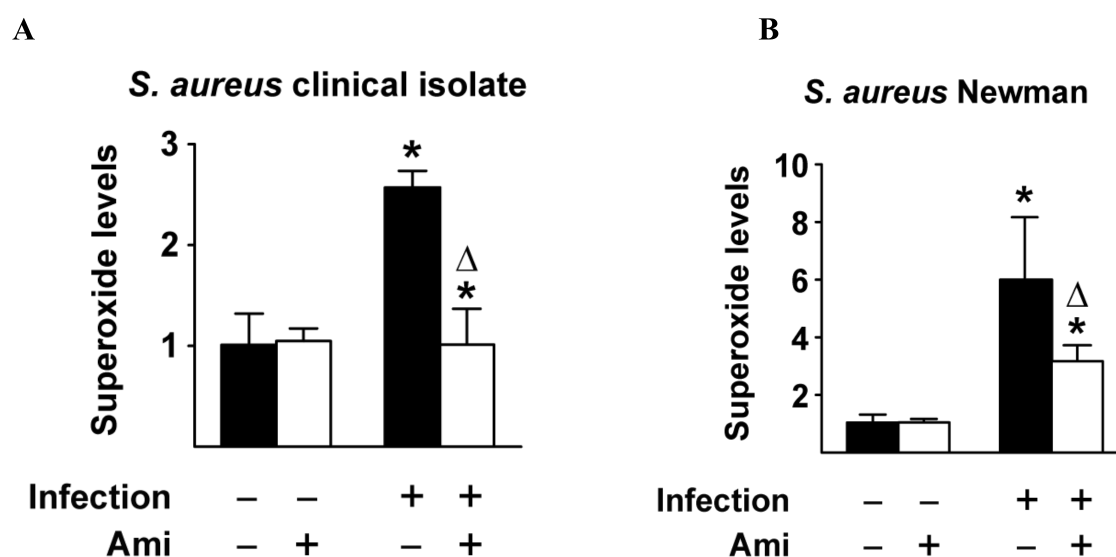


Figure 4.2.3. ***S. aureus*-induced ROS, a process that depends on the Asm**

EOMA cells were preincubated with amitriptyline (20 µM) for 20 min and then infected with a clinical *S. aureus* strain (MOI 200:1) (A) or the *S. aureus* Newman strain (MOI 200:1) (B) for 7.5 min. The production of ROS was quantified by electron spin resonance. Relative O_2^- levels were used to indicate ROS accumulation. Data for panels A and B are mean ± SD of four independent experiments. Significant differences between infected and non-infected controls were determined by t-test and are indicated by asterisk (* P<0.05). Significant differences between untreated samples and amitriptyline-treated samples were determined by t-test and are indicated by delta ($^{\Delta}$ P<0.05).

4.2.4. Infection of endothelial cells with *S. aureus* activates Asm and leads to the production of ROS in a positive feedback loop

To further define the interaction of *S. aureus*-induced Asm activation and ROS production, we pre-incubated of EOMA cells with the functional Asm inhibitor amitriptyline (Ami) or with antioxidant Tiron or NAC for 20 min, and infected EOMA cells with a clinical *S. aureus* strain at a lower multiplicity of infection (MOI= 10:1) for another 20 min and determined Asm activity and ROS production. The results revealed a similar but slightly delayed time course of Asm activation (**Figure 4.2.4.A.)** and ROS release (**Figure 4.2.4.B.)**. Pre-incubation of EOMA cells with the antioxidants Tiron or N-acetylcysteine (NAC) reduced Asm activation by *S. aureus* (**Figure 4.2.4.A.).** Pre-incubation of EOMA cells with the functional Asm inhibitor amitriptyline (Ami) or with Tiron or NAC also inhibited ROS release (**Figure 4.2.4.B.)** suggesting a positive feedback loop of *S. aureus*–induced Asm activation and ROS release.

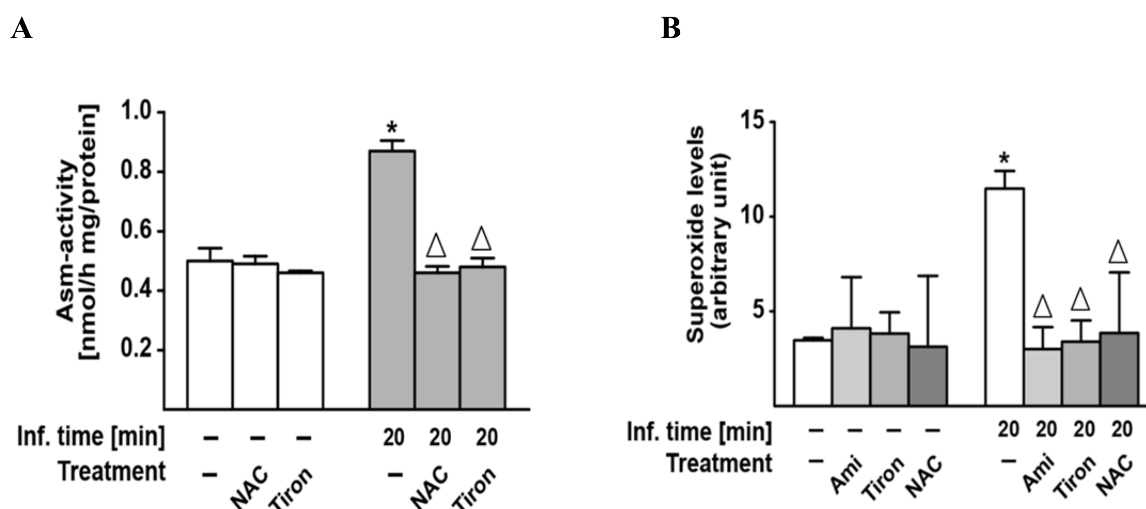


Figure 4.2.4. ***S. aureus*-induced Asm activation and ROS production form a positive feedback loop**

EOMA cells were pre-incubated with Ami or NAC or Tiron (20 µM) for 20 min and then infected with a clinical *S. aureus* strain (MOI 10:1) for 20 min. Ceramide concentrations (A) and ROS production (B) were measured. Data for panels A and B are mean ± SD of four independent experiments. Significant differences between infected and non-infected controls were determined by t-test and are indicated by asterisk (* $P<0.05$). Significant differences between untreated samples and amitriptyline-treated samples were determined by t-test and are indicated by delta ($^{\Delta}$ $P<0.05$).

4.3. *S. aureus* induces degradation of junctional proteins via the Asm/ceramide system

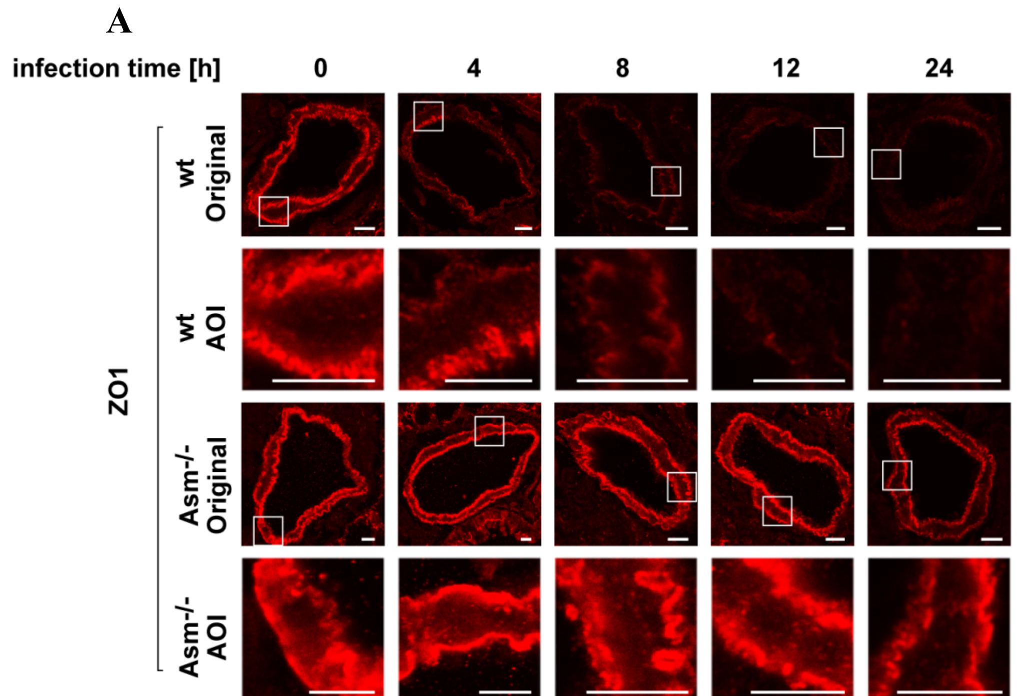
4.3.1. Asm deficiency prevents degradation of junctional proteins upon *S. aureus* infection *in vivo*

During a severe *S. aureus* infection, the bacteria and their toxins may spread in the bloodstream and affect the integrity of endothelial cells, thereby resulting in increased vascular permeability (Hocke et al 2006, Seeger et al 1990). It is well documented that endothelial activation plays a major role in the cellular immune response to sepsis (Aird 2003, Ait-Oufella et al 2010, Boos et al 2006).

To gain insight into the mechanism by which Asm and ceramide mediate endothelial dysfunction and lung edema after *S. aureus* infection, we determined whether systemic infection with *S. aureus* induces the breakdown of junctional proteins in pulmonary endothelial cells *in vivo*, and, if so, whether this process depends on the Asm/ceramide system. To this end, we systemically infected wt and Asm-deficient mice with $5x10^6$ a clinical *S. aureus* strain. We then obtained lung sections and stained them with Cy3-labeled antibodies against ZO1, ZO2, Occludin, or E-cadherin. Confocal microscopy showed that infection with *S. aureus* induces dramatic degradation of ZO1, ZO2, Occludin, and E-cadherin junctional proteins in a time-dependent manner in endothelial cells from blood vessels in wt lungs but not in endothelial cells from the lungs of Asm-deficient mice (**Figure 4.3.1.A-D.**). Thus, the disruption of junctional proteins, which is caused by *S. aureus* infection *in vivo*, requires functional Asm.

Co-stainings of lung sections with Cy3-coupled antibodies against junctional proteins and FITC isolectin B4, which is a marker for endothelial cells, confirmed that junctional proteins are only degraded in lung endothelial cells of wt mice upon infection with S. aureus, but not in Asm-deficient endothelial cells (**Figure 4.3.1.E-H.**).

A



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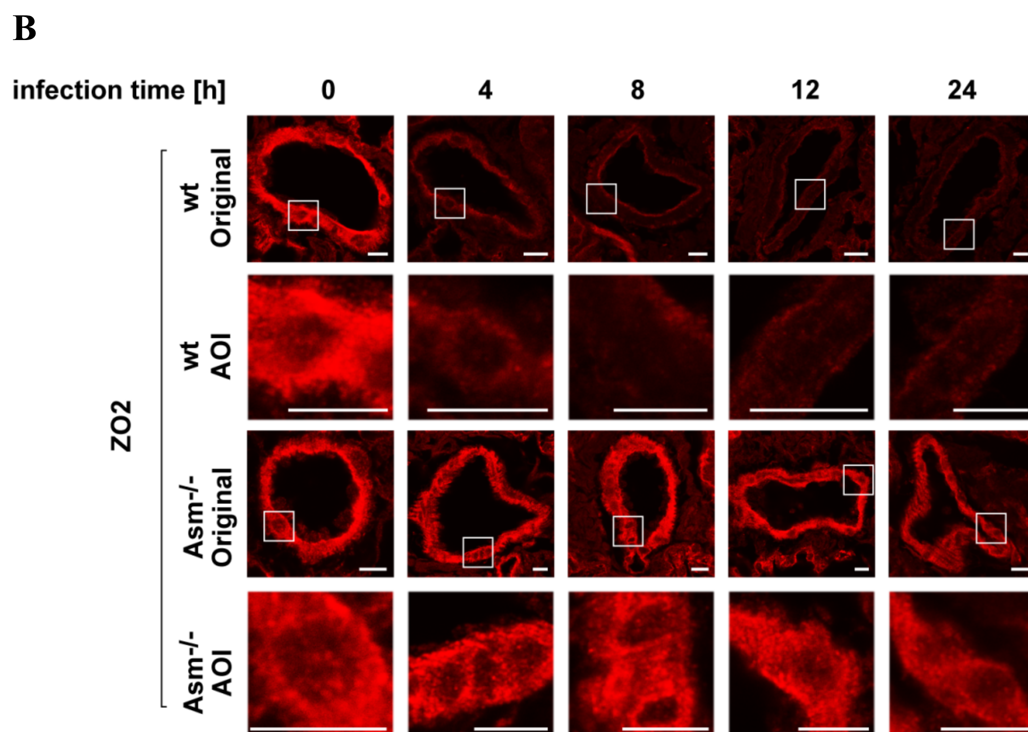
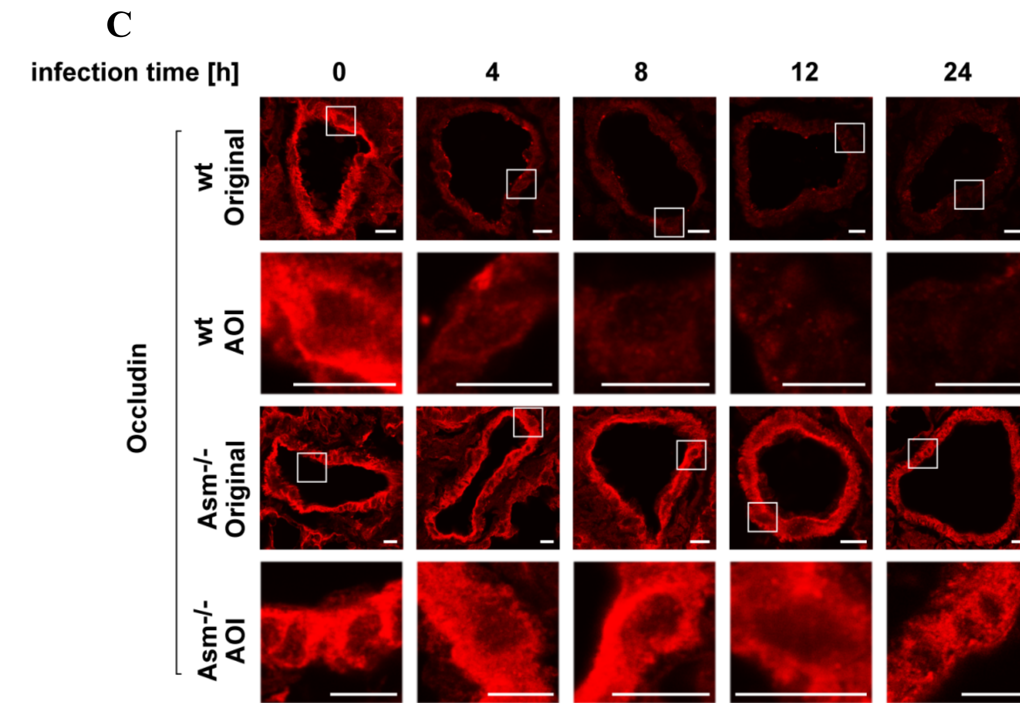
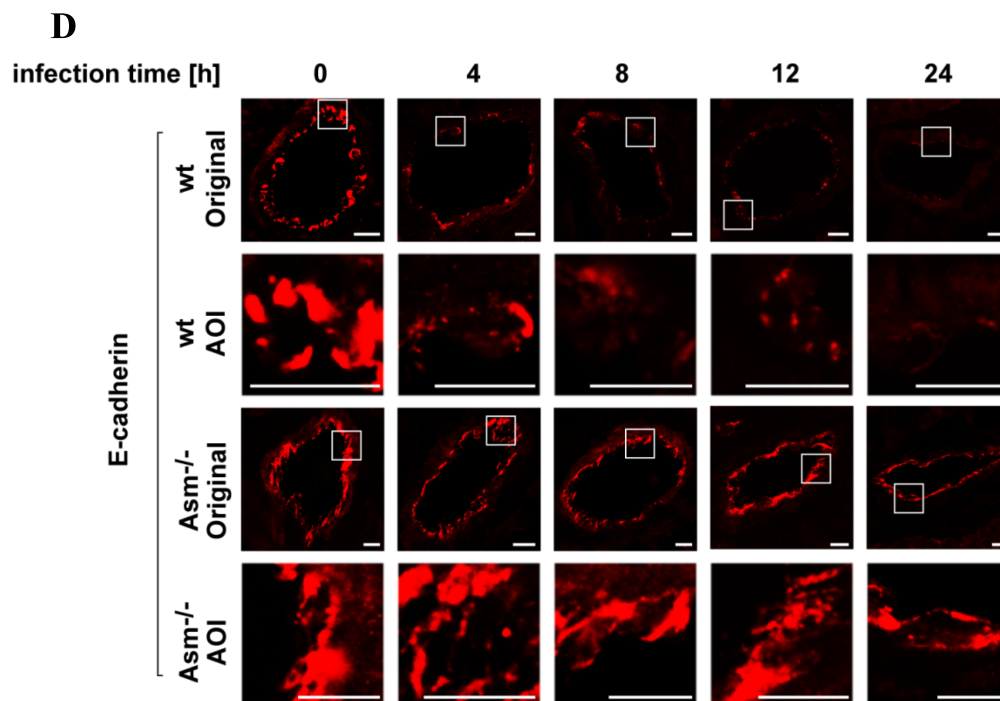
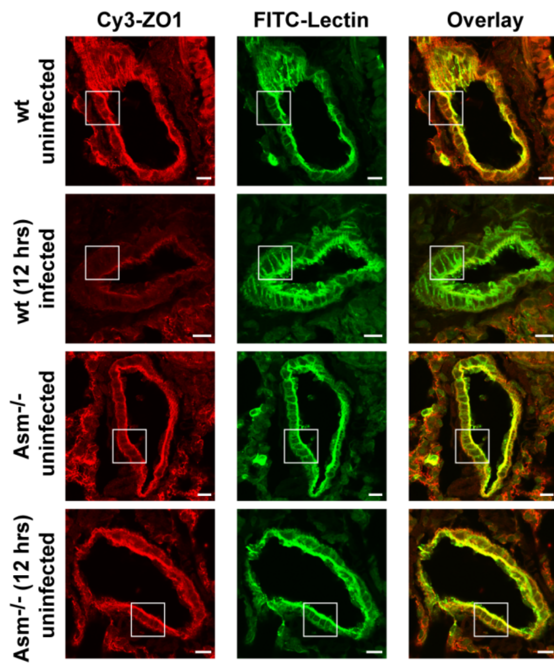


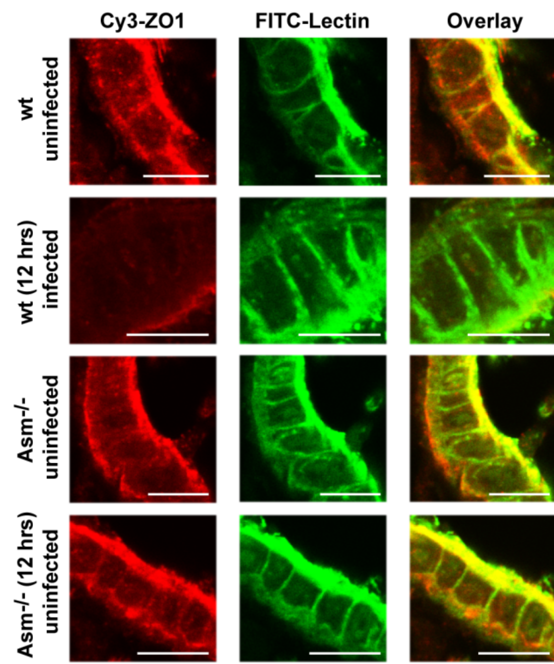

C
infection time [h]
0
4
8
12
24
Occludin
wt Original
wt AOI
Asm-/- Original
Asm-/- AOI


D
infection time [h]
0
4
8
12
24
E-cadherin
wt Original
wt AOI
Asm-/- Original
Asm-/- AOI

E-1



E-2



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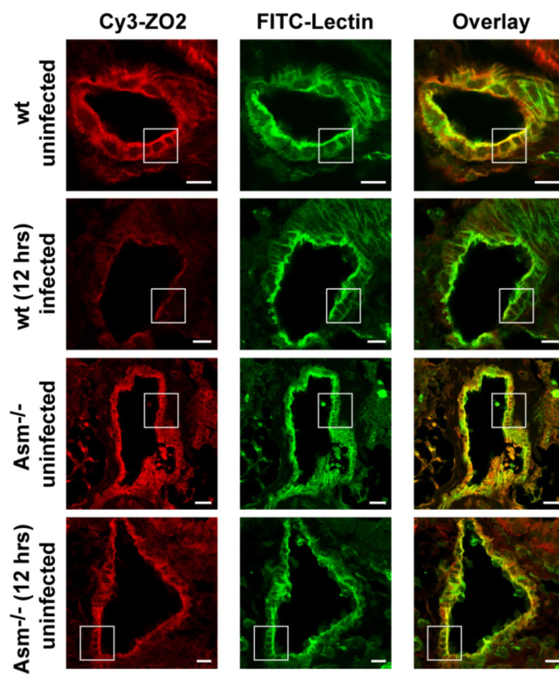

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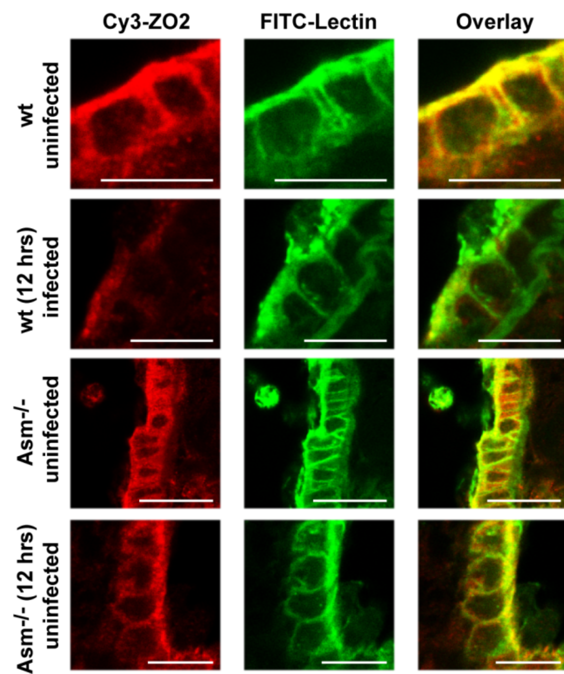

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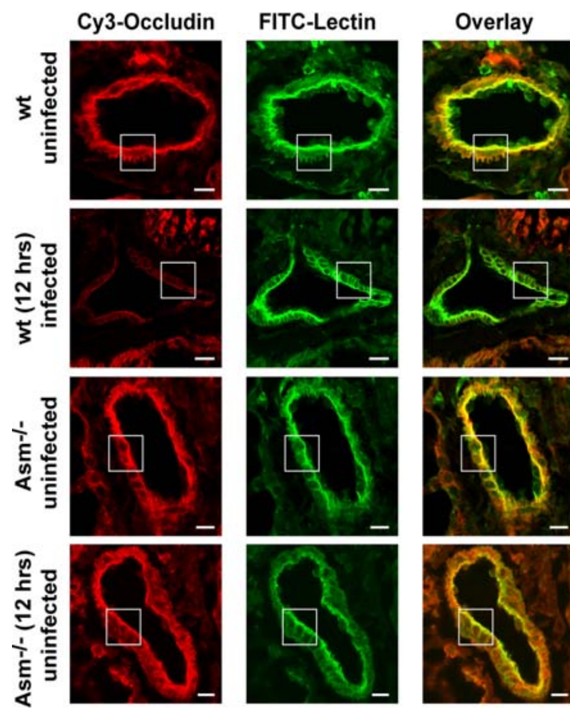

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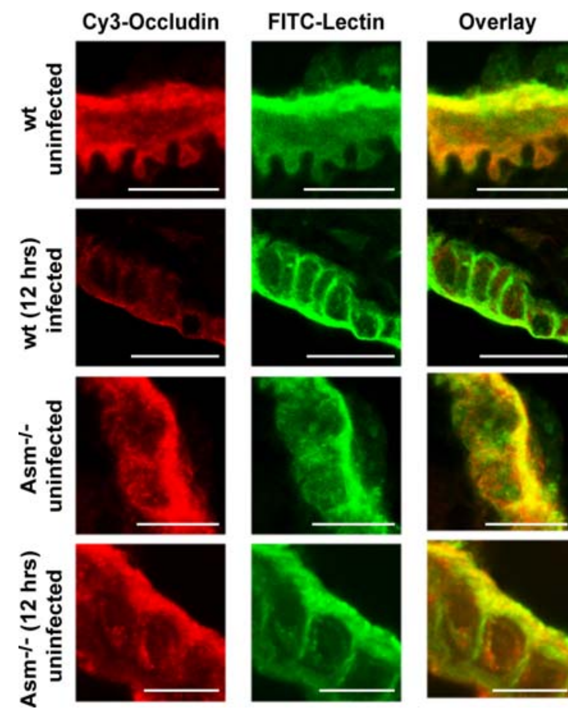

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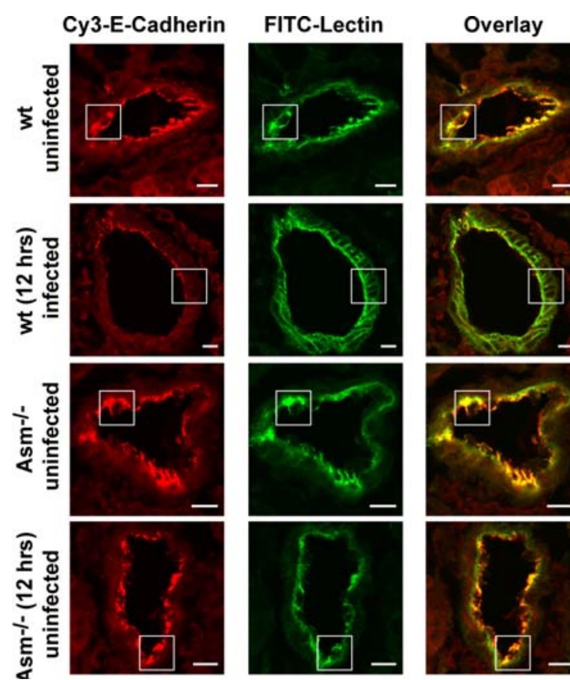


H-2

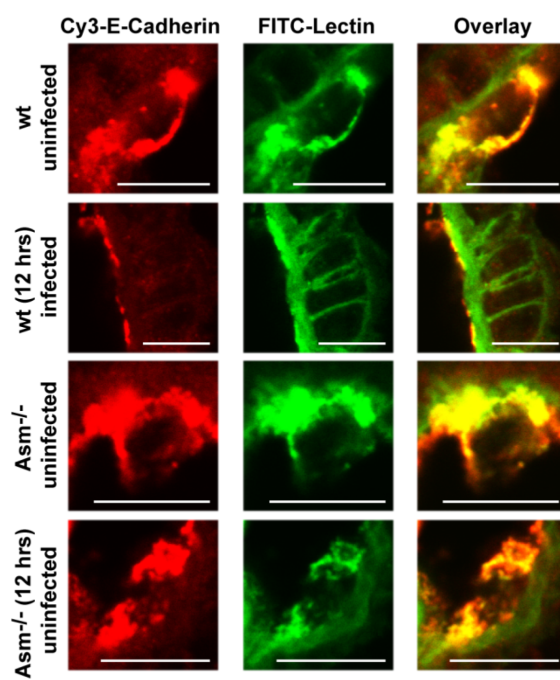


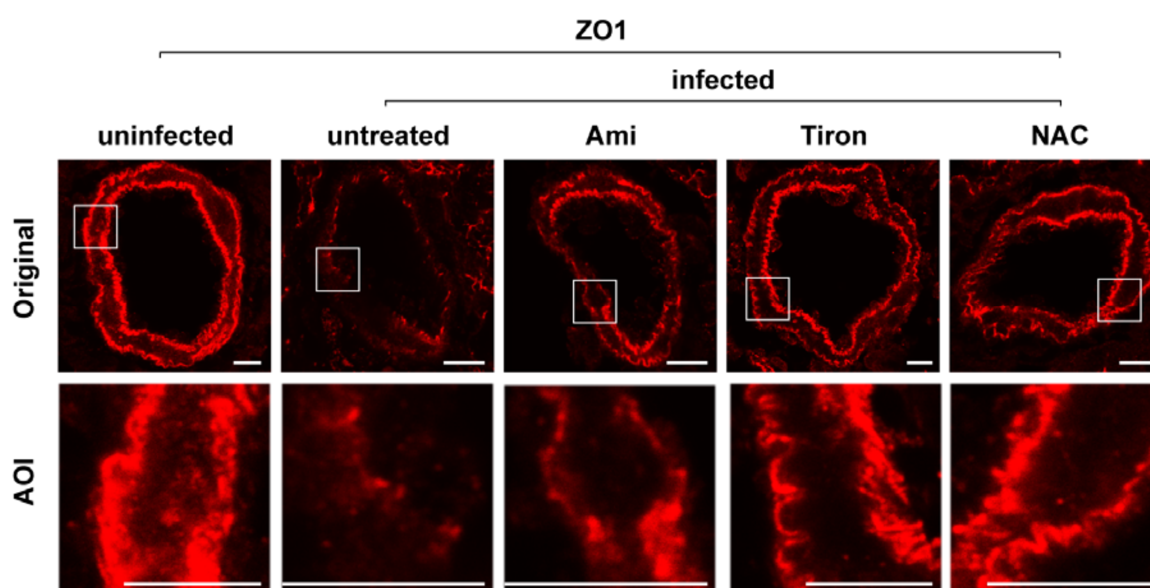
Figure 4.3.1. Effect of Asm deficiency on degradation of junctional proteins upon *S. aureus* infection *in vivo*

Wild-type and Asm-deficient mice were left uninfected or were infected with *S. aureus* for different time points. The lungs were removed, fixed, dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm. The lung sections were stained with Cy3-labeled anti-ZO1 (A and E), anti-ZO2 (B and F), anti-Occludin (C and G) or anti-E-cadherin (D and H) antibodies (magnification, 40×). Representative fluorescence images from three independent experiments are shown (original image and an area of interest [AOI]). Scale bar is 10 µM.

4.3.2. Asm and ROS are necessary to degradation of junctional proteins induced by *S. aureus* infection *in vivo*

In order to further confirm that Asm is involved in regulating junctional proteins degradation upon *S. aureus* infection *in vivo* and whether junctional proteins degradation presupposes the production of ROS, we pretreated wt mice with intraperitoneal injections of amitriptyline or Tiron and then infected them with a clinical *S. aureus* strain. Confocal microscopy analysis demonstrated that the inhibition of Asm or ROS also protects junctional proteins in lung endothelial cells from degradation after systemic *S. aureus* infection *in vivo* (**Figure 4.3.2.A-D.**).

A



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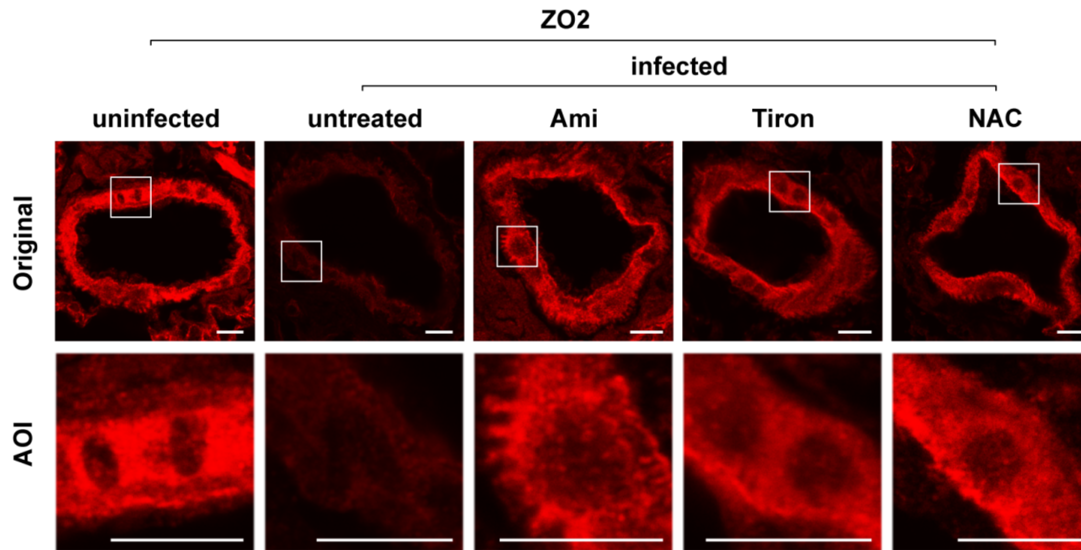

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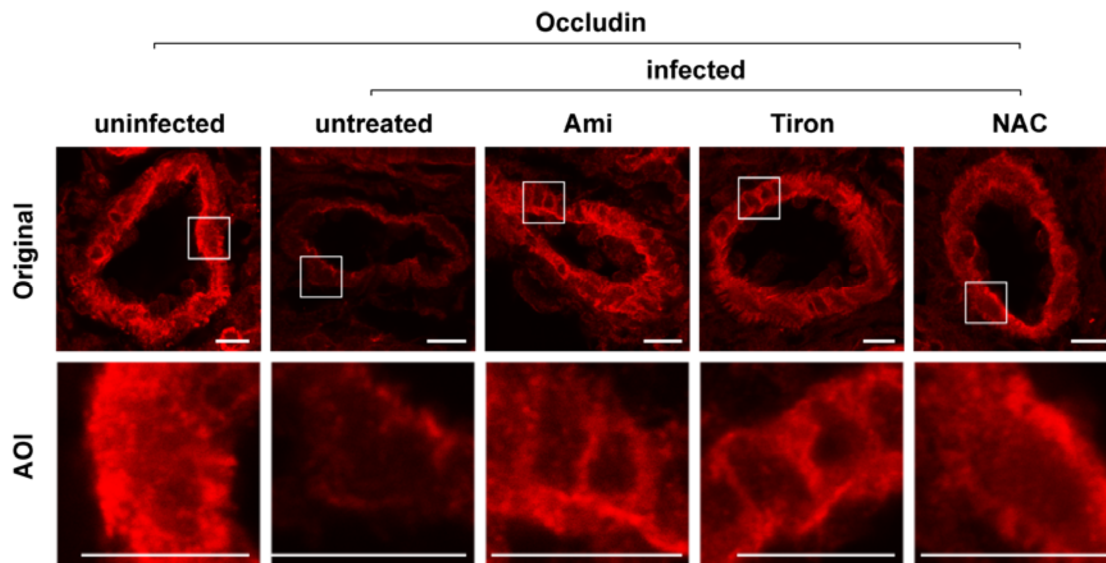


D

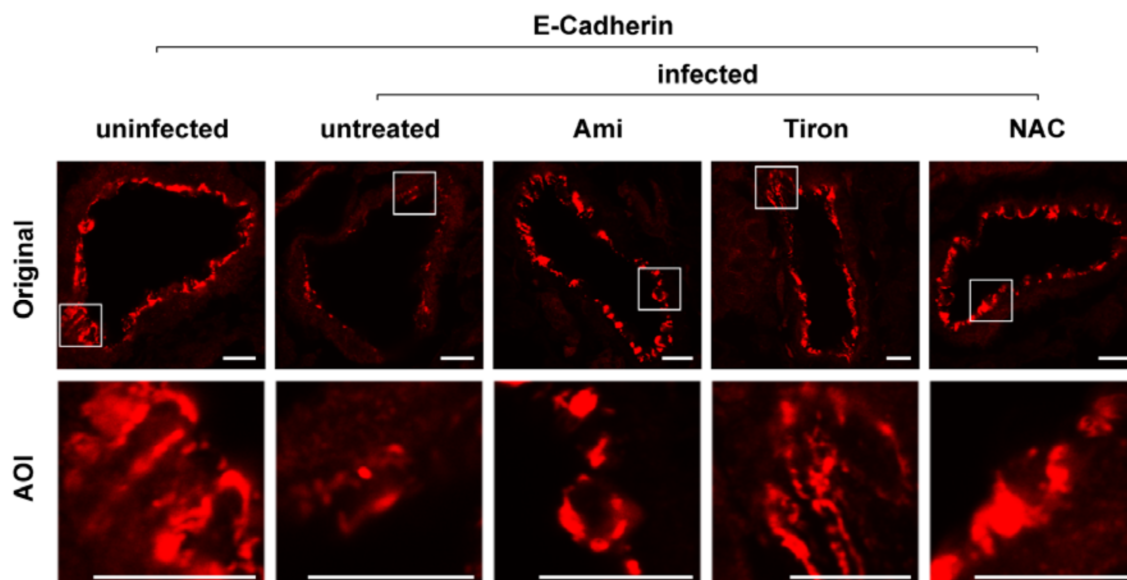


Figure 4.3.2. Inhibition of Asm and ROS prevents *S. aureus*-induced degradation of junctional proteins *in vivo*

Wild type C57BL/6J mice were pretreated with 10 mg/kg amitriptyline, 100 mg/kg Tiron or 100 mg/kg NAC by intraperitoneal injection, twice daily for 2.5 days, and then infected with *S. aureus* for 12 hrs. The lungs were removed, fixed, dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm. The lung sections were stained with Cy3-labeled anti-ZO1 (A), anti-ZO2 (B), anti-Occludin (C) or anti-E-cadherin (D) antibodies (magnification, 40×). Representative fluorescence images from three independent experiments are shown (original image and an area of interest [AOI]). Scale bar is 10 µM.

4.3.3. Asm and ROS are necessary to degradation of junctional proteins induced by *S. aureus* infection *in vitro*

To further confirm the degradation of junctional proteins is a consequence of Asm activation and ROS production, we pre-incubated EOMA cells with 20 µM amitriptyline, 10 mM Tiron or 10 mM NAC before infection with a clinical *S. aureus* strain (MOI 10:1). We found that inhibition of Asm or ROS protects junctional proteins from degradation after *S. aureus* infection in the EOMA cells (**Figure 4.3.3.**).

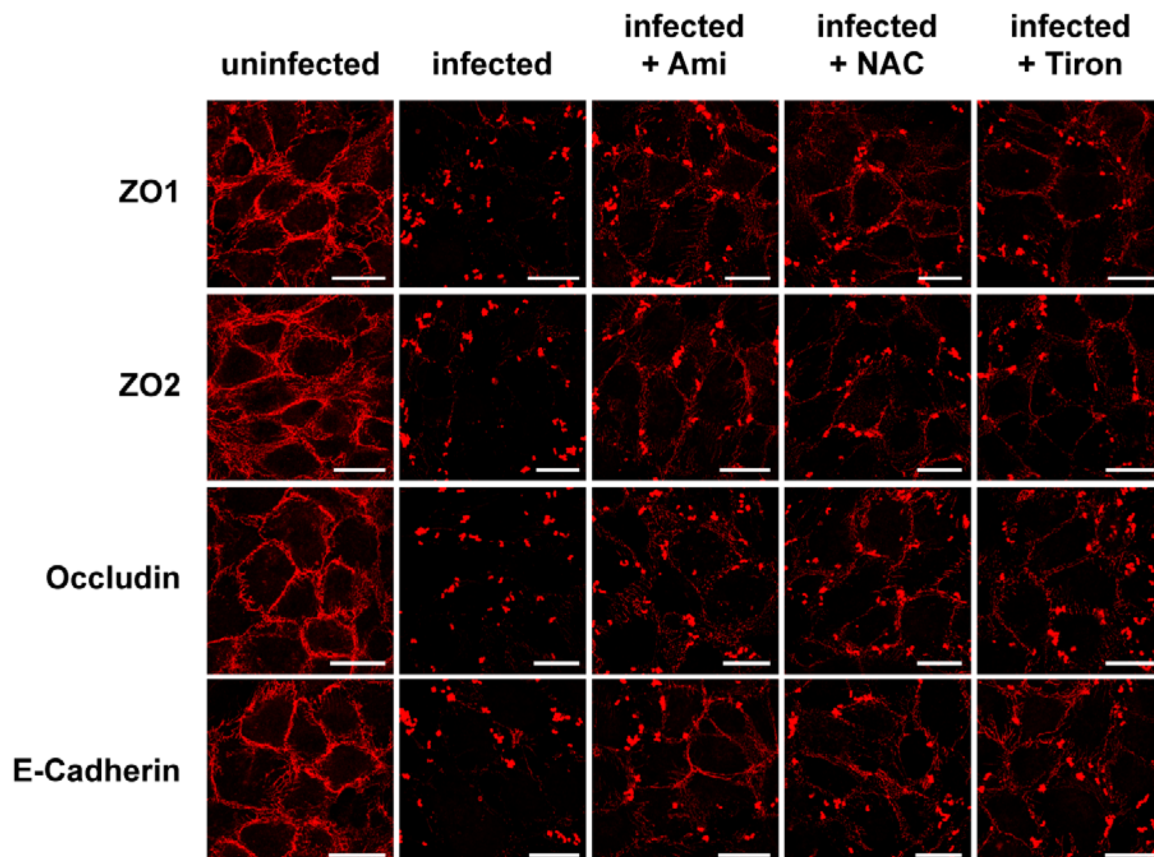


Figure 4.3.3. Inhibition of Asm and ROS prevents *S. aureus*-induced degradation of junctional proteins *in vitro*

EOMA cells were pretreated for 20 min with amitriptyline (20 µM), Tiron (10 mM) or NAC (10 mM) before being infected with a clinical *S. aureus* strain (MOI 10:1) for 2hrs. Immunoflourescence stainings were performed with antibodies against ZO1, ZO2, Occludin, or E-cadherin for determination of the degradation of these junctional proteins. The presented pictures are representative of the results of at least three independent experiments (magnification, 40×). Scale bar is 25 µM.

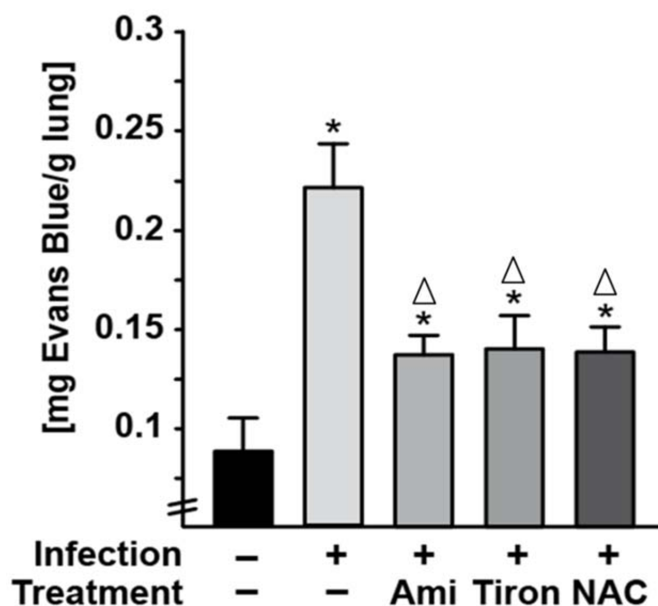
The data presented above demonstrate that *S. aureus* infection leads to the degradation of tight junctional proteins *in vitro* and *in vivo*. Genetic deficiency or pharmacological inhibition of Asm prevents *S. aureus*-induced degradation of junctional proteins. Moreover, inhibition of ROS also protects junctional proteins from degradation. Collectively, these findings indicate that Asm mediates the *S. aureus*–induced breakdown of junctional proteins by ROS.

4.4. Pharmacologic inhibition of Asm or ROS before systemic infection with *S. aureus* prevents lung edema

4.4.1. Pretreatment with amitriptyline, Tiron or NAC alleviates pulmonary edema upon *S. aureus* infection

To test the significance of the pathway from the Asm via ROS to the degradation of junctional proteins for the development of lung edema, we treated wt mice with intraperitoneal injections of amitriptyline, Tiron or NAC before systemic infection with a clinical *S. aureus* strain and then measured lung edema. *S. aureus* infection induced severe lung edema (**Figure 4.4.1.A. and B.**), events that were prevented by pretreatment with amitriptyline, Tiron or NAC (**Figure 4.4.1.A. and B.**). These findings show that lung edema induced by *S. aureus* via the pathway through Asm and ROS can be prevented by pretreatment with pharmacologic inhibitors of this pathway.

A



B

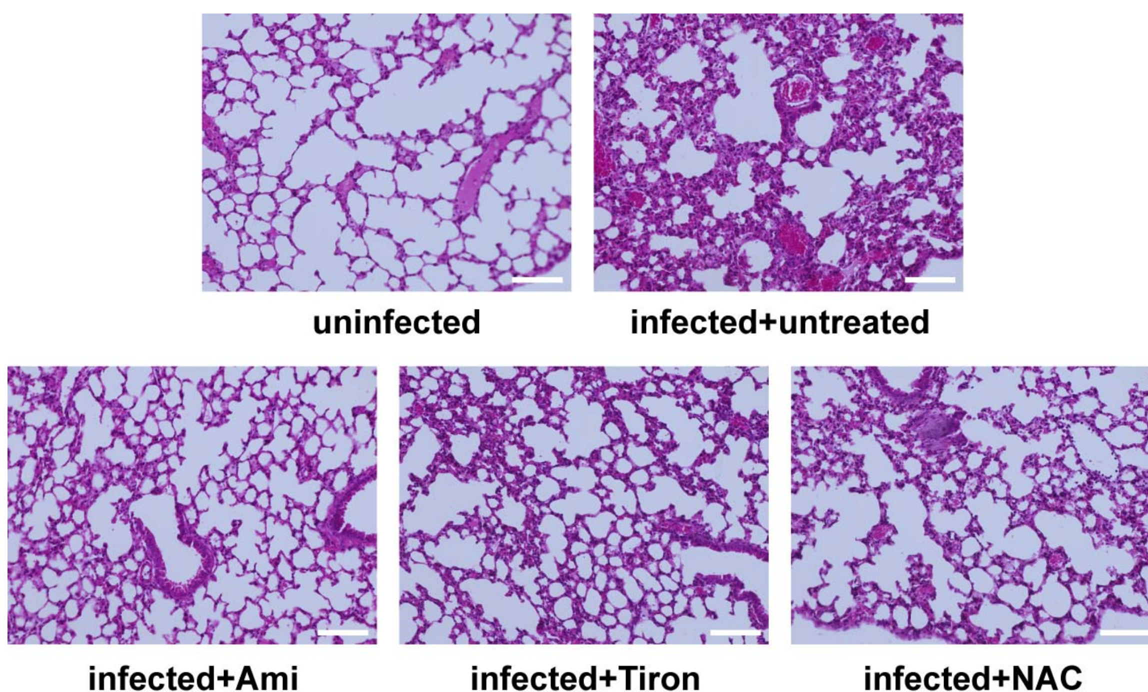


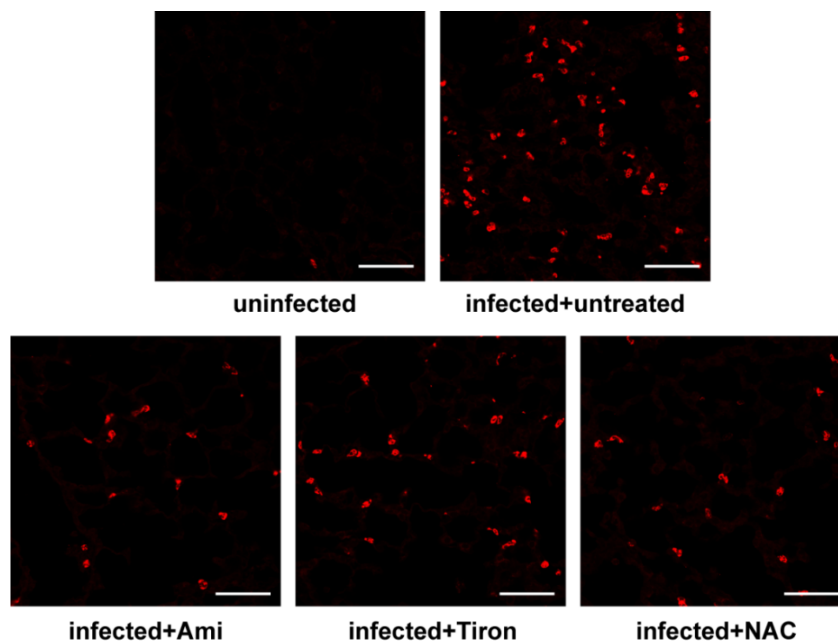
Figure 4.4.1. Effect of pharmacological Asm inhibition and neutralization of ROS on pulmonary edema upon *S. aureus* infection

Wild-type mice were pretreated by intraperitoneal injection of 10 mg/kg amitriptyline, 100 mg/kg Tiron or 100 mg/kg NAC, twice daily for 2.5 days. Mice were infected with *S. aureus* for 12 hrs. Lung edema was determined by extravasation of Evans Blue (A) and by staining with H&E (scale bar is 100 μM, magnification, 20×) (B). Representative images from three independent experiments are shown. *, significant differences between uninfected and infected samples; Δ, significant differences between treated and untreated samples (all $P<0.05$, t-test).

4.4.2. Pretreatment with amitriptyline, Tiron or NAC decreases neutrophil trafficking into the lung

Next, we tested whether Asm and ROS play an important role on neutrophil trafficking to the lung induced by *S. aureus* infection as suggested by their marked effect on the integrity of junctional proteins and lung edema. To this end, we treated wt mice with intraperitoneal injections of amitriptyline, Tiron or NAC before systemic infection with a clinical *S. aureus* strain and then measured neutrophil influx. The results revealed that pretreatment with amitriptyline, Tiron or NAC prevented influx of neutrophils into the lung (**Figure 4.4.2.A. and B.**). This data indicates that Asm and ROS facilitate neutrophil trafficking following into lung tissue upon *S. aureus* infection.

A



B

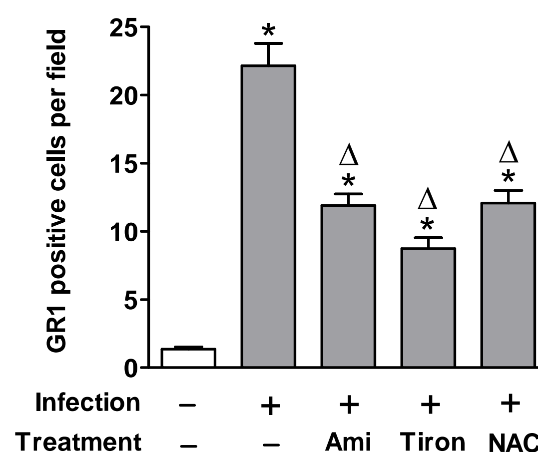


Figure 4.4.2. Effect of amitriptyline, Tiron or NAC on neutrophil trafficking into lung tissue induced by *S .aureus* infection

Wild-type mice were pretreated by intraperitoneal injection of 10 mg/kg amitriptyline, or 100 mg/kg Tiron or 100 mg/kg NAC twice daily for 2.5 days. Mice were infected with *S. aureus* for 12 hrs. Neutrophil emigration was determined by staining of lung sections with Cy3-labeled anti-GR1 antibody (scale bar is 50 µM) followed by fluorescence microscopy (A). Shown are representative images from three independent experiments. Neutrophil trafficking was quantified by analysis of 50 fields per group (B). Displayed is the average of GR1-positive cells per field of 63x magnification. *, significant differences between uninfected and infected samples; Δ, significant differences between treated and untreated samples (all $P<0.05$, t-test).

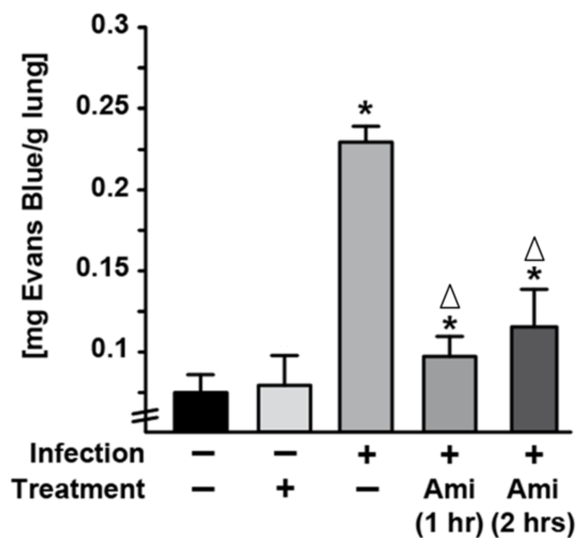
The results from **4.4.1** and **4.4.2** indicate pharmacologic inhibition of Asm or ROS before systemic infection with *S. aureus* prevents lung edema and influx of neutrophils into the lung.

4.5. Treatment of already septic mice with amitriptyline prevents the development of lung edema

4.5.1. Treatment with amitriptyline 1 hr or 2 hrs post *S. aureus*-infection reduces pulmonary edema

The finding that both Asm-deficiency and pre-incubation with the Asm inhibitor amitriptyline protect mice from lung edema induced by *S. aureus* infection led us question whether the administration of amitriptyline reduces the severity of pulmonary edema in mice that were already infected with *S. aureus*. If so, amitriptyline administration might be a clinically relevant therapeutic option for the treatment of *S. aureus*–induced pulmonary edema. To this end, we infected wt mice with a clinical *S. aureus* strain and treated them with amitriptyline 1 or 2 hrs later. Twelve hours after infection, the mice were sacrificed and Evans Blue extravasation was determined or lung sections were stained with H&E. As shown in **Figure 4.5.1.A. and B.,** treatment with amitriptyline reduced the severity of pulmonary edema even after the onset of systemic infection with *S. aureus*.

A



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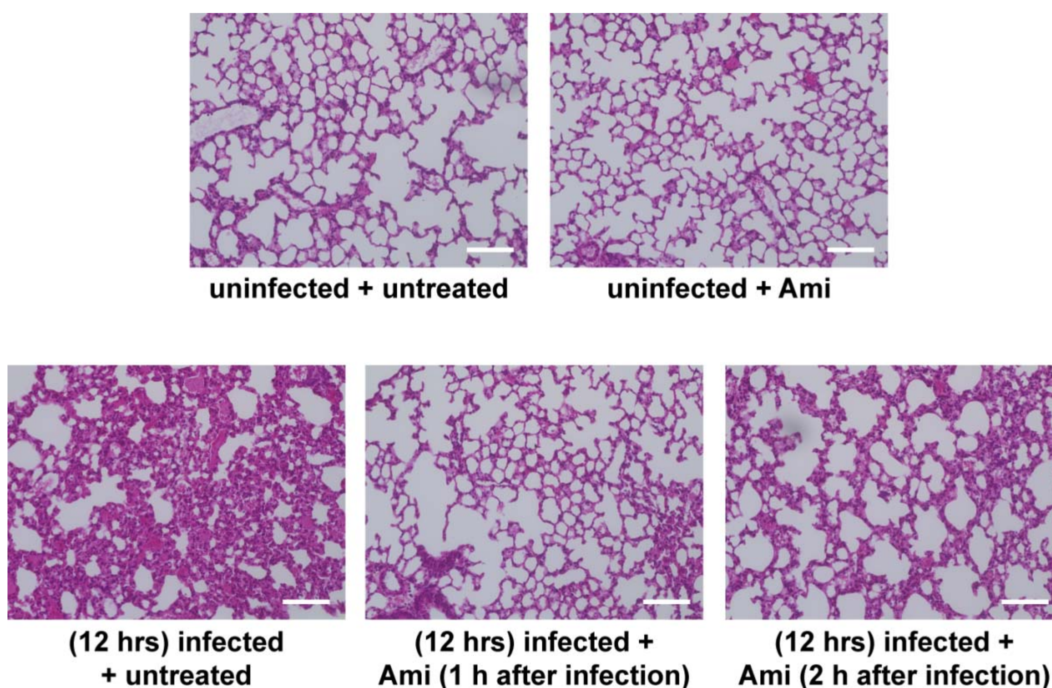


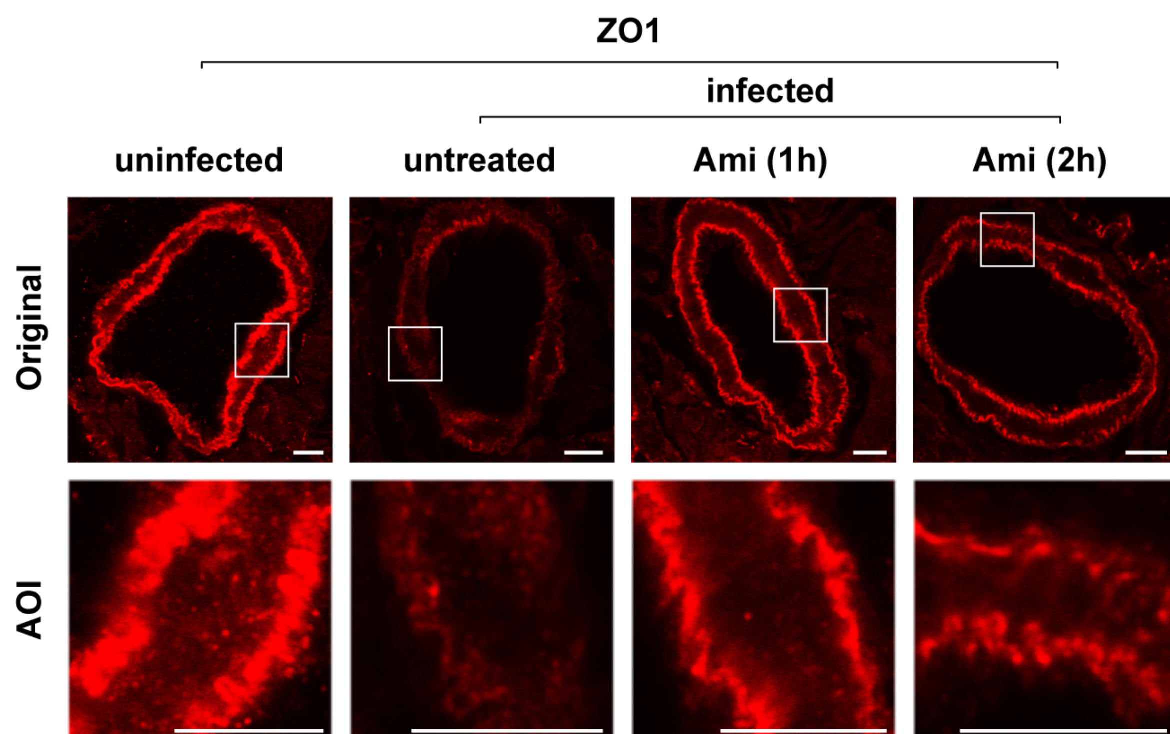
Figure 4.5.1. Amitriptyline treatment prevents lung edema in *S. aureus*-infected mice even after onset of the systemic infection

Wild-type mice were infected with *S. aureus*. 1 h or 2 hrs later they were i.p. injected with 16 mg/kg amitriptyline. The mice were sacrificed 12 hrs after infection. Lung edema was determined by extravasation of Evans Blue (A) and by staining with H&E (scale bar is 100 µM, magnification, 20×) (B). Panel A shows the mean ± SD from 4 mice. Images in B are representative from three independent experiments. *, significant differences between uninfected and infected samples; Δ, significant differences between treated and untreated samples (all $P<0.05$, t-test).

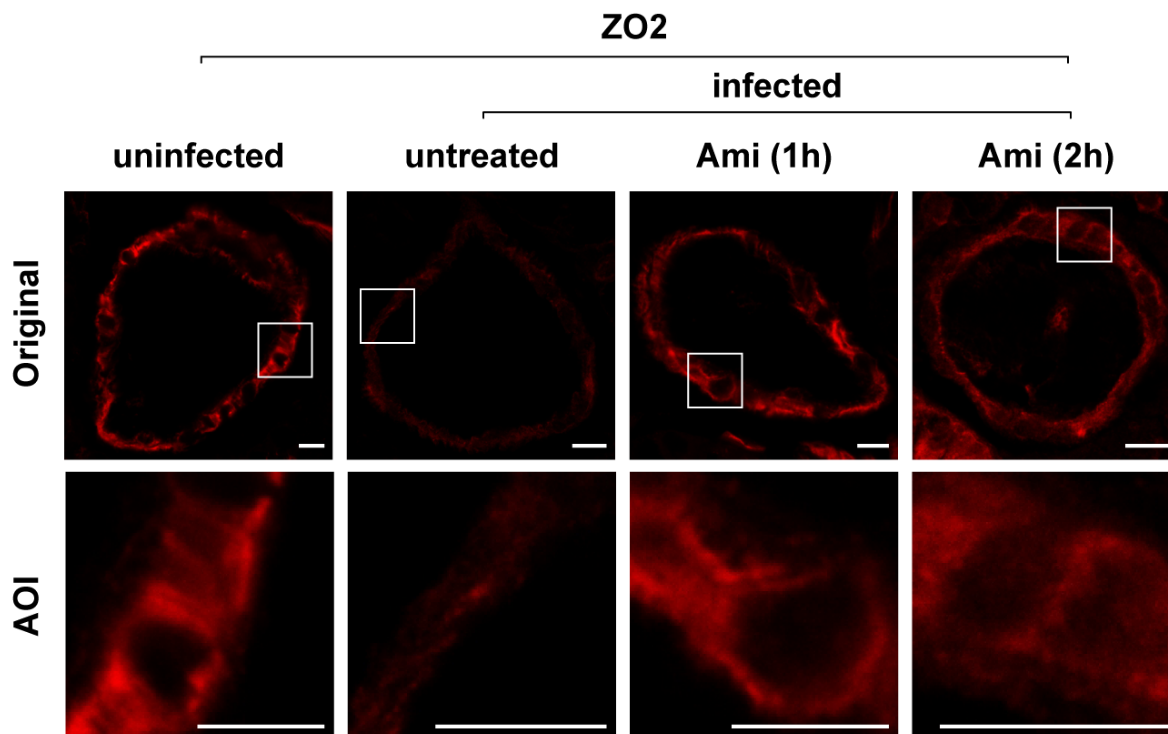
4.5.2. Treatment with amitriptyline 1 hr or 2 hrs after *S. aureus*-infection prevents degradation of junctional proteins

To determine whether pharmaceutical blockade of Asm by amitriptyline even after infection with *S. aureus* also protects the mice from degradation of junctional proteins *in vivo*, wild-type C57BL/6J mice were infected with a clinical *S. aureus* strain. The mice were treated with i.p. injection of 16 mg/kg amitriptyline 1 hr or 2 hrs after infection, respectively. The mice were sacrificed 12 hrs after infection and the lung sections were stained with anti-ZO1, anti-ZO2, anti-Occludin or anti-E-cadherin antibodies and analyzed by confocal fluorescence microscopy. The results showed amitriptyline-treatment reduced the degradation of junctional proteins even when the drug was applied 1 hr or 2 hrs post infection with *S. aureus* (**Figure 4.5.2. A-D.**).

A



B



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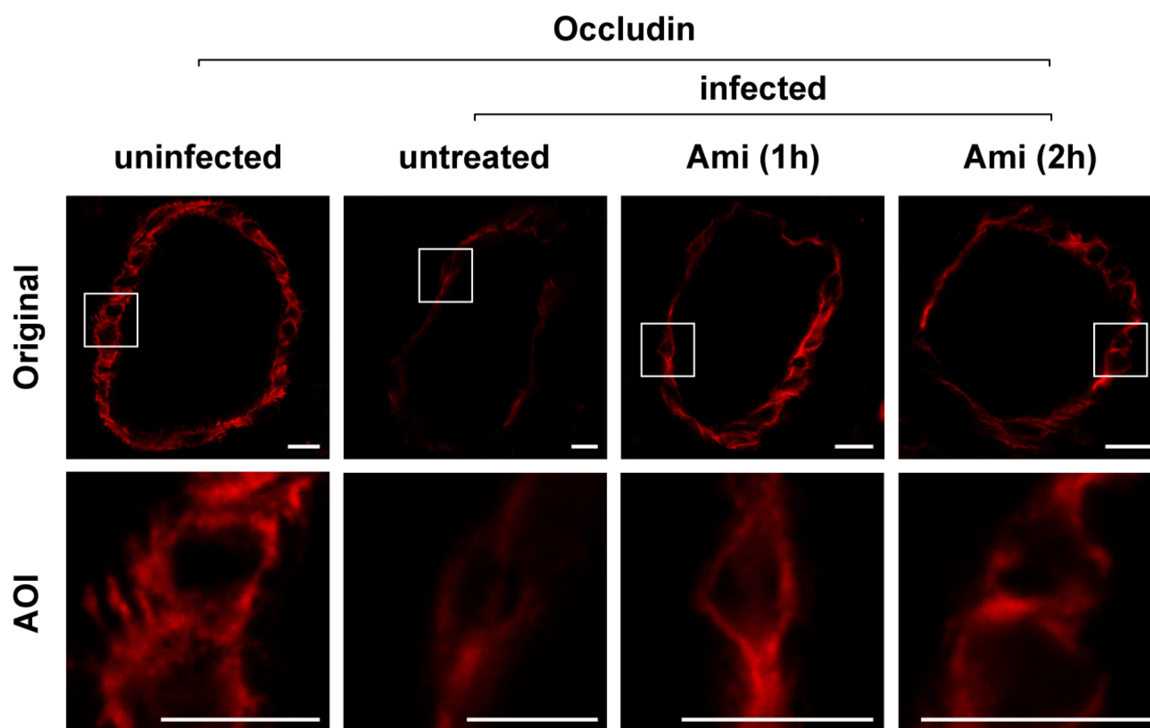

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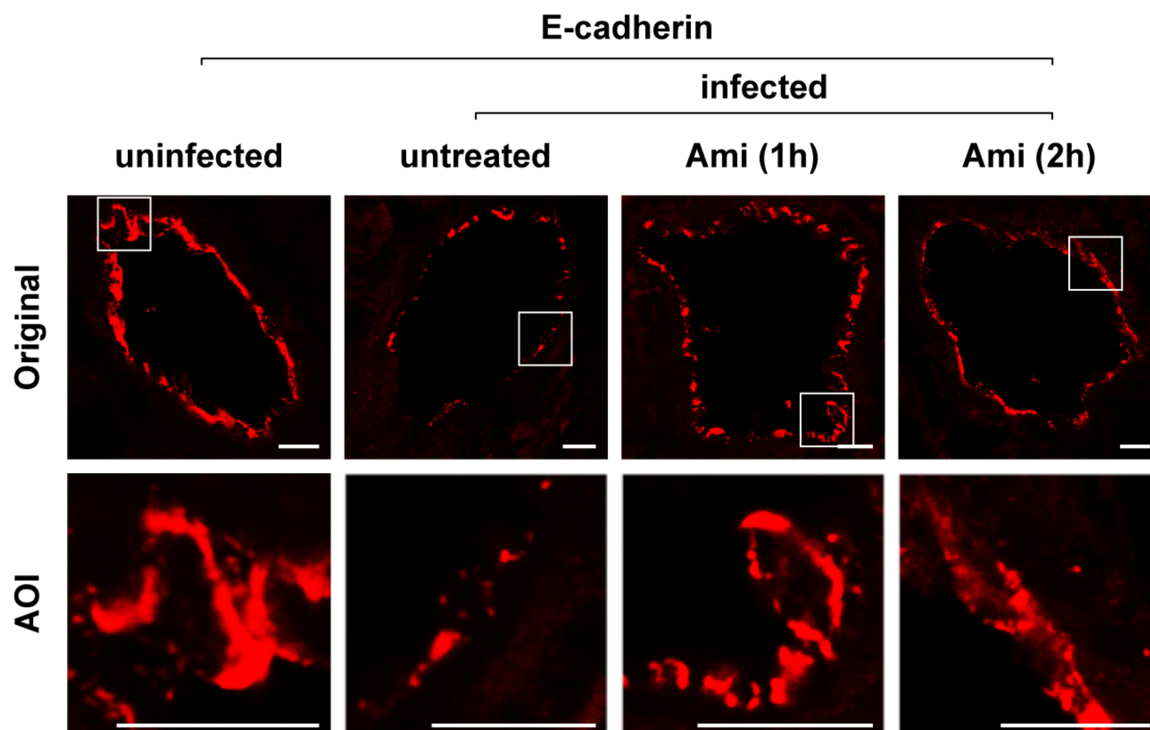


Figure 4.5.2. Amitriptyline treatment prevents degradation of junctional proteins even if administered after *S. aureus* infection

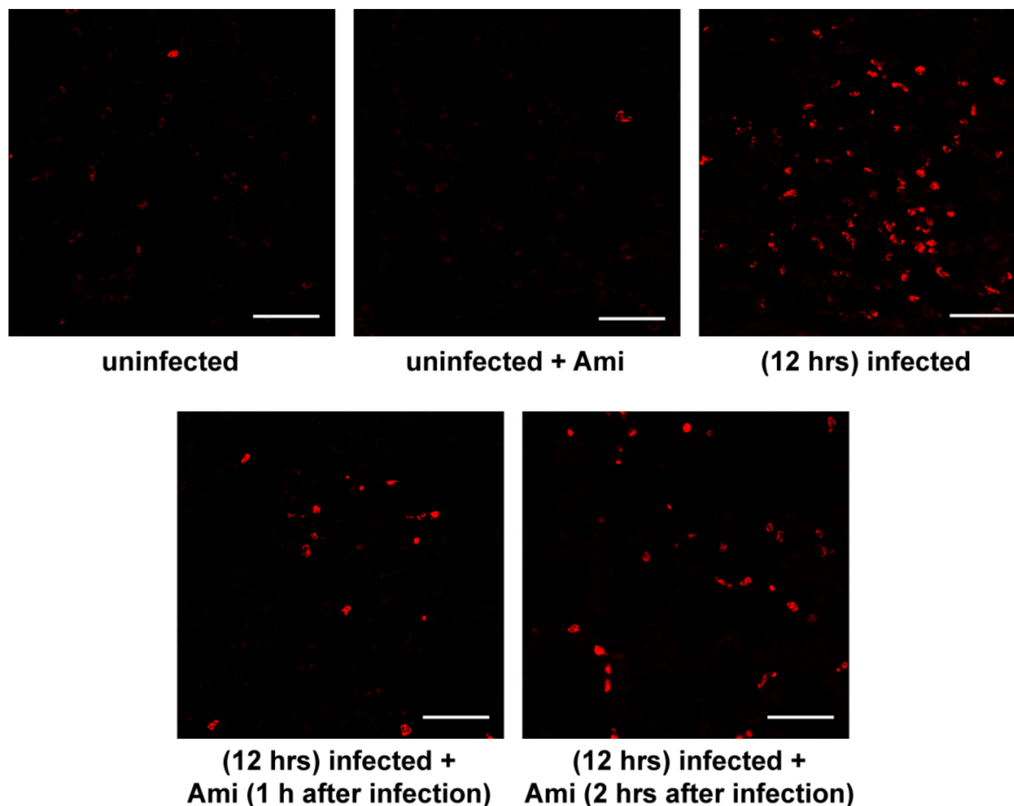
Wild-type mice were infected with *S. aureus* and were treated 1 h or 2 hrs after infection with i.p. amitriptyline. Twelve hours after infection, the mice were sacrificed. Lung sections were stained with Cy3-labeled antibodies against ZO1 (A), ZO2 (B), Occludin (C) or E-cadherin (D). Images were obtained by confocal microscopy and are representative of three independent experiments (magnification, 40×). The original image and an area of interest (AO1) are shown. Scale bar is 10 µM.

4.5.3. Treatment with amitriptyline 1hr or 2 hrs after infection prevents neutrophil trafficking into the lung tissue

To determine whether pharmacological inhibition of Asm with amitriptyline also reduces neutrophil trafficking after infection of mice with *S. aureus*, wild-type C57BL/6J mice were infected with *S. aureus.* Again, the mice received 16 mg/kg amitriptyline by i.p. injected 1 hr or 2 hrs after infection. The mice were sacrificed 12 hrs after infection and the lung sections were stained with anti-GR1 antibody. The results demonstrated that amitriptyline treatment reduced pulmonary influx of neutrophils even if injected 1 hr or 2 hrs after infection, which

confirms that Asm play an important role in *S. aureus*-induced neutrophil trafficking (**Figure 4.5.3. A. and B.**).

A



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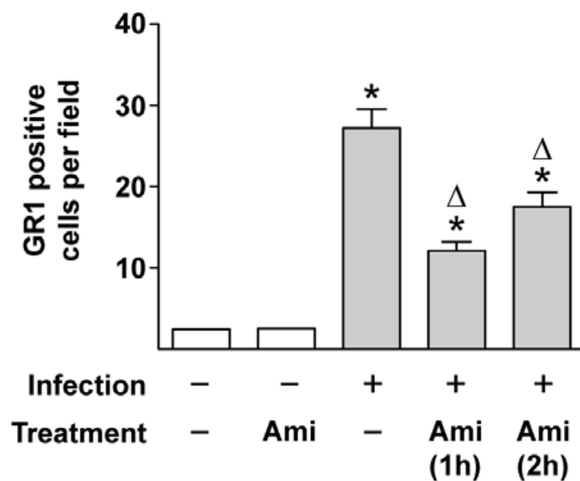


Figure 4.5.3. Amitriptyline treatment reduces *S. aureus*-induced neutrophil trafficking

Wild-type mice were infected with *S. aureus* for 1 hr or 2 hrs, and then treated with i.p. injection of 16 mg/kg amitriptyline. The mice were sacrificed 12 hrs after infection and the lungs were removed, fixed, dehydrated and embedded. The lung sections were stained with Cy3-labeled anti-GR1 antibody (scale bar is 50 μM, magnification, 63×) (A). Representative images from three independent experiments are shown. The average number of GR1-positive cells per field of 63x magnification was quantified by analysis of 50 fields per group (B). Data are shown as mean ± SD, n = 3. *, significant differences between uninfected and infected samples; Δ, significant differences between treated and untreated samples (all $P<0.05$, t-test).

This indicates that amitriptyline treatment after systemic infection with *S. aureus* protects junctional proteins degradation and reduces pulmonary influx of neutrophils, finally preventing pulmonary edema. Thus, amitriptyline might be a novel therapy to *S. aureus*-induced sepsis.

4.6. The combination of amitriptyline and antibiotics in the very early time can be a novel therapy to *S. aureus*-induced sepsis

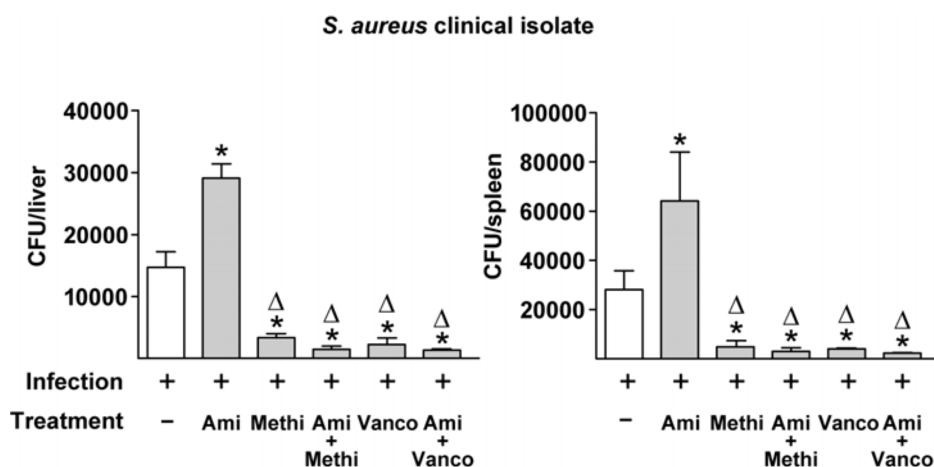
4.6.1. The combination of amitriptyline and antibiotics contributes to bacteria killing

Sepsis remains a challenge for intensive care physicians and is one of the leading causes of death nowadays. Although improvements in supportive care of patients with sepsis (eg, more effective and less damaging mechanical ventilation, improved fluid resuscitation, and broad-spectrum antibiotic coverage) have improved survival rates, sepsis remains a condition with high mortality ranging from 20% to 50% of severely affected patients (Creamer et al 2012, Kanafani et al 2009, Moore et al 2011).

Clinically, treating *S. aureus* sepsis with antibiotics often achieves only limited success, and severe lung edema often still develops even with appropriate antibiotic treatment (Kanafani et al 2009, Moore et al 2011, Moore et al 2012). Thus, with the aim of developing novel efficient therapeutic approaches to the treatment of *S. aureus*–induced sepsis and lung edema, we examined the effect of a combination of amitriptyline and antibiotics on bacterial killing and lung edema after systemic *S. aureus* infection. To this end, we infected wt mice with a

clinical *S. aureus* strain or the *S. aureus* Newman strain. One hour after infection with the incidence of first clinical symptoms, amitriptyline (Ami) or methicillin (Methi) or vancomycin (Vanco) or a combination of amitriptyline and either methicillin or vancomycin were injected. The injection of methicillin or vancomycin was repeated after 9 hrs. Control mice were left uninfected. The mice were sacrificed 12 hrs after infection, and bacterial numbers were determined in liver and spleen. Only antibiotics alone or in combination with amitriptyline kill bacteria in liver and spleen (**Figure 4.6.1. A-B.**). Treatment with amitriptyline alone can't kill bacteria and even shows more bacterial numbers in liver and spleen compared to untreated wt mice after infection (**Figure 4.6.1. A-B.**).

A



B

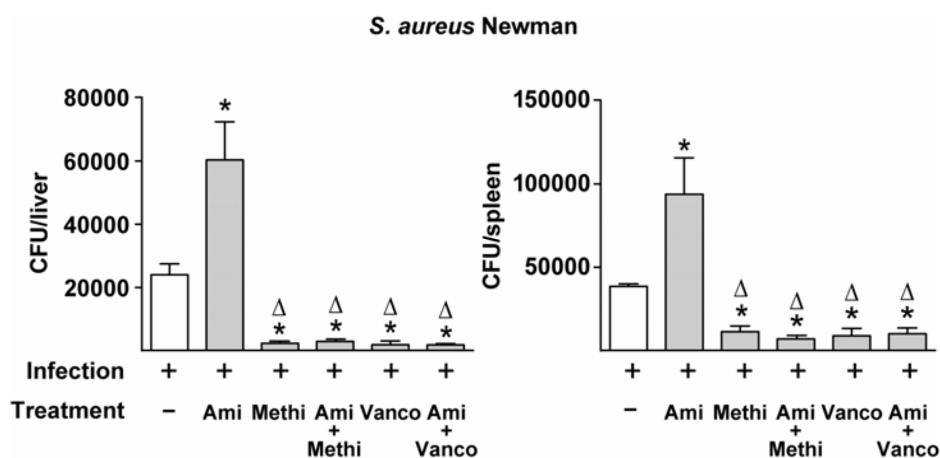


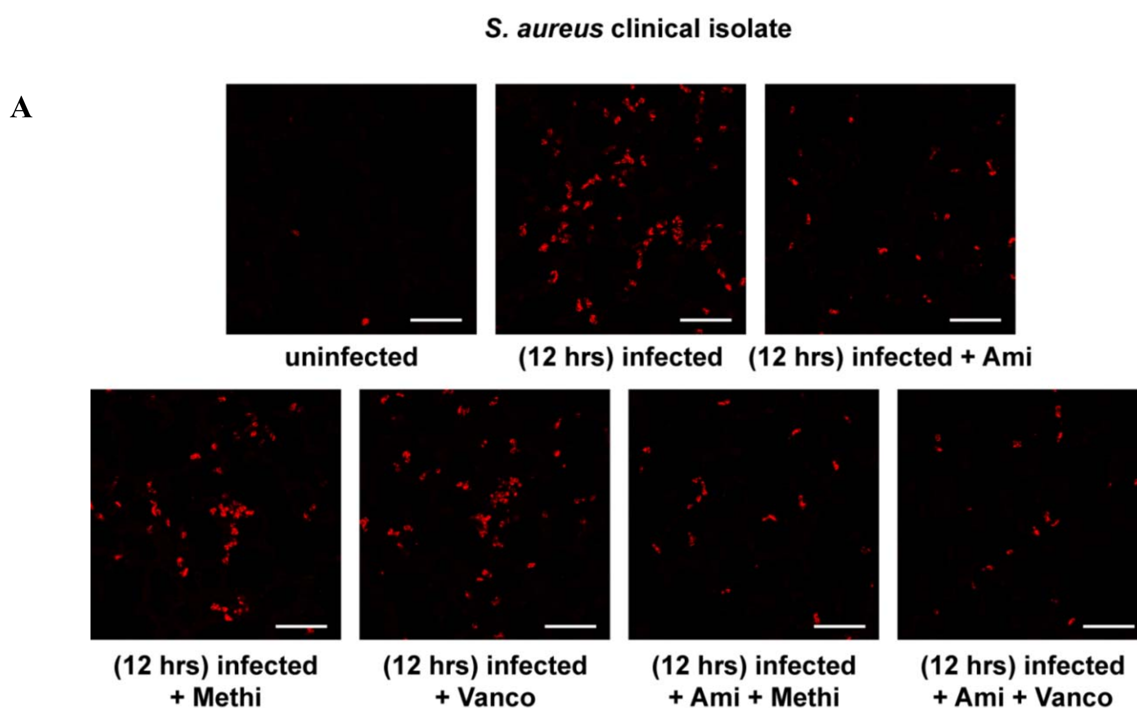
Figure 4.6.1. Effect of the combination of amitriptyline and antibiotics on bacteria killing

Wild-type mice were infected with a clinical *S. aureus* strain (A) or the *S. aureus* Newman strain (B). They were then left untreated; treated with an i.p. injection of amitriptyline (16 mg/kg) 1 h after

infection; treated with either methicillin or vancomycin (both 100 mg/kg) 1 h and 9 hrs after infection; or treated with the combination of amitriptyline and methicillin or vancomycin. The mice were sacrificed 12 hrs after infection. The liver and spleen were removed, homogenized, and lysed in saponin for the quantification of intracellular and extracellular bacteria (CFU) on lysogeny broth (LB) plates. Data shown are mean ± SD of three independent experiments. *, significant differences between uninfected and infected samples; Δ, significant differences between treated and untreated samples ($P<0.05$, t-test).

4.6.2. The combination of amitriptyline and antibiotics inhibits neutrophil trafficking into the lung

To define the effect of the combination of amitriptyline with antibiotics on neutrophil trafficking into the lung upon *S. aureus* infection, we infected wt mice with a clinical *S. aureus* strain or the *S. aureus* Newman strain. One hour after infection with the incidence of first clinical symptoms, amitriptyline or methicillin or vancomycin or a combination of amitriptyline and either methicillin or vancomycin were injected. The injection of methicillin or vancomycin was repeated after 9 hrs. Control mice were left uninfected. The mice were sacrificed 12 hrs after infection and the left lungs were removed. Obtained lung sections were stained with anti-GR1 antibody and analysed by confocal fluorescence microscopy. The results showed *S. aureus*-induced neutrophil trafficking into the lung is abrogated by treatment with amitriptyline alone or combining with antibiotics, but not treatment with methicillin or vancomycin alone (**Figure 4.6.2. A-B.**).



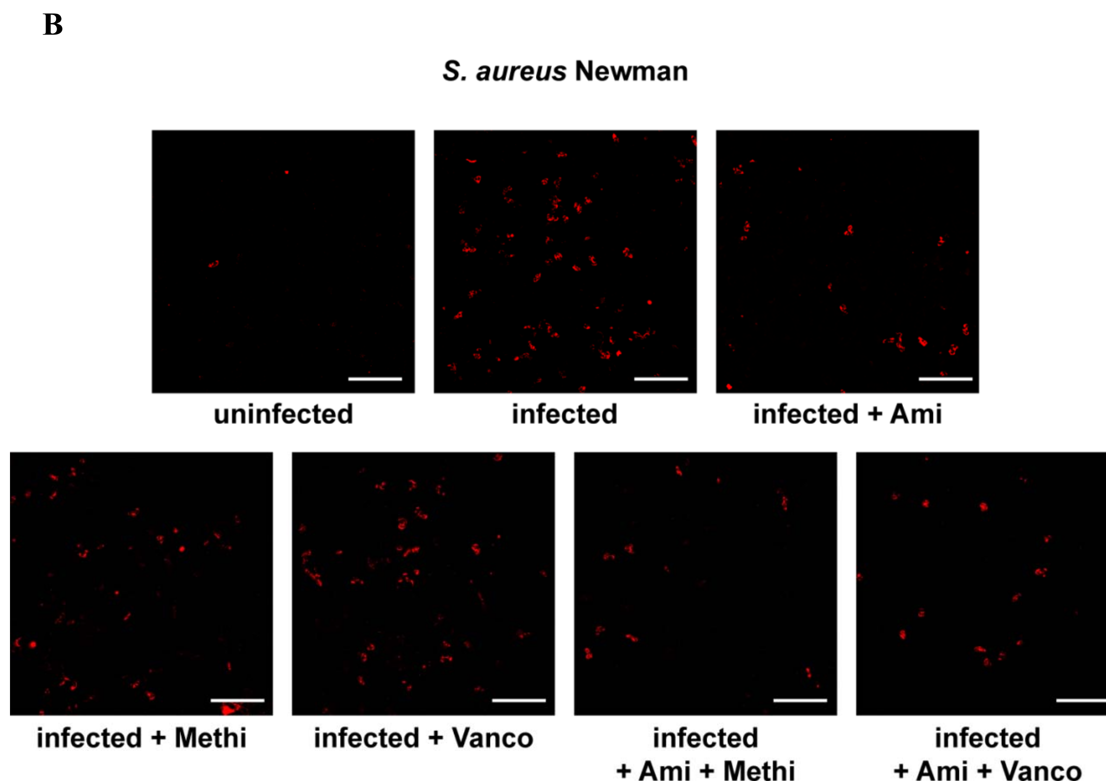


Figure 4.6.2. The combination of amitriptyline and antibiotics abrogates *S. aureus*-induced neutrophil trafficking into the lung

Wild-type mice were infected with a clinical *S. aureus* strain (A) or the *S. aureus* Newman strain (B). They were then left untreated; treated with an i.p. injection of amitriptyline (16 mg/kg) 1 h after infection; treated with either methicillin or vancomycin (both 100 mg/kg) 1 h and 9 hrs after infection; or treated with the combination of amitriptyline and methicillin or vancomycin. 12 hrs after infection, four mice per group were sacrificed and the left lungs were removed, fixed, dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm. The lung sections were stained with Cy3-labeled anti-GR1 antibody (scale bar is 50 µM, magnification, 63×). Representative fluorescence images from three independent experiments are shown.

4.6.3. The combination of amitriptyline and antibiotics protects mice from tight junctional proteins degradation after *S. aureus* infection

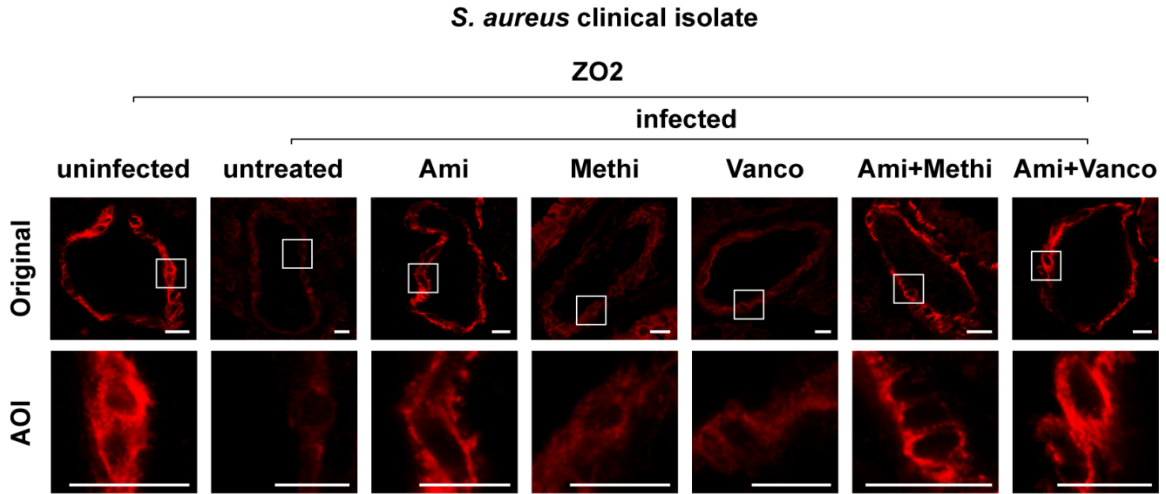
To determine the effect of the combination of amitriptyline with antibiotics on degradation of pulmonary junctional proteins upon *S. aureus* infection, we infected wt mice with a clinical *S. aureus* strain or the *S. aureus* Newman. One hour after infection with the incidence of first clinical symptoms, amitriptyline or methicillin or vancomycin or a combination of amitriptyline and either methicillin or vancomycin were injected. The injection of methicillin or vancomycin was repeated after 9 hrs. Control mice were left uninfected. The mice were sacrificed 12 hrs after infection and the left lungs were removed. Lung sections were stained

with anti-ZO1, anti-ZO2, anti-Occludin or anti-E-cadherin antibodies. The stainings were analysed by confocal fluorescence microscopy. The results showed that only treatment with amitriptyline alone or combining with antibiotics, but not treatment with methicillin or vancomycin alone, prevented breakdown of TJ proteins *in vivo* after infection of mice with *S. aureus* (**Figure 4.6.3. A-D.**).

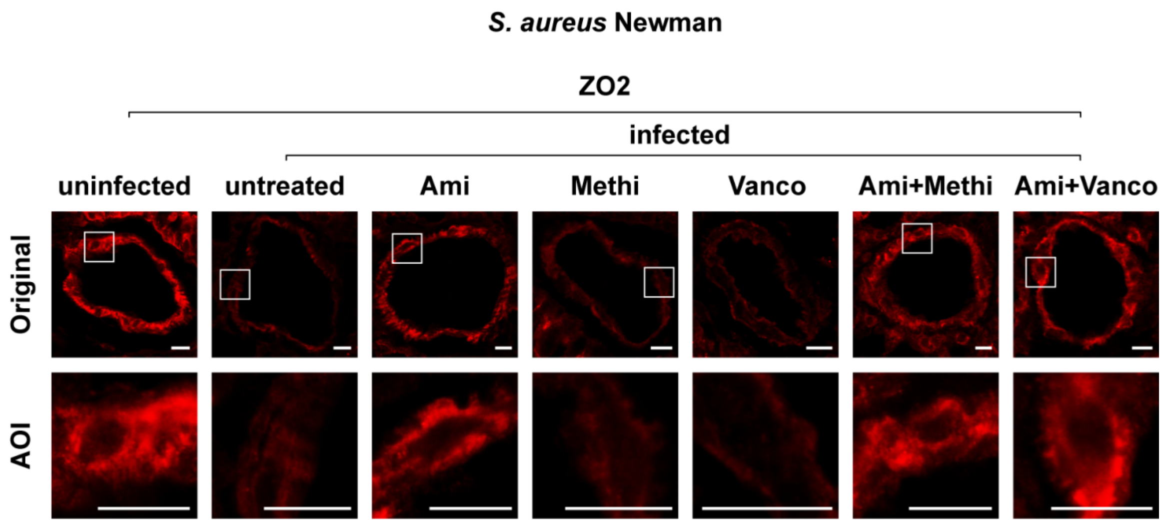
A-1

A-2

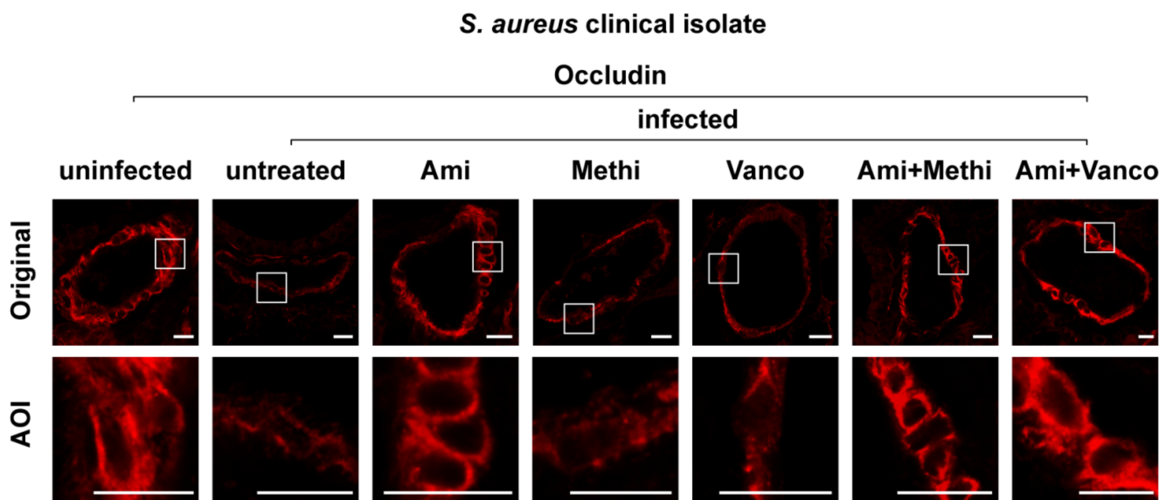
B-1



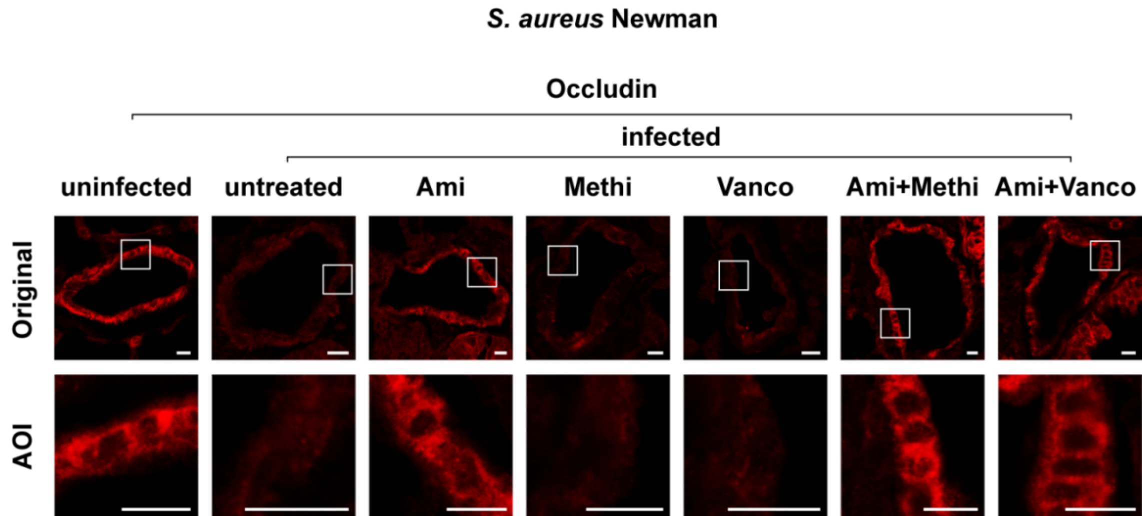
B-2



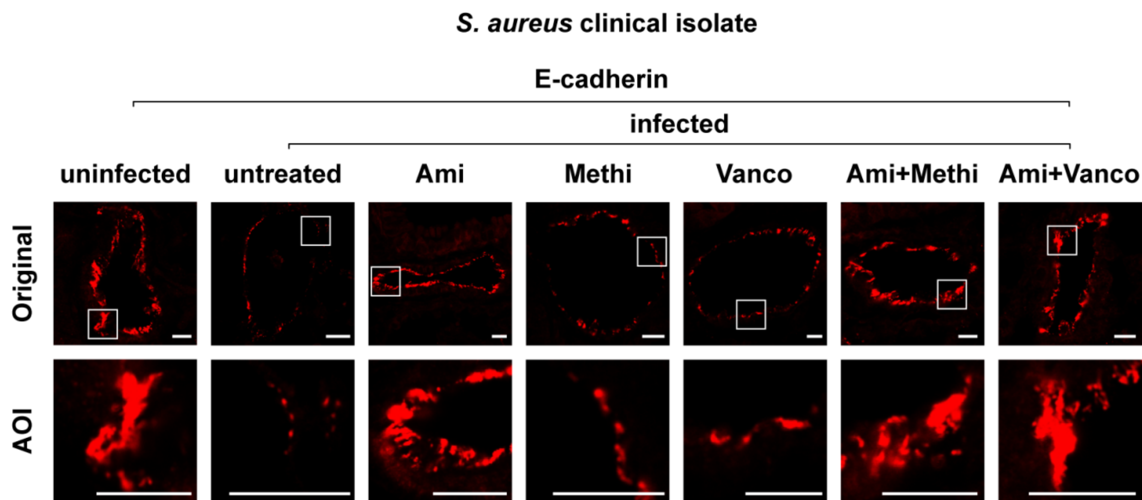
C-1



C-2



D-1



D-2

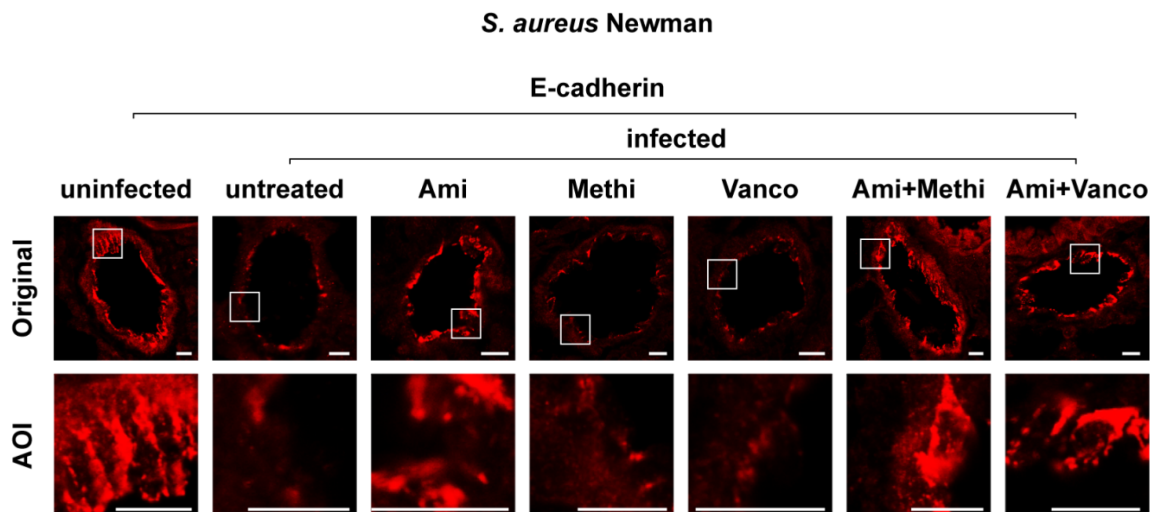


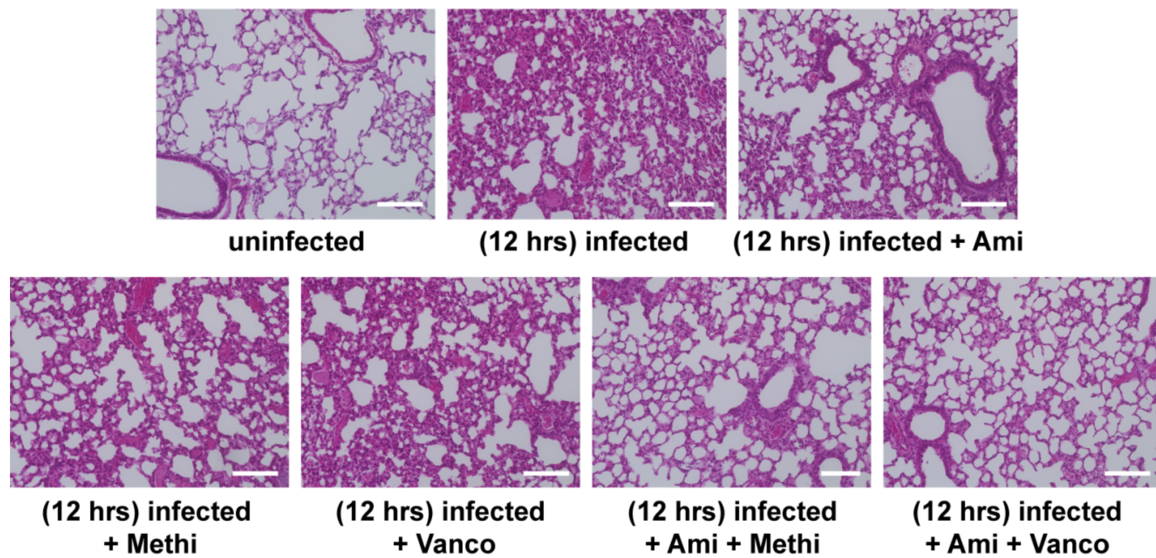
Figure 4.6.3. The combination of amitriptyline and antibiotics inhibits degradation of pulmonary tight junctional proteins upon *S. aureus* infection

Wild-type mice were infected with a clinical *S. aureus* strain (A-D-1) or the *S. aureus* Newman strain (A-D-2). They were then left untreated; treated with an i.p. injection of amitriptyline (16 mg/kg) 1 h after infection; treated with either methicillin or vancomycin (both 100 mg/kg) 1 h and 9 hrs after infection; or treated with the combination of amitriptyline and methicillin or vancomycin. After 12 hrs infection, four mice per group were sacrificed and the left lungs were removed, fixed, dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm. The lung sections were stained with Cy3-labeled anti-ZO1 (A), anti-ZO2 (B), anti-Occludin (C) or anti-E-cadherin (D) antibodies (scale bar is 10 µM, magnification, 40×). Representative fluorescence images from three independent experiments are shown.

4.6.4. The combination of amitriptyline and antibiotics rescues mice form *S. aureus*-induced lung edema

To determine the effect of the combination of amitriptyline with antibiotics on pulmonary edema upon *S. aureus* infection, we infected wt mice with a clinical *S. aureus* strain or the *S. aureus* Newman strain. One hour after infection, amitriptyline or methicillin or vancomycin or a combination of amitriptyline and either methicillin or vancomycin were injected. The injection of methicillin or vancomycin was repeated after 9 hrs. Control mice were left uninfected. The mice were sacrificed 12 hrs after infection and the left lungs were removed. Lung sections were stained with Hematoxylin-Eosin and analysed by confocal fluorescence microscopy. The results showed that only treatment with amitriptyline alone or combining with antibiotics, but not treatment with methicillin or vancomycin alone, prevented lung edema *in vivo* after infection of mice with *S. aureus* (**Figure 4.6.4.A. and B.**).

A



B

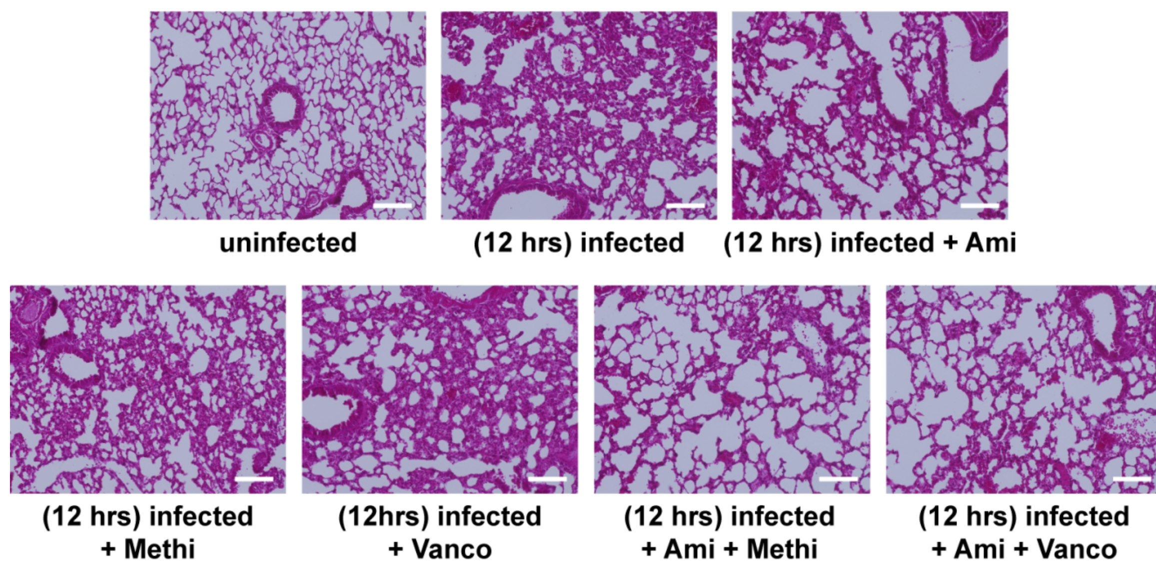


Figure 4.6.4. The combination of amitriptyline and antibiotics inhibits lung edema upon *S. aureus* infection

Wild-type mice were infected with a clinical *S. aureus* strain (A) or the *S. aureus* Newman strain (B). They were then left untreated; treated with an i.p. injection of amitriptyline (16 mg/kg) 1 h after infection; treated with either methicillin or vancomycin (both 100 mg/kg) 1 h and 9 hrs after infection; or treated with the combination of amitriptyline and methicillin or vancomycin. After 12 hrs infection, four mice per group were sacrificed and the left lungs were removed, fixed, dehydrated and embedded

in paraffin for sectioning at a thickness of 6 μm. The lung sections were stained with Hematoxylin-Eosin (scale bar is 100 μM, magnification, 20×). Representative fluorescence images from three independent experiments are shown.

4.7. The pharmacological treatment of lung edema and bacterial burden protects from lethality of *S. aureus* sepsis

To investigate the link between bacterial burden and sepsis-induced lethality, we performed mortality experiments with untreated and pharmacologically treated wt and Asm-deficient mice after infecting them intravenously with 5×10^6 CFU *S. aureus*. Wt mice died between 26 and 52 h after infection. Treatment of wt mice with amitriptyline delayed the death of the mice and the mice died between 50 and 85 h after infection. A very similar time course was observed for Asm-deficient mice that died between 50 and 80 h of infection. Treatment of wt mice with methicillin or vancomycin (1 and 9 h after infection and then twice daily) alone only rescued 50 % mice (11-day observation period). In contrast, the treatment of wt mice with a combination of amitriptyline and antibiotics rescued 100 % of infected wt mice, and no deaths were observed. Likewise, Asm-deficient mice under antibiotic intervention were also completely protected from sepsis-induced lethality (**Figure 4.7.**).

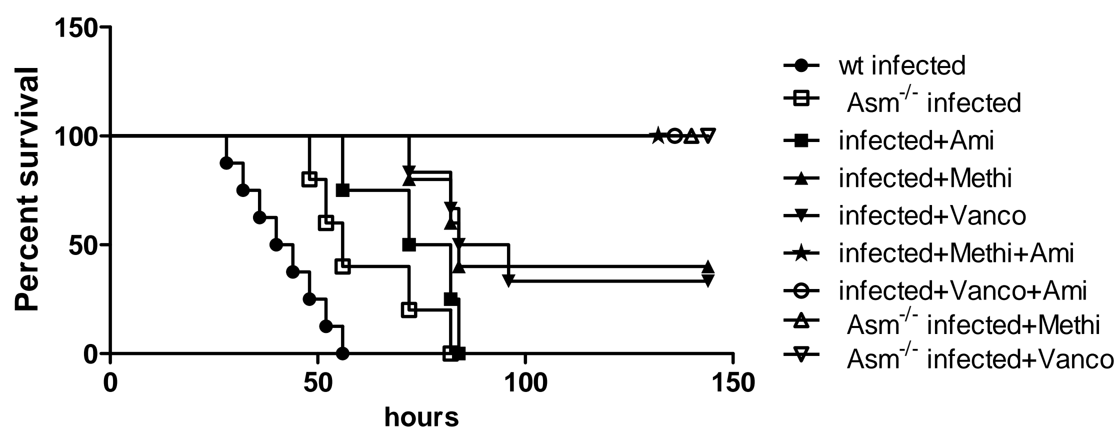


Figure 4.7. The pharmacological treatment of lung edema and bacterial burden protects from lethality of *S. aureus* sepsis

Wild-type (wt) and Asm-deficient ($Asm^{-/-}$) mice were infected intravenously with 5×10^6 CFU *S. aureus*. Mice were then left untreated or were pharmacologically treated with amitriptyline (Ami) (1 h after infection, then twice daily), methicillin (Methi), or vancomycin (Vanco) (1 and 9 h after infection

and then twice daily) or with a combination of amitriptyline and antibiotics. Survival was observed for up to 11 days. Data are shown in percent survival. Significance was determined by log-rank (Mantel–Cox) test.

In summary, these data indicate that the inhibition of lung edema in Asm-deficient or amitriptyline-treated mice together with a sufficient antibiotic treatment, which reduces the number of bacteria, is able to completely protect from lethality of *S. aureus* sepsis.

4.8. Control studies

4.8.1. Amitriptyline, Tiron or NAC has no effect on EOMA cells

To eliminate the effect of the amitriptyline, Tiron or NAC on junctional proteins degradation in EOMA cells, we treated endothelial cells with 20 µM amitriptyline, 10 mM Tiron or 10 mM NAC for 140 min and stained the cells with the antibodies against ZO1, ZO2, Occludin, and E-cadherin. We found that amitriptyline, Tiron or NAC alone is without effect on TJs degradation in EOMA cells (**Figure 4.8.1.**).

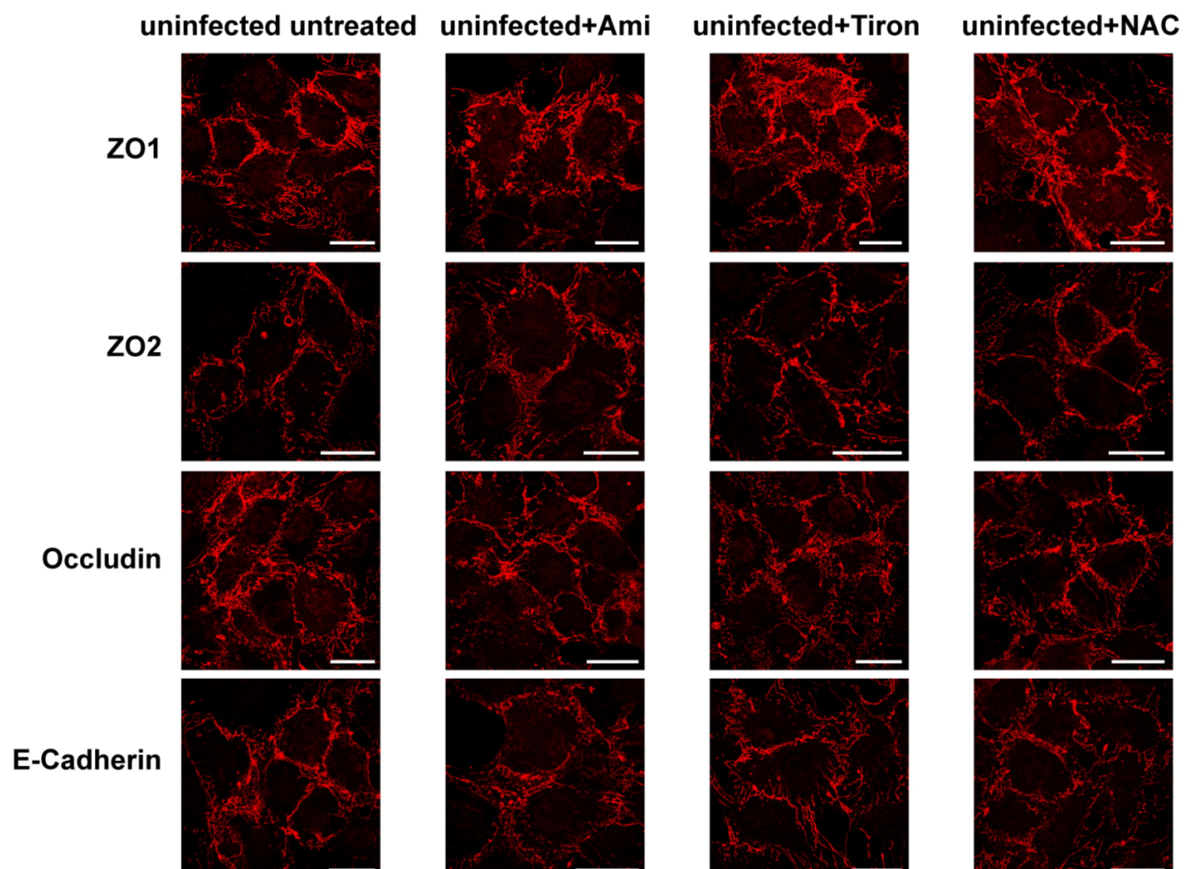


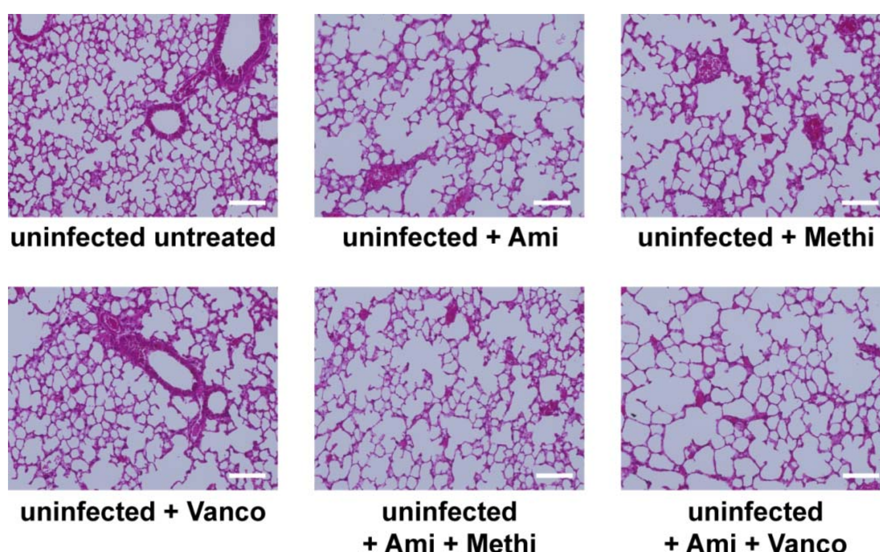
Figure 4.8.1. Effect of amitriptyline, Tiron or NAC on the distribution of ZO1, ZO2, Occludin and E-cadherin in EOMA cells

EOMA cells were treated for 140 min with amitriptyline (20 μM), Tiron (10 mM) or NAC (10 mM). Immunoflourescence stainings were performed with antibodies against ZO1, ZO2, Occludin, or E-cadherin for determination of the degradation of these tight junction proteins. The presented pictures are representative of the results of at least three independent experiments (magnification, 40×). Scale bar is 25 μM.

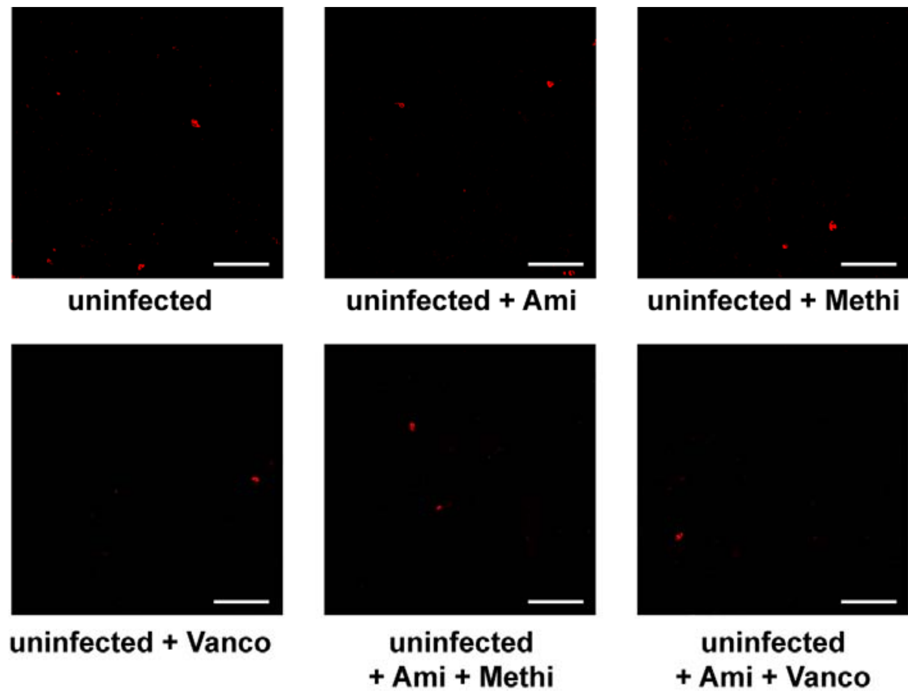
4.8.2. The drugs have no effect on lung parameters

To eliminate the effect of the drugs on lung parameters in uninfected mice, amitriptyline or methicillin or vancomycin or a combination of amitriptyline and either methicillin or vancomycin were injected. The injection of methicillin or vancomycin was repeated after 8 hrs. Control mice were left untreated. The mice were sacrificed 11 hrs after treatments. Lung sections were stained with Hematoxylin-Eosin (**Figure 4.8.2.A.**), anti-GR1 antibody (**Figure 4.8.2.B.**) and anti-ZO1 (**Figure 4.8.2.C.**), anti-ZO2 (**Figure 4.8.2.D.**), anti-Occludin (**Figure 4.8.2.E.**) or anti-E-cadherin (**Figure 4.8.2.F.**) antibodies. The stainings were analysed by confocal fluorescence microscopy. The studies confirmed that the drugs were without effect on lung parameters.

A



B



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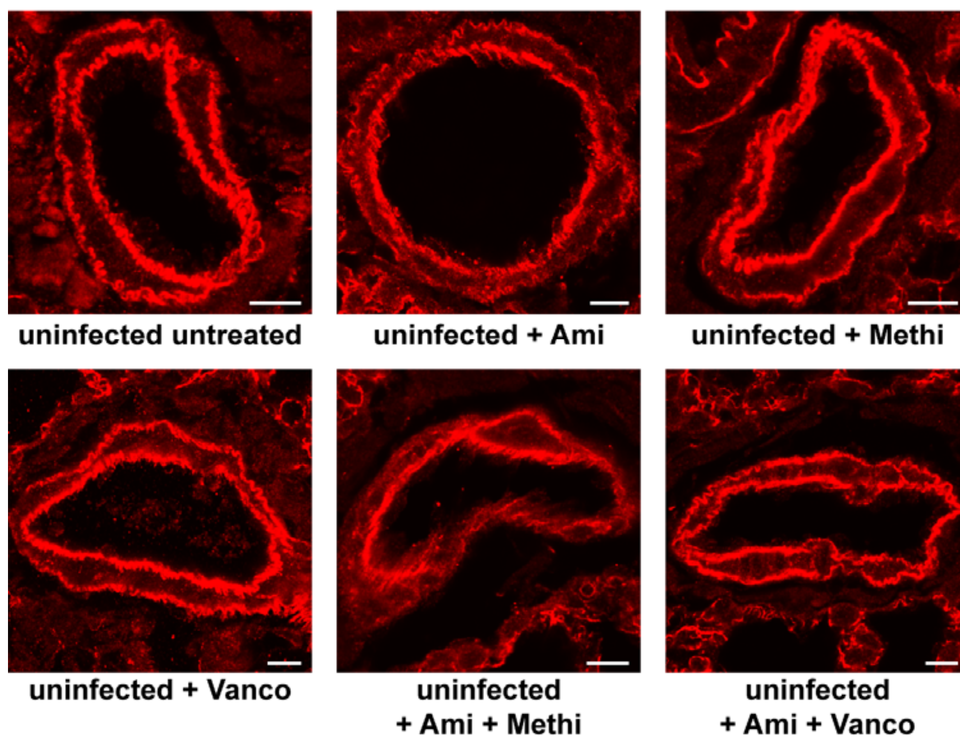

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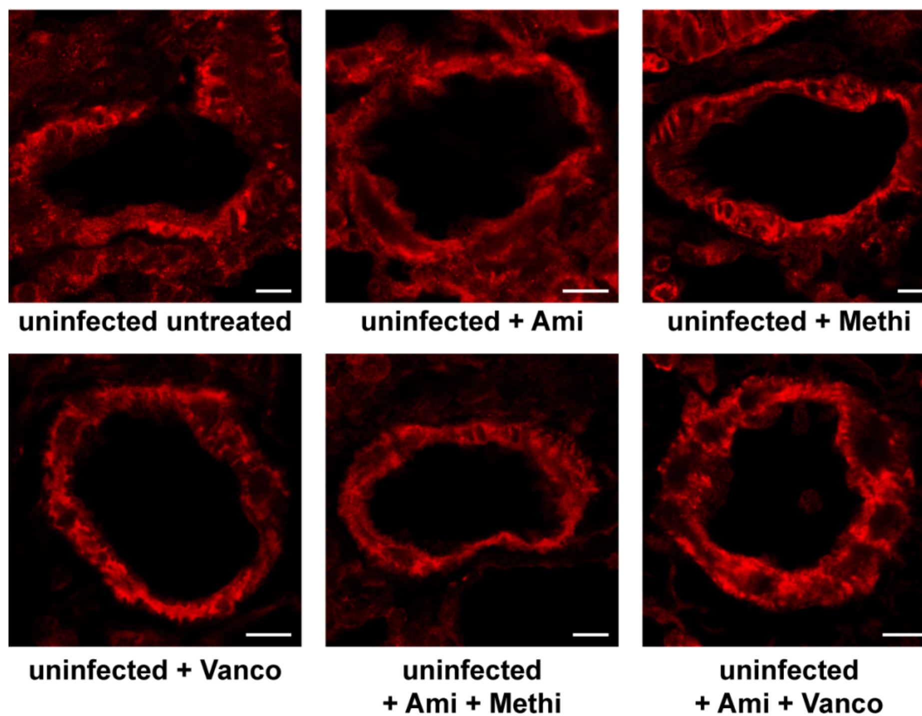
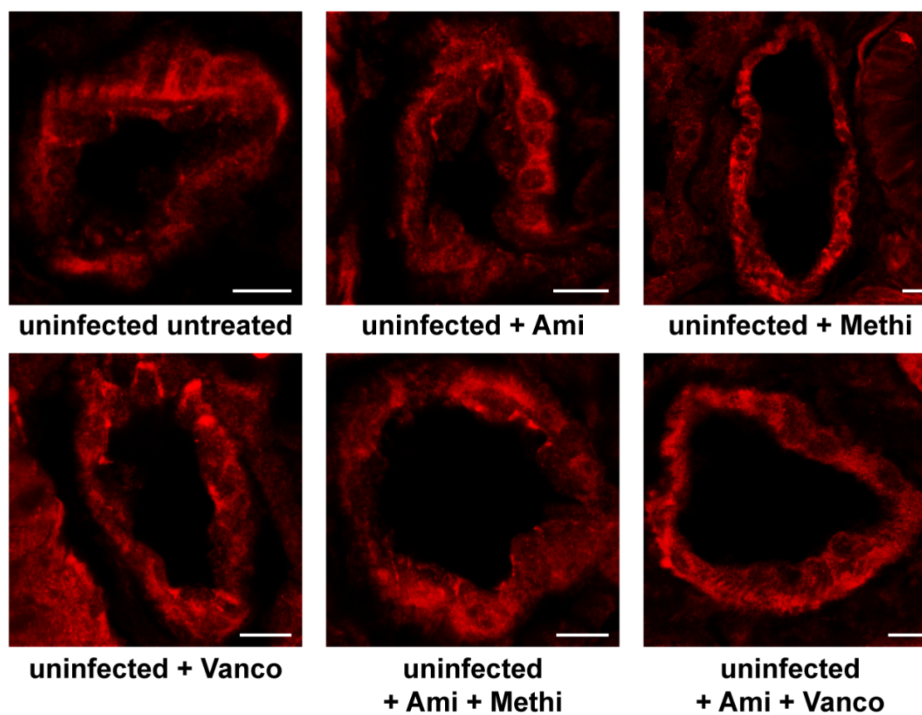


E



F

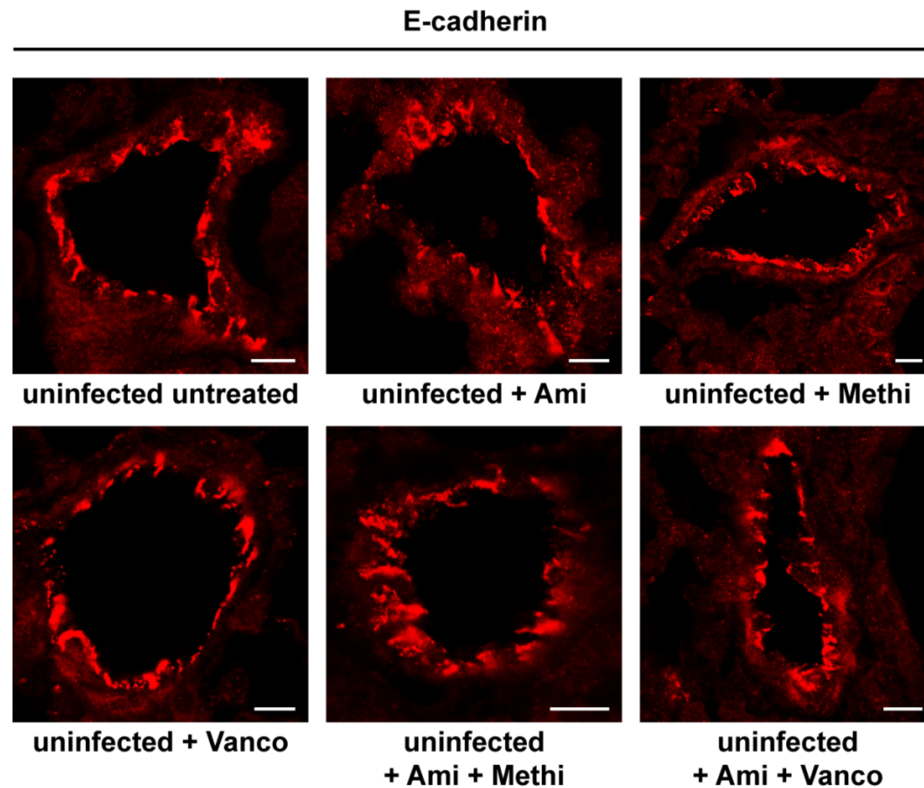


Figure 4.8.2. Effect of the drugs on lung parameters

Wild-type mice were treated with an i.p. injection of amitriptyline (16 mg/kg), either methicillin or vancomycin (both 100 mg/kg) or with a combination of amitriptyline and Methicillin or vancomycin. Injection of the antibiotics was repeated after 8 hrs. After 11 hrs treatments, four mice per group were sacrificed and the left lungs were removed, fixed, dehydrated and embedded in paraffin for sectioning at a thickness of 6 µm. The lung sections were stained with Hematoxylin-Eosin (A) (scale bar is 100 µM, magnification, 20×), Cy3-labeled anti-GR1 antibody (B) (scale bar is 50 µM, magnification, 63×), or Cy3-labeled anti-ZO1 (C), anti-ZO2 (D), anti-Occludin (E) or anti-E-cadherin (F) antibodies (scale bar is 10 µM, magnification, 40×). Representative fluorescence images from three independent experiments are shown.

5. DISCUSSION

5.1. Discussion of the Methods

5.1.1. Determination of Asm activity in cell lysates

Different methods are used to determine sphingomyelinase enzymatic activity, such as assays of pure enzyme and assays of cell extracts. In these assays the enzymatic activity of sphingomyelinase can be assayed by determining the conversion of sphingomyelin to ceramide and phosphorylcholine.

For assaying purified sphingomyelinase, natural or synthetic sphingomyelin is prepared, pure or mixed with other lipids, in the form of extruded large unilamellar vesicles (LUVs) approximately 100 nm in diameter (Richards et al 1986). When LUVs are assayed with sphingomyelinase, ceramide production in the bilayers leads to vesicle aggregation, which in turn produces an increase in turbidity or light scattering in the suspension. Thus the reaction can be followed in real time just by measuring the increase in turbidity (absorbance at 500 nm) or in light scattering (e.g. with a fluorometer with both the excitation and emission monochromators adjusted at 500nm).

For assaying sphingomyelinase activity in cell lysates, sphingomyelin need to be labeled, radioactively, fluorescently or otherwise. In the present study, the enzymatic activity was measured as the degradation of radioactive [^{14}C]-sphingomyelin to ceramide and [^{14}C]-phosphorylcholine. Because [^{14}C]-phosphorylcholine is soluble in water, it is easily separated from the substrate [^{14}C]-sphingomyelin and ceramide, which remains in the organic phase following extraction. Sphingomyelin can serve as substrate for three forms of sphingomyelinases that manifest acid, neutral or basic pH optima for maximal enzyme activity (Hannun 1996). In the present study Asm activity was discriminated from the neutral or basic sphingomyelinase activity by performing the assay at pH 5.0. Because of the difficulty in bringing together the enzyme and substrate molecules in the presence of cell homogenates, a suitable detergent, such as Triton X-100 was used. It should be noted that,

apart from emulsifying the substrate, the detergents bind and modify the enzyme activity, thus detergent concentration and initial detergent:substrate ratio was kept constant for reproducibility of assays. Finally, it has been suggested that the Asm may be located in detergent-resistant/insoluble fractions; thus, for determination of Asm activity in whole-cell lysates, no centrifugation step was performed after lysis and sonication of the cells to prevent pelleting and loss of the Asm.

5.1.2. Ceramide measurement by diacylglycerol (DAG) kinase assay

The crucial role of ceramide in numerous cellular processes and particularly stress responses, has led to the necessity of developing rapid and quantitative assays for ceramide determination. In the literature, there are several methods reported for quantifying ceramide such as normal phase high performance liquid chromatography (HPLC) analysis after derivatization with a fluorescent tag (Couch et al 1997, Iwamori et al 1979, Previati et al 1996, Yano et al 1998), evaporative lightscattering detection (McNabb et al 1999), high performance thin layer chromatography (HPTLC) analysis (Motta et al 1994), or using cells labeled with radioactive precursors (Tepper et al 2000). Ceramide molecular species can be determined following hydrolysis and analysis of the liberated and derivatized sphingoid bases by means of HPLC (Nishimura et al 1985, Smith et al 1995) and fatty acids by means of GC/MS (Samuelsson et al 1969). New methods for quantitative analysis of ceramide molecular species have been developed and are based on HPLC or reversed phase HPLC (RP-HPLC) separation of their fluorescent analogs prepared after derivatization with anthroyl cyanide (Yano et al 1998), benzoyl chloride (Couch et al 1997), or benzoic anhydride (Iwamori et al 1979). Moreover, mass spectrometry methodologies have been developed for the detection of molecular species of ceramide (Couch et al 1997, Gu et al 1997, Kalhorn et al 1999, Karlsson et al 1998, Liebisch et al 1999, Mano et al 1997, Watts et al 1999). However, most of these methods require long periods of processing and/or analysis.

The DAG kinase assay is a widely used method for the rapid quantification of ceramide. The primary advantages of the DAG kinase assay are the measurement of total mass levels of ceramide; the use of crude lipid extracts in the assay; and the ability of process a large number

of samples in a rapid manner. DAG kinase activity was originally reported by Hokin and Hokin (Hokin et al 1959). The enzyme was validated as an analytical tool in measuring diglyceride levels by the demonstration of a linear relationship between the amount of diglyceride added to an *in vitro* assay and the amount of product (phosphatidic acid) formed. Ceramides share structural similarities with diglycerides, and Schneider and Kennedy reported that bacterial DAG kinase can utilize ceramide as a substrate with a K_m nearly five times greater than that for diglyceride (Schneider et al 1973). Early attempts to use DAG kinase to quantify ceramide revealed a linear but non-quantitative relationship between substrate added and product formed. Further modification of the assay demonstrated that DAG kinase could also be used for quantitative conversion of ceramide to ceramide-1-phosphate over a range of 25 pmol to 2 nmol (Van Veldhoven et al 1995). These refinements have required special emphasis on the protocol of lipid extraction, purity of the reagents used for the preparation of the mixed micelles and on the development of high levels of recombinant DAG kinase.

The nonpolar properties of ceramide require that it be extracted from cells in organic solvents. Thus, the cells are lysed in a solution containing chloroform and methanol. Acidification of lysates with hydrochloric acid helps extraction of shorter acyl chain ceramide-1-phosphates or hydroxylated ceramides, thus being important to gain optimal usage of the exogenously added ceramides or internal standards. In the DAG kinase reaction, it is critical that the substrate is in a soluble form for optimal conversion to product by the enzyme. Mixed micelles containing a non-ionic detergent, such as n-octyl-β-glucopyranoside, and a phosholipid, such as cardiolipin, are utilized for this purpose. Of particular importance is the level of ceramide conversion to ceramide-1-phosphate. For the DAG kinase assay to yield reliable quantitative results, the reaction must go to completion with total conversion of DAG and ceramide. Otherwise, the results become sensitive to the effects of the efficiency of the reaction (K_m and V_{max} consideration of the DAG kinase) and to possible ‘competition’ between DAG and ceramide as substrates. In the present study, an excess of enzyme and ATP was used which

allowed linear and quantitative conversion and was sufficient for the phosphorylation of cellular ceramides as well as exogenously added ceramide.

5.1.3. Detection of lung edema by Evans Blue Dye

Evans Blue dye is widely used to study *in vitro* cellular permeability (Patterson et al 1992) and *in vivo* vascular leakage (Ferrero 2004). Historically, the dye was introduced for its utility in blood volume determinations by the dye dilution technique (Gibson et al 1937). Eventually, because the very high affinity of the dye for albumin was discovered (Freedman et al 1969, Rawson 1943), it began to be used as a surrogate marker for serum albumin flux across the luminal barrier in many *in vivo* experimental situations. Although subsequent investigations have revealed that the dye-albumin conjugate is not covalent in nature (Le et al 1947), it continues to be used today in situations where use of radioactively labeled albumin is not feasible or for histological examinations of injured tissue (Finck et al 1989, Moitra et al 2007, Saria et al 1983).

In the present study, pulmonary vascular permeability was estimated by using the Evans blue method. Evans blue dye, which strongly binds to albumin, is a well-known marker of protein extravasation in models of acute lung injury (Turnage et al 1995). A critical point to detect lung edema is to allow Evans blue dye to circulate for some time, which can ensure that albumin-bound Evans blue moves into the lung parenchyma through compromised vascular barrier in the lung. The major problem in detection of vascular leakage is the possibility of intravascular Evans blue dye residual. This would lead to an amplification of the optical density signal a false-positive result. To avoid this artifact, the lungs were perfused free of blood with phosphate-buffered saline via the pulmonary artery. Another crucial point is that total amount of dye should be calculated by means of a standard calibration curve. In the present study, results were expressed as µg/g of wet tissue.

5.1.4. Measurement of production of ROS by Electron Spin Resonance (ESR)

ROS formation and signaling are of major importance and regulate a number of processes in physiological conditions, which has led to the necessity of developing specific and sensitive assays for ROS determination. In the literature, there are several methods reported to allow measurement of generation and accumulation of different ROS, particularly H_2O_2, $O_2^{\cdot-}$, and NO, in cells and/or organisms, such as fluorescent ROS dye technologies (Chen et al 2010, Zielonka et al 2010), genetically encoded ROS reporters (Ostergaard et al 2001, Schwarzlander et al 2011), nanoparticle delivery systems (Koo et al 2007, Lee et al 2009), and nanotube ROS probes (Kim et al 2011, Leeuw et al 2007). However, there are two major problems associated with even the most recently developed ROS fluorescent probes that still persist: reversibility and reaction rate.

ESR was first observed in Kazan State University by Soviet physicist Yevgeny Zavoisky in 1944, and was developed independently at the same time by Brebis Bleaney at the University of Oxford. ESR spectroscopy is a technique for studying materials with unpaired electrons. The basic concepts of ESR are analogous to those of nuclear magnetic resonance (NMR), but it is electron spins that are excited instead of the spins of atomic nuclei. ESR is the most direct technique and an effective and unique way to detect free radicals in biological samples. ESR spectroscopy is particularly useful for studying organic radicals.

Although unpaired electrons of species such as NO, ·OH, or $O_2^{\cdot-}$ are too low in concentration and short-lived to be directly detected by ESR in biological systems, this dilemma can be circumvented by ESR measurement of more stable secondary radical species formed by adding exogenous spin-traps—molecules that react with primary radical species to give longer-lasting radical adducts with characteristic ESR signatures that can accumulate to levels permitting detection (Blodig et al 1999, Chen 2008). As we known, the concentration of ROS is very low and this would lead to the difficulty for detecting by ESR. To avoid this artifact, enough cells need to be prepared. Finally, since superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals ($O_2^{\cdot-} + O_2^{\cdot-}$ + 2H+ -- $O_2 + H_2O_2$) (McCord et al 1969). In the present study, SOD-inhibited fraction of the signal was used to calibrate the system.

5.1.5. Asm-deficient animals

To determine the crucial role of Asm in the present study, Asm-deficient mice were used. The Asm knock-out mouse was originally generated in the laboratory of Dr. E. Schuchman (Horinouchi et al 1995) and has been proven to be a valuable tool in investigating the role of Asm in different cellular processes. Unfortunately, a study from Nix and Stoffel (2000) reported marked biochemical alterations and membrane dysfunction in cells derived from their line of Asm knock-out mice such as: increase of sphingomyelin and gycosphingolipids in the plasma membrane of hepatocytes, reduction of caveolin levels in embryonic fibroblasts, reduced signalling through tyrosine kinases in T lymphocytes, lymphopenia, the absence of proliferation of T cells in response to anti-CD3, reduced expression of the anti-apoptotic adapter FLIP, and a paradoxical increase in apoptosis of anti-CD3 pre-treated splenocytes upon activation of CD95 (Nix et al 2000). Therefore, the authors concluded that the previously reported apoptotic abnormalities in Asm-deficient cells and tissues (Cifone et al 1995, Lin et al 2000, Morita et al 2000, Pena et al 2000, Perez et al 1997, Santana et al 1996, Zundel et al 1998, Zundel et al 2000) did not result merely from Asm deficiency, but rather were impacted by disruption of membrane microdomains in response to altered sphingolipid metabolism (Nix et al 2000).

However, Lozano and co-workers pointed out that the phenotype of the Asm-deficient mouse line used in the study of Nix and Stoffel was different from the mouse line generated in the laboratory of Dr. Schuchman, which displayed, up to a certain age (12-16 weeks), only a minimal increase in sphingomyelin content, unchanged levels of caveolin-1, normal MAP-kinase signalling and tyrosine phosphorylation patterns, no lymphopenia, normal T cells proliferation and no decrease in FLIP levels (Lozano et al 2001). Furthermore, the life expectancy of around 9-10 months (Pena et al 2000, Santana et al 1996) was in contrast with that of the mice generated by Stoffel and co-workers, who reported that the life span of their Asm-deficient mice was maximally 4 months, with mice succumbing to advanced Niemann-Pick disease type A(Otterbach et al 1995). The mice used in the present study show

the earliest clinical manifestation of Niemann-Pick disease type A between 12-16 weeks of age; therefore, all the Asm-deficient mice involving in our experiments were carried out with animals younger than 10 weeks of age, before any biochemical, histological or clinical manifestations of Niemann-Pick disease type A were apparent. This excluded that the effects observed in the Asm-deficient cells were due to altered cellular processes (as described above) but instead, were completely dependent on the lack of Asm.

5.2. Discussion of the Results

5.2.1. Role of the Asm/ceramide system in redox signaling

Our findings indicate that infecting endothelial cells with *S. aureus* activates Asm and thereby triggers the release of ceramide. Asm leads to the production of ROS by endothelial cells. Asm plays a critical role in *S. aureus*-triggered ROS release. *S. aureus* induces ROS accumulation in endothelial cells. These events were all absent in amitriptyline-treated mice that have a reduced Asm activity or in mice lacking the Asm genetically. Consistently, Reinehr and co-workers demonstrated that inhibiting Asm blocks the release of ROS, a finding suggesting that ROS functions downstream of Asm in hepatocytes (Reinehr et al 2006). Similarly, inhibiting Asm attenuates the ceramide and ROS production induced by histone deacetylase/perifosin (Rahmani et al 2005), fenretinide (Lovat et al 2004), sodium nitroprusside (Sanvicens et al 2006), or *P. aeruginosa* (Manago et al 2015, Zhang et al 2007, Zhang et al 2008). Taken together, these data confirmed the notion that Asm activation and ceramide production are upstream signals of ROS production.

NADPH oxidases (Nox), which are localized to various cellular membranes, are classically known as important ROS producers (Geiszt et al 2000, Lambeth 2004, Suh et al 1999). Ceramide induces the activation of ROS-generating enzymes, including NADPH oxidase, xanthine oxidase, NO synthase, and the mitochondrial respiratory chain (Corda et al 2001, Lecour et al 2006). In particular, ceramide has been shown to activate NADPH oxidase and to increase the production of ROS in a variety of mammalian cells, including human aortic smooth muscle cells (Bhunia et al 1997), endothelial cells (Zhang et al 2007), macrophages

(Zhang et al 2008) and erythrocytes (LaRocca et al 2014). The precise mechanism that how ceramide activates NADPH oxidase is not well understood. Because many stimuli activate NADPH oxidase by translocation and aggregation, it has been proposed that ceramide mediates the fusion of small raft domains to ceramide-enriched membrane platforms, which facilitate the aggregation of subunits of NADPH oxidase and enhances interactions between subunits of NADPH oxidase, thereby stimulating the production of ROS (**Figure 5.2.1.**) (Boini et al 2010, Jin et al 2008, Li et al 2007, Zhang et al 2006). Yi and colleagues demonstrated that ceramide in platforms may also directly enhance NADPH oxidase activity by activating small G protein Rac1/2 by activation of guanine nucleotide exchange factors (GEFs) such as Vav2 (**Figure 5.2.1.**) (Li et al 2007, Yi et al 2007).

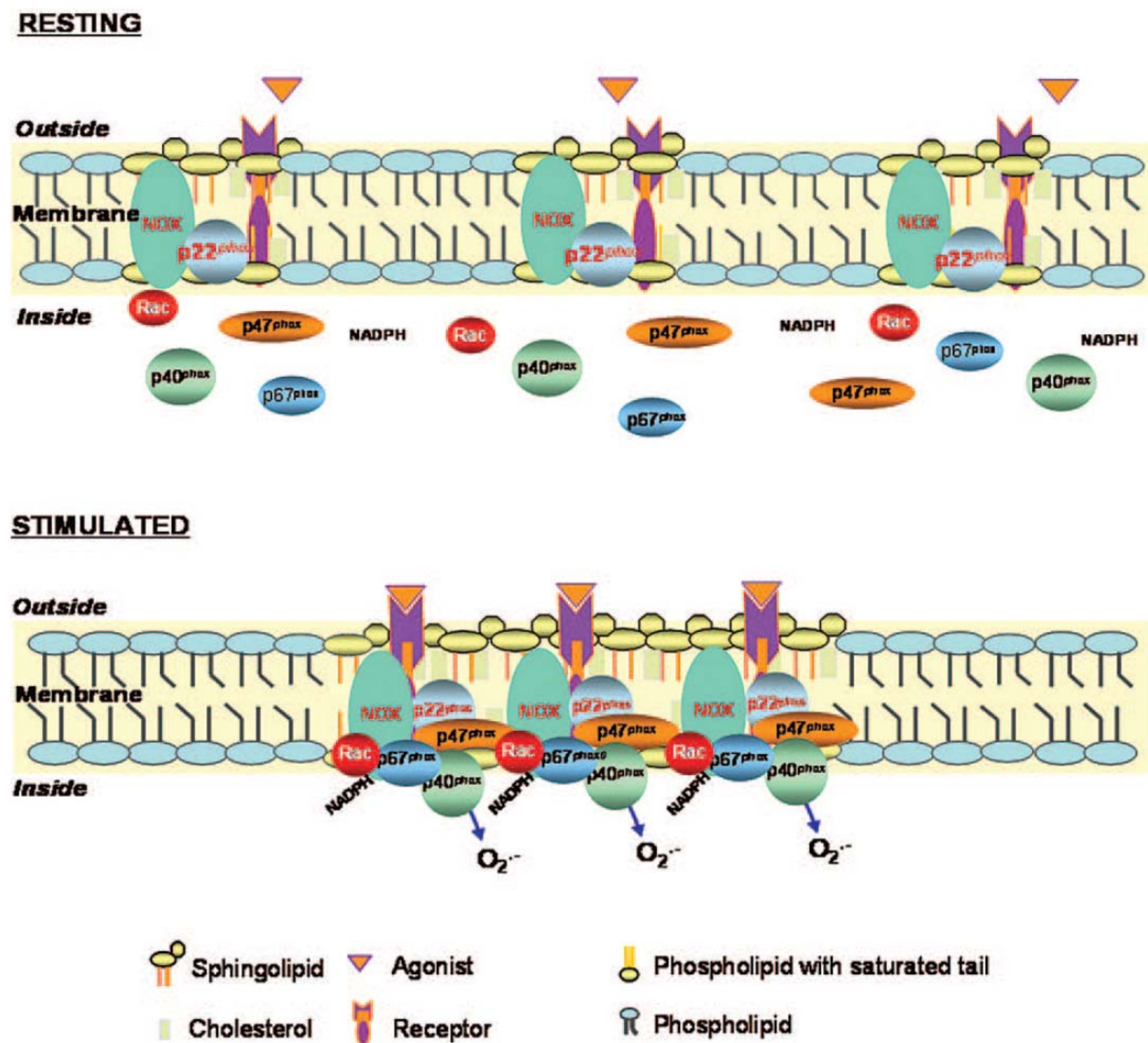


Figure 5.2.1. A hypothetic model showing lipid rafts (LRs) and LRs clustering to form a redox signaling platform

Under resting condition, individual LRs with attached receptors are present in the membrane of ECs (panel for resting cells). These individual LRs are dynamic microdomains and carry several membrane-bound or attached proteins or enzymes such as G-proteins, protein kinases, or the subunits of NADPH oxidase $gp91^{phox}$ and $p47^{phox}$. When ligands or agonists bind to their receptors on individual LRs, the clustering is activated to form a number of LR macrodomains or platforms with aggregation or recruitment of receptors, NADPH oxidase subunits, and other proteins such as Rac GTPase. Clustering of these proteins and enzymes leads to activation of NADPH oxidase and production of $O_2^{\cdot-}$, which results in a prominent amplification of the transmembrane signal (panel for stimulated cells) (Li et al 2007).

5.2.2. Role of ROS in Asm activation

Our findings demonstrated that pretreating endothelial cells with the antioxidants N-acetylcysteine (NAC) and Tiron substantially inhibited Asm activation and the signaling events downstream of Asm activation, such as junctional proteins degradation induced by systemic *S. aureus* infection, thus indicating that ROS release is required for Asm activation. Moreover, several recent studies have indicated that the generation of ROS may be involved in the activation of Asm in response to various stimuli (Charruyer et al 2005, Dumitru et al 2006, Scheel-Toellner et al 2004), which seems to conflict with the above conclusion that Asm activation results in ROS production. This conundrum is discussed below and an amplifying concept is presented.

Scheel-Toellner and colleagues demonstrated that Asm activation, ceramide generation, and CD95 clustering play a crucial role in the spontaneous apoptosis of neutrophils; apoptosis was substantially delayed in Asm-deficient mice (Scheel-Toellner et al 2004). These events were pretreated by antioxidants indicating the dependence of the Asm on ROS. In accordance, pretreatment with the antioxidant pyrrolidine dithiocarbamate (PDTC) abolished Asm activation by ultraviolet (UV)-C light in U937 cells, a finding suggesting that ROS functions downstream of Asm (Charruyer et al 2005).

In addition, Dumitru and colleagues also demonstrated the involvement of ROS in TNF-related apoptosis-inducing ligand (TRAIL)-induced activation of Asm and apoptosis (Dumitru et al 2006). Stimulation with TRAIL/DR5 led to activation of Asm and the

subsequent formation of ceramide-enriched membrane platforms, DR5 clustering, and consequent apoptosis. Pretreatment with antioxidants NAC and Tiron substantially inhibited TRAIL-induced Asm activation, ceramide/DR5 clustering, and apoptosis, demonstrating that ROS play a crucial role in TRAIL-induced Asm/ceramic activation (Dumitru et al 2006). Finally, studies investigating the cellular effects of Cu^{2+} showed that Cu^{2+} also promotes the ROS-dependent activation of Asm and leads to the death of hepatocytes (Lang et al 2007). It was shown that the accumulation of Cu^{2+}, as occurred in Wilson disease, activates Asm in hepatocytes and triggers the release of ceramide in these cells. This process leads to Cu^{2+}-induced hepatocyte death, which can be prevented by a deficiency in Asm (Lang et al 2007).

In summary, these studies demonstrate that Asm activity is regulated by ROS upon systemic *S. aureus* infection, but the enzyme also stimulates ROS release. Therefore, we proposed a positive feed-back loop and a vicious cycle of–induced Asm activation and ROS release. In this amplification model, initial activation of Asm results in ROS production, which further enhance activation of Asm thus forms a feed-forward loop for ROS production. This finding is similar to the results of previous studies showing a positive feedback loop between the Asm and ROS after infection of macrophages with *P. aeruginasa* (Zhang et al 2008).

5.2.3. Role of the Asm/ceramide system and ROS for lung edema induced by systemic *S. aureus* infection

The results of the present study demonstrate that genetic deficiency or pharmacologic inhibition of the Asm/ceramide system in mice protects against lung edema induced by systemic *S. aureus* infection. The Asm/ceramide system triggered the formation of ROS, resulting in degradation of tight junction proteins and neutrophil trafficking, followed by lung edema. Pretreatment of mice or treatment of already infected mice with amitriptyline, a potent functional inhibitor of Asm, both protected mice from lung edem caused by systemic *S. aureus* infection. All the data presented above indicate that Asm/ceramide is a novel key molecule for the induction of lung edema by systemic *S. aureus* infection. Furthermore, pretreatment of mice with the antioxidants N-acetylcysteine (NAC) and Tiron substantially

inhibited the events downstream of Asm activation, such as junctional proteins degradation, neutrophil trafficking and lung edema, thus indicating that ROS release is also required for lung edema induced by systemic *S. aureus* infection.

It has been speculated that membrane rafts (MRs) and ROS may constitute an amplification system of redox signals and ceramide signaling cell membranes, which insures the efficiency of signal transduction (Li et al 2013a). The formation of such feed forward amplifying loop for MR redox and ceramide signaling may also be responsible for the temporospatial regulation of a complex signalosome that precisely and efficiently control cell function. If the activity of this regulatory loop is excessively enhanced, excessive production of both ROS and ceramide may result in the progress and development of different diseases or pathological processes (Li et al 2013a).

With respect to the role of these domains for the regulation of vascular endothelial functions, it has been described by numerous studies. Zhang and colleagues demonstrated that transfection of Asm siRNA markedly attenuated CD95 ligand in isolated small bovine coronary arteries, and induced inhibition of endothelium-dependent vasorelaxation (a response to bradykinin) by 60 % (Zhang et al 2007). The results suggest that Asm, the release of ceramide, and MR-derived ceramide-enriched membrane platforms are involved in the activation of NADPH oxidase in response to cytokines in coronary ECs, consequently leading to endothelial dysfunction (Jin et al 2007, Zhang et al 2006, Zhang et al 2007).

Moreover, the MR redox signaling platform associated with NADPH oxidase has been demonstrated to be responsible for endothelial dysfunction induced by various stimuli such as death receptor activation, homocysteine, cytokines, or adipokines (Jin et al 2008, Xia et al 2011, Zhang et al 2006). As a commonly used functional study, endothelium-dependent vasodilation (EDVD) response in isolated perfused arteries was tested. It was found that various stimulations which led to the formation of MR redox signaling platforms such as CD95 ligand, endostatin, homocysteine, and visfatin all led to impairment of EDVD. This

impairment was homeostatically recovered by NADPH oxidase inhibition using apocynin, or Asm siRNA, suggesting that MR redox signaling platforms with NADPH oxidase participate in the impairment of endothelial function (Jin et al 2008, Zhang et al 2007).

In addition, platelet-activating factor (PAF), lipopolysaccharide (LPS) or acid instillation treatment induces Asm-dependent production of ceramide and results in pulmonary edema and has a key role in ALI. Agents that interfere with PAF-induced ceramide release, such as steroids or the xanthogenate D609, attenuate pulmonary edema formation induced by PAF, endotoxin or acid instillation. These results identify Asm and ceramide as possible therapeutic targets in acute lung injury (Goggel et al 2004).

During sepsis, ALI results from activation of innate immune cells and endothelial cells by endotoxins, leading to systemic inflammation through pro-inflammatory cytokine overproduction, oxidative stress, and intracellular Ca^{2+} overload. Recent data by Gandhirajan and co-workers indicate that ROS-driven Ca^{2+} signaling promotes vascular barrier dysfunction and induce pulmonary edema (Gandhirajan et al 2013).

All the experiments confirmed the notion that feed forward amplifying loop of Asm/ceramic and ROS is involved in systemic *S. aureus*–induced pulmonary edema.

5.2.4. Role of junctional proteins degradation for lung edema induced by systemic *S. aureus* infection

As we known endothelial barrier dysfunction occurs during stimulation by inflammatory agents, pathogens, activated blood cells, or disease states. The pathophysiology is characterized by excessive flux of plasma across the exchange micro-vessel wall into the surrounding tissues. Traditionally, compromised endothelial cell–cell junctional integrity is considered to account for the leak response.

A principal hallmark of lung edema is degradation of junctional proteins, which induces the disruption of endothelial cell barrier, followed by excessive flux, neutrophil trafficking and

consequently lung edema. Clinicians have long recognized the problem of vascular leak but had no tools to reverse it.

Out data presented here indicate that systemic *S. aureus* infection induces lung edema via degradation of junctional proteins. Genetic deficiency of the Asm/ceramide system in mice protects against junctional proteins degradation and reduces lung edema induced by systemic *S. aureus* infection. Furthermore, pre-treatment of the mice or treatment of already infected mice with amitriptyline both protected mice from junctional proteins degradation and reduces lung edema. Likewise, pretreatment of mice with the antioxidants N-acetylcysteine (NAC) and Tiron substantially inhibited junctional proteins degradation and reduces lung edema.

The experiments further suggest, on one hand, the feed forward amplifying loop for Asm/ceramide and ROS is required for degradation of junctional proteins induced by systemic *S. aureus* infection; on the other hand, junctional proteins degradation is responsible for lung edema induced by systemic *S. aureus* infection. This concept is consistent with findings that superoxide can directly down-regulate TJ proteins and indirectly activate matrix metalloproteinases (MMPs) that contribute to disrupt the integrity of endothelial cell layers (Gu et al 2011). Moreover, superoxide directly activates several inflammatory cytokines, which in turn activate MMPs (Abdul-Muneer et al 2015, Gu et al 2011, Rochfort et al 2014). Furthermore, in some pathological conditions, ROS induce the degradation of tight junctional proteins, following by vascular leakage and neutrophil trafficking with consequent pulmonary edema (Catanzaro et al 2015, Naik et al 2014).

All the data presented above indicate that antidepressant amitriptyline and antioxidants N-acetylcysteine (NAC) and Tiron might be very useful for future therapies for lung edema induced by degradation of junctional proteins. At the same time, the development of agents or mediators that reinforce intercellular junctional proteins should be a goal of drug research.

5.2.5. Role of neutrophil recruitment for lung edema induced by systemic *S. aureus* infection

Lung edema, endothelial and epithelial injury are accompanied by an influx of neutrophils into the interstitium and broncheoalveolar space. Neutrophils are considered to play a key role in the progression of ALI/ARDS since activation and transmigration of neutrophils is a hallmark event in the progression of ALI/ARDS (Abraham 2003).

The importance of neutrophils in ALI/ARDS is affirmed by clinical data and animal models. In patients with ARDS, the concentration of neutrophils in the bronchoalveolar lavage (BAL) fluid correlates with severity of ARDS and outcome (Matthay et al 1984, Parsons et al 1985, Steinberg et al 1994). Furthermore, depletion of neutrophil in mice reduces the severity of lung injury (Abraham et al 2000). Interestingly, blocking interleukin-8 (IL-8), a major chemoattractant for neutrophils, protects rabbits from acid aspiration-induced lung injury (Folkesson et al 1995). Further to emigration, neutrophils are irreplaceable in bacterial clearance, much of which is mediated by phagocytosis and intracellular bacterial killing (Soehnlein 2009). Even so, ALI/ARDS can occur in children and adults with neutropenia (Laufe et al 1986, Ognibene et al 1986, Sivan et al 1990), which indicates that neutrophil-independent mechanisms alone allow for development of ALI/ARDS under specific conditions. Despite that, a multitude of experimental and clinical data point at the causative role of neutrophils in lung injury.

The present study indicates that neutrophil trafficking plays a key role in lung edema induced by systemic *S. aureus* infection. Genetic deficiency of the Asm/ceramide system in mice reduces neutrophils trafficking, consequently protects the mice against lung edema induced by systemic *S. aureus* infection. Furthermore, pre-treatment of the mice or treatment of already infected mice with amitriptyline both protected mice from neutrophil trafficking and reduces lung edema. Furthermore, pretreatment of mice with the antioxidants N-acetylcysteine (NAC) and Tiron substantially inhibited neutrophils trafficking and reduces lung edema.

The experiments further suggest, on one hand, the feed forward amplifying loop for Asm/ceramide and ROS is required for neutrophil trafficking induced by systemic *S. aureus* infection; on the other hand, neutrophil trafficking is involved in lung edema induced by systemic *S. aureus* infection. Consistently, it has recently been shown that recruitment of neutrophils is a key event in development of ALI, linking to plasma leakage and deterioration of oxygenation (Grommes et al 2011, Ware et al 2000). LPS inhalation mimics human Gram-negative ALI, inducing neutrophil recruitment, pulmonary edema and finally impairment of gas exchange (Matute-Bello et al 2008). Moreover, the importance of neutrophils in ALI is supported by studies where lung injury is abolished or reversed by depletion of neutrophils (Looney et al 2006, Soehnlein et al 2008).

Hence, antidepressant amitriptyline and antioxidants N-acetylcysteine (NAC) and Tiron might be very useful for future therapies for lung edema induced by neutrophils trafficking. At the same time, the development of agents or mediators that decrease neutrophils recruitment should be a goal of drug research.

5.2.6. Clinical significance of combination of amitriptyline and antibiotic

S. aureus is a leading cause of septic infections, and *S. aureus*–induced sepsis is one of the most serious infections acquired in hospitals or in the community. However, even with the use of appropriate antibiotics, fatal lung edema often develops (Cortes Garcia et al 2012, Moore et al 2011, Moore et al 2012). Despite many clinical trials, no FDA-approved drug is available for use in sepsis, a lack that underscores the importance of future sepsis research. Interestingly, our studies with wt and Asm-deficient mice seem to mimic the situation in hospitals showing a high lethality in septic *S. aureus* infections even if adequately treated with antibiotics (Cortes Garcia et al 2012, Moore et al 2011, Moore et al 2012).

Our findings demonstrate that treating mice with amitriptyline 1 or 2 h after infection reduces *S. aureus*–induced pulmonary edema and also inhibits myeloid cell trafficking and the degradation of junctional proteins. The reduced capacity of mice treated with amitriptyline or Asm-deficient mice to kill *S. aureus* is consistent with the previous notion that myeloid cells

lacking Asm are unable to cluster and activate NADPH oxidases resulting in a defect of the production of superoxide and a reduced killing of pathogens. Finally, amitriptyline-treated or Asm-deficient mice died by the inability to eliminate the bacteria. In contrast, antibiotics kill the bacteria, but did not reduce lung edema. Thus, the combination of amitriptyline and antibiotics combines the advantages of inhibiting lung edema and eliminating systemic bacteria, protecting mice from lethality.

It is very interesting that Goggel and co-workers demonstrate that steroids or the xanthogenate D609, which can interfere with PAF-induced ceramide synthesis, inhibit pulmonary edema formation induced by platelet-activating factor (PAF), lipopolysaccharide (LPS) or acid instillation (Goggel et al 2004). These results indicate that Asm/ceramide signaling plays an important role in pulmonary edema induced by PAF instillation.

However, the molecular mechanisms how the Asm/ceramide system induces lung edema is largely unknown. According to previous studies, eNOS/eNO might be involved in this role. It has been reported that pharmacological inhibition of the Asm pathway with imipramine, D609 or dexamethasone blocks the PAF-induced increase of caveolin-1 and eNOS in caveolae, and the decreases in eNO production and edema formation in rat lung (Yang et al 2010). These results suggest that inhibition of eNOS/eNO signaling decreases PAF-induced lung edema.

More recently, Samapati and colleagues indicate PAF increases lung edema and endothelial Ca^{2+}. This response is abrogated by inhibitors of Asm or in Asm-deficient mice, and replicates by lung perfusion with exogenous Asm or C_2-ceramide. Further experiments demonstrate that PAF increases the caveolar abundance of TRPC6 channels via Asm activation, subsequently induces increases in lung endothelial Ca^{2+}, vascular filtration coefficient, and edema formation , which were attenuated by the TRPC inhibitor SKF96365 and in TRPC6-deficient mice, whereas direct activation of TRPC6 replicated the Ca^{2+} and edema response to PAF. The exogenous NO donor PapaNONOate or the cyclic guanosine

3′,5′-monophosphate analog 8Br-cGMP blocked the endothelial Ca^{2+} and permeability response to PAF (Samapati et al 2012).

According to our data, the Asm/ceramide system induces lung edema by junctional proteins degradation and neutrophil trafficking, which is a novel viewpoint to explain how the Asm/ceramide system induces lung edema upon systemic *S. aureus* infection.

5.2.7. Possible additional roles of Asm as activator of ADAM 10

S. aureus expresses multiple toxins, such as alpha-toxin, Panton-Valentine leukocidin, and enterotoxin B and A, which cause membrane damage, infiltration of myeloid cells and macrophages, cytokine production, and increased vascular permeability resulting in severe pulmonary edema and lung injury (Bhakdi et al 1984, Diep et al 2010, Mattix et al 1995, Menoret et al 2012).

Pore-forming cytotoxins (PFTs) are a family of bacterial virulence factors that cause eukaryotic cell injury and death (Gonzalez et al 2008). *S. aureus* encodes multiple PFTs, the best-studied being α-hemolysin (Hla), which is expressed by almost all strains (Tomita et al 1997). Hla is involved in the pathogenesis of skin infections, pneumonia, corneal infection and toxic shock syndrome (Brosnahan et al 2009, Bubeck Wardenburg et al 2007a, Bubeck Wardenburg et al 2007b, Kennedy et al 2010, O'Callaghan et al 1997), including those caused by MRSA.

Both of the *S. aureus* strains used in our study produce several hemolysins. It is possible that *S. aureus* toxins mediate the activation of the Asm observed in the present study after infection of endothelial cells with *S. aureus*. However, at present, it is unknown whether purified alpha-toxin activates the Asm in human and mouse monocytic cells. Recently, it was reported that the binding of Hla to its eukaryotic receptor A-disintegrin and metalloprotease 10 (ADAM10) leads to the up-regulation of ADAM10 activity, which is required for Hla-induced cytotoxicity (Inoshima et al 2011, Powers et al 2012, Wilke et al 2010).

Increased ADAM10 activity in epithelial and endothelial cells and following signaling cascades disrupts the cell barrier function, and this disruption contributes to the pathogenesis of lethal lung edema. However, it is unknown whether the Asm and ADAM10 function in the same signaling cascade or are independent pathways that are both required for the cellular effects of alpha-toxin.

5.2.8. Possible additional roles of Asm as activator of the Nalp3 inflammasome

Microorganisms that invade the cytosol can be recognized by cytoplasmatic pattern-recognition receptors (PRRs), most notably the nucleotide-binding and oligomerization domain, leucine-rich repeat (also known as NOD-like receptors, both abbreviated to NLR) (Kawai et al 2010, Schroder et al 2010). NLRs, which detect microbial components in the cytosol and trigger the assembly of large caspase-1-activating complexes termed inflammasomes, are further subcategorized based on differences in the N-terminal domains (Schroder et al 2010, Stutz et al 2009). Amongst the various inflammasomes, the NALP3 inflammasome is particularly qualified to response to various activators, leading to caspase activation. NLRP3 inflammasome activation induces caspase-1 activation, which causes the processing of the pro-inflammatory cytokines IL-1β and IL-18 and triggers the inflammatory stress (Schroder et al 2010, Stutz et al 2009). IL-1 β is an early cytokine in ALI patients and induces alveolar permeability and causes production of other cytokines such as IL-6 and TNF-a (Ganter et al 2008).

S. aureus is a potent activator of the inflammasome in macrophages resulting in upregulation of caspase-1 upon its interaction with Asc and NALP3 (Mariathasan et al 2006). Moreover, α-hemolysin activates the NALP3-inflammasome during *S. aureus* pneumonia, inducing necrotic pulmonary injury. Moreover, Nalp3$^{-/-}$ mice have less-severe pneumonia (Kebaier et al 2012). In addition, purified α-hemolysin activates the NALP3-inflammasome, ultimately leading to the secretion of the pro-inflammatory cytokines IL-1β and IL-18 and the resultant tissue necrosis and inflammation (Craven et al 2009).

Recently, it was reported that the inflammasome protein complex activates caspase-1 to promote the processing and secretion of IL-1β, which is responsible for alveolar epithelial permeability. In addition, it is shown that inflammasome inhibition blocks hyperoxia-induced alveolar permeability and cytokine production (Kolliputi et al 2010). Later, Kolliputi and colleagues further reveal that ceramide causes NALP3-inflammasome activation, induction of caspase-1, IL-1β cleavage, release of pro-inflammatory cytokines, and ultimately alveolar epithelial permeability. Short-hairpin RNA silencing of NALP3-inflammasome components abrogated ceramide-induced secretion of pro-inflammatory cytokines and abolished ceramide-induced alveolar epithelial permeability in *in vitro* (Kolliputi et al 2012). However, the role of inflammasome in relation to *S. aureus*-induced Asm/ceramide activation and inflammatory cytokine production leading to endothelial permeability remains unknown.

5.2.9. Significances and perspectives

In the present study, we demonstrated that the combination of amitriptyline and antibiotics effectively protects mice from lung edema and bacteremia during sepsis. Amitriptyline is a well-known antidepressant that has been widely used in clinical practice for more than 50 years and is associated with only mild adverse effects at therapeutic doses. Thus, the major significance of the research work in this dissertation is to indicate that inhibition of the Asm/ceramide system in combination with antibiotics could be a novel approach to treat severe systemic and often lethal infections and to inhibit lung injury in patients with incipient sepsis. The results presented in this dissertation increases our understanding of the signaling mechanisms responsible for the acute ROS response in endothelial cells during host-pathogen interaction and link the function of Asm with ceramide redox signaling. The notion that Asm deficiency or antioxidants prevent *S. aureus*-induced Asm/ceramide activation, ROS release, junctional proteins degradation, neutrophil trafficking and lung edema, which may contribute to the understanding how systemic *S. aureus* infections induce lung edema. This may direct the development of new therapeutic strategy for treatment of this disease.

6. SUMMARY

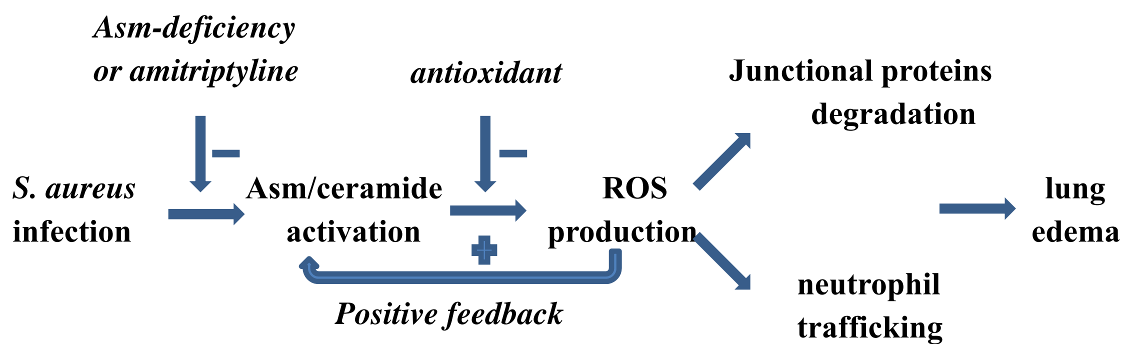
Pulmonary edema associated with increased vascular permeability is a severe complication of *S. aureus*–induced sepsis and an important cause of human pathology and death. Antibiotics alone are often insufficient to cure patients with *S. aureus*–induced sepsis. Although treatment with effective doses of bactericidal antibiotics indeed prevents the bacterial burden, antibiotics often fail to prevent fatal lung edema after septic infection with *S. aureus*.

The present study investigated the role of the Asm/ceramide system in the development of lung edema caused by *S. aureus*. Furthermore, the present study identified signaling mechanisms responsible for lung edema caused by *S. aureus.* Most importantly, the present study identifies a novel approach to patients with *S. aureus*-induced sepsis with bactericidal antibiotics applied in combination with amitriptyline. The major findings are:

- *S. aureus* rapidly activates Asm and induces ceramide release in the plasma membranes.
- The Asm/ceramide system mediates *S. aureus*-induced ROS production.
- The Asm/ceramide system and ROS form a positive feedback loop.
- Asm and ceramide activation are crucial for *S. aureus*-induced lung edema.
- ROS is essential for *S. aureus*-induced lung edema.
- The Asm/ceramide system and ROS activation lead to junctional proteins degradation.
- The Asm/ceramide system and ROS activation lead to neutrophil trafficking.
- Both junctional proteins degradation and neutrophil trafficking contribute to lung edema induced by *S. aureus* infections.
- Genetic deficiency or pharmacologic inhibition of the Asm/ceramide system in mice protects against lung edema induced by systemic *S. aureus* infection.

- Pretreating endothelial cells with the antioxidants N-acetylcysteine (NAC) or Tiron substantially inhibited Asm activation and the signaling events downstream of Asm activation.
- The combination of antibiotics and amitriptyline reduced both pulmonary edema and bacteremia protecting mice from lethal sepsis and lung dysfunction.

Therefore, the proposed signaling pathway is depicted as bellow:



7. REFERENCES

1 Abais J.M., Xia M., Li G., Gehr T.W., Boini K.M., Li P.L. (2014). Contribution of endogenously produced reactive oxygen species to the activation of podocyte NLRP3 inflammasomes in hyperhomocysteinemia. Free radical biology & medicine 67: 211-220.

2 Abbott N.J., Patabendige A.A., Dolman D.E., Yusof S.R., Begley D.J. (2010). Structure and function of the blood-brain barrier. Neurobiology of disease 37: 13-25.

3 Abdul-Muneer P.M., Chandra N., Haorah J. (2015). Interactions of oxidative stress and neurovascular inflammation in the pathogenesis of traumatic brain injury. Molecular neurobiology 51: 966-979.

4 Abraham E., Baughman R., Fletcher E., Heard S., Lamberti J., Levy H. et al (1999). Liposomal prostaglandin E1 (TLC C-53) in acute respiratory distress syndrome: a controlled, randomized, double-blind, multicenter clinical trial. TLC C-53 ARDS Study Group. Critical care medicine 27: 1478-1485.

5 Abraham E., Carmody A., Shenkar R., Arcaroli J. (2000). Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. American journal of physiology Lung cellular and molecular physiology 279: L1137-1145.

6 Abraham E. (2003). Neutrophils and acute lung injury. Critical care medicine 31: S195-199.

7 Abraham E., Laterre P.F., Garg R., Levy H., Talwar D., Trzaskoma B.L. et al (2005). Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. The New England journal of medicine 353: 1332-1341.

8 Abraham E.P.a.E.C. (1940). An enzyme from bacteria able to destroy penicillin. . Nature 146: 837-842.

9 Adams J.M., Hauser C.J., Livingston D.H., Lavery R.F., Fekete Z., Deitch E.A. (2001). Early trauma polymorphonuclear neutrophil responses to chemokines are associated with development of sepsis, pneumonia, and organ failure. The Journal of trauma 51: 452-456; discussion 456-457.

10 Adhikari N., Burns K.E., Meade M.O. (2004). Pharmacologic therapies for adults with acute lung injury and acute respiratory distress syndrome. The Cochrane database of systematic reviews: CD004477.

11 Aird W.C. (2003). The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101: 3765-3777.

12 Aires de Sousa M., de Lencastre H. (2004). Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant Staphylococcus aureus clones. FEMS immunology and medical microbiology 40: 101-111.

13 Ait-Oufella H., Maury E., Lehoux S., Guidet B., Offenstadt G. (2010). The endothelium: physiological functions and role in microcirculatory failure during severe sepsis. Intensive care medicine 36: 1286-1298.

14 Alberti C., Brun-Buisson C., Burchardi H., Martin C., Goodman S., Artigas A. et al (2002). Epidemiology of sepsis and infection in ICU patients from an international multicentre cohort study. Intensive care medicine 28: 108-121.

15 Alcaide P., Newton G., Auerbach S., Sehrawat S., Mayadas T.N., Golan D.E. et al (2008). p120-Catenin regulates leukocyte transmigration through an effect on VE-cadherin phosphorylation. Blood 112: 2770-2779.

16 Anas A.A., Wiersinga W.J., de Vos A.F., van der Poll T. (2010). Recent insights into the pathogenesis of bacterial sepsis. The Netherlands journal of medicine 68: 147-152.

17 Anderson B.O., Bensard D.D., Harken A.H. (1991). The role of platelet activating factor and its antagonists in shock, sepsis and multiple organ failure. Surgery, gynecology & obstetrics 172: 415-424.

18 Anderson E.J., Efird J.T., Davies S.W., O'Neal W.T., Darden T.M., Thayne K.A. et al (2014). Monoamine oxidase is a major determinant of redox balance in human atrial myocardium and is associated with postoperative atrial fibrillation. Journal of the American Heart Association 3: e000713.

19 Angus D.C., Linde-Zwirble W.T., Lidicker J., Clermont G., Carcillo J., Pinsky M.R. (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Critical care medicine 29: 1303-1310.

20 Ashbaugh D.G., Bigelow D.B., Petty T.L., Levine B.E. (1967). Acute respiratory distress in adults. Lancet 2: 319-323.

21 Azevedo L.C., Janiszewski M., Soriano F.G., Laurindo F.R. (2006). Redox mechanisms of vascular cell dysfunction in sepsis. Endocrine, metabolic & immune disorders drug targets 6: 159-164.

22 Azevedo L.C., Janiszewski M., Pontieri V., Pedro Mde A., Bassi E., Tucci P.J. et al (2007). Platelet-derived exosomes from septic shock patients induce myocardial dysfunction. Critical care 11: R120.

23 Bachofen M., Weibel E.R. (1977). Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. The American review of respiratory disease 116: 589-615.

24 Bao J.X., Jin S., Zhang F., Wang Z.C., Li N., Li P.L. (2010). Activation of membrane NADPH oxidase associated with lysosome-targeted acid sphingomyelinase in coronary endothelial cells. Antioxidants & redox signaling 12: 703-712.

25 Barbui C., Hotopf M. (2001). Amitriptyline v. the rest: still the leading antidepressant after 40 years of randomised controlled trials. The British journal of psychiatry : the journal of mental science 178: 129-144.

26 Barenholz Y., Thompson T.E. (1980). Sphingomyelins in bilayers and biological membranes. Biochimica et biophysica acta 604: 129-158.

27 Becker K.A., Grassmé H., Zhang Y., Gulbins E. (2010a). Ceramide in Pseudomonas aeruginosa infections and cystic fibrosis. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 26: 57-66.

28 Becker K.A., Riethmuller J., Luth A., Doring G., Kleuser B., Gulbins E. (2010b). Acid sphingomyelinase inhibitors normalize pulmonary ceramide and inflammation in

cystic fibrosis. American journal of respiratory cell and molecular biology 42: 716-724.

29 Becker K.A., Henry B., Ziobro R., Tummler B., Gulbins E., Grassmé H. (2012). Role of CD95 in pulmonary inflammation and infection in cystic fibrosis. Journal of molecular medicine 90: 1011-1023.

30 Bernard G.R., Luce J.M., Sprung C.L., Rinaldo J.E., Tate R.M., Sibbald W.J. et al (1987). High-dose corticosteroids in patients with the adult respiratory distress syndrome. The New England journal of medicine 317: 1565-1570.

31 Bernard G.R., Artigas A., Brigham K.L., Carlet J., Falke K., Hudson L. et al (1994). The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. American journal of respiratory and critical care medicine 149: 818-824.

32 Bernard G.R., Wheeler A.P., Arons M.M., Morris P.E., Paz H.L., Russell J.A. et al (1997). A trial of antioxidants N-acetylcysteine and procysteine in ARDS. The Antioxidant in ARDS Study Group. Chest 112: 164-172.

33 Bernard G.R., Vincent J.L., Laterre P.F., LaRosa S.P., Dhainaut J.F., Lopez-Rodriguez A. et al (2001). Efficacy and safety of recombinant human activated protein C for severe sepsis. The New England journal of medicine 344: 699-709.

34 Bhakdi S., Suttorp N., Seeger W., Fussle R., Tranum-Jensen J. (1984). [Molecular basis for the pathogenicity of S. aureus alpha-toxins]. Immunitat und Infektion 12: 279-285.

35 Bhunia A.K., Han H., Snowden A., Chatterjee S. (1997). Redox-regulated signaling by lactosylceramide in the proliferation of human aortic smooth muscle cells. The Journal of biological chemistry 272: 15642-15649.

36 Blanco J., Muriel-Bombin A., Sagredo V., Taboada F., Gandia F., Tamayo L. et al (2008). Incidence, organ dysfunction and mortality in severe sepsis: a Spanish multicentre study. Critical care 12: R158.

37 Blodig W., Smith A.T., Winterhalter K., Piontek K. (1999). Evidence from spin-trapping for a transient radical on tryptophan residue 171 of lignin peroxidase. Archives of biochemistry and biophysics 370: 86-92.

38 Bock J., Szabo I., Gamper N., Adams C., Gulbins E. (2003). Ceramide inhibits the potassium channel Kv1.3 by the formation of membrane platforms. Biochemical and biophysical research communications 305: 890-897.

39 Boini K.M., Zhang C., Xia M., Han W.Q., Brimson C., Poklis J.L. et al (2010). Visfatin-induced lipid raft redox signaling platforms and dysfunction in glomerular endothelial cells. Biochimica et biophysica acta 1801: 1294-1304.

40 Bone R.C., Fisher C.J., Jr., Clemmer T.P., Slotman G.J., Metz C.A., Balk R.A. (1989). Sepsis syndrome: a valid clinical entity. Methylprednisolone Severe Sepsis Study Group. Critical care medicine 17: 389-393.

41 Boos C.J., Goon P.K., Lip G.Y. (2006). The endothelium, inflammation, and coagulation in sepsis. Clinical pharmacology and therapeutics 79: 20-22.

42 Boyce J.M., Cookson B., Christiansen K., Hori S., Vuopio-Varkila J., Kocagoz S. et al (2005). Meticillin-resistant Staphylococcus aureus. The Lancet infectious diseases 5: 653-663.

43 Briel M., Meade M., Mercat A., Brower R.G., Talmor D., Walter S.D. et al (2010). Higher vs lower positive end-expiratory pressure in patients with acute lung injury and acute respiratory distress syndrome: systematic review and meta-analysis. JAMA : the journal of the American Medical Association 303: 865-873.

44 Brosnahan A.J., Mantz M.J., Squier C.A., Peterson M.L., Schlievert P.M. (2009). Cytolysins augment superantigen penetration of stratified mucosa. Journal of immunology 182: 2364-2373.

45 Brown D.A., London E. (1998). Functions of lipid rafts in biological membranes. Annual review of cell and developmental biology 14: 111-136.

46 Brugger B., Glass B., Haberkant P., Leibrecht I., Wieland F.T., Krausslich H.G. (2006). The HIV lipidome: a raft with an unusual composition. Proceedings of the National Academy of Sciences of the United States of America 103: 2641-2646.

47 Brun-Buisson C., Minelli C., Bertolini G., Brazzi L., Pimentel J., Lewandowski K. et al (2004). Epidemiology and outcome of acute lung injury in European intensive care units. Results from the ALIVE study. Intensive care medicine 30: 51-61.

48 Bubeck Wardenburg J., Bae T., Otto M., Deleo F.R., Schneewind O. (2007a). Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nature medicine 13: 1405-1406.

49 Bubeck Wardenburg J., Patel R.J., Schneewind O. (2007b). Surface proteins and exotoxins are required for the pathogenesis of Staphylococcus aureus pneumonia. Infection and immunity 75: 1040-1044.

50 Burgoyne J.R., Mongue-Din H., Eaton P., Shah A.M. (2012). Redox signaling in cardiac physiology and pathology. Circulation research 111: 1091-1106.

51 Butcher E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67: 1033-1036.

52 Calderon R.O., DeVries G.H. (1997). Lipid composition and phospholipid asymmetry of membranes from a Schwann cell line. Journal of neuroscience research 49: 372-380.

53 Catanzaro D., Rancan S., Orso G., Dall'Acqua S., Brun P., Giron M.C. et al (2015). Boswellia serrata Preserves Intestinal Epithelial Barrier from Oxidative and Inflammatory Damage. PloS one 10: e0125375.

54 Cavanagh S.P., Gough M.J., Homer-Vanniasinkam S. (1998). The role of the neutrophil in ischaemia-reperfusion injury: potential therapeutic interventions. Cardiovascular surgery 6: 112-118.

55 Chalfant C.E., Szulc Z., Roddy P., Bielawska A., Hannun Y.A. (2004). The structural requirements for ceramide activation of serine-threonine protein phosphatases. Journal of lipid research 45: 496-506.

56 Chance B., Sies H., Boveris A. (1979). Hydroperoxide metabolism in mammalian organs. Physiological reviews 59: 527-605.

57 Charruyer A., Grazide S., Bezombes C., Muller S., Laurent G., Jaffrezou J.P. (2005). UV-C light induces raft-associated acid sphingomyelinase and JNK activation and translocation independently on a nuclear signal. The Journal of biological chemistry 280: 19196-19204.

58 Chen X., Zhong Z., Xu Z., Chen L., Wang Y. (2010). 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. Free radical research 44: 587-604.

59 Chen Y.R. (2008). EPR spin-trapping and nano LC MS/MS techniques for DEPMPO/OOH and immunospin-trapping with anti-DMPO antibody in mitochondrial electron transfer system. Methods in molecular biology 477: 75-88.

60 Chongtrakool P., Ito T., Ma X.X., Kondo Y., Trakulsomboon S., Tiensasitorn C. et al (2006). Staphylococcal cassette chromosome mec (SCCmec) typing of methicillin-resistant Staphylococcus aureus strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCCmec elements. Antimicrobial agents and chemotherapy 50: 1001-1012.

61 Cifone M.G., Roncaioli P., De Maria R., Camarda G., Santoni A., Ruberti G. et al (1995). Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. The EMBO journal 14: 5859-5868.

62 Cohen J. (2002). The immunopathogenesis of sepsis. Nature 420: 885-891.

63 Corada M., Mariotti M., Thurston G., Smith K., Kunkel R., Brockhaus M. et al (1999). Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proceedings of the National Academy of Sciences of the United States of America 96: 9815-9820.

64 Corda S., Laplace C., Vicaut E., Duranteau J. (2001). Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-alpha is mediated by ceramide. American journal of respiratory cell and molecular biology 24: 762-768.

65 Cortes Garcia M., Sierra Moros M.J., Santa-Olalla Peralta P., Hernandez-Barrera V., Jimenez-Garcia R., Pachon I. (2012). Clinical characteristics and outcomes of diabetic patients who were hospitalised with 2009 pandemic influenza A H1N1 infection. The Journal of infection 64: 218-224.

66 Couch L.H., Churchwell M.I., Doerge D.R., Tolleson W.H., Howard P.C. (1997). Identification of ceramides in human cells using liquid chromatography with detection by atmospheric pressure chemical ionization-mass spectrometry. Rapid communications in mass spectrometry : RCM 11: 504-512.

67 Courvalin P. (2006). Vancomycin resistance in gram-positive cocci. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42 Suppl 1: S25-34.

68 Craven R.R., Gao X., Allen I.C., Gris D., Bubeck Wardenburg J., McElvania-Tekippe E. et al (2009). Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PloS one 4: e7446.

69 Creamer E., Galvin S., Dolan A., Sherlock O., Dimitrov B.D., Fitzgerald-Hughes D. et al (2012). Evaluation of screening risk and nonrisk patients for methicillin-resistant Staphylococcus aureus on admission in an acute care hospital. American journal of infection control 40: 411-415.

70 Cremesti A., Paris F., Grassmé H., Holler N., Tschopp J., Fuks Z. et al (2001). Ceramide enables fas to cap and kill. The Journal of biological chemistry 276: 23954-23961.

71 Dellinger R.P., Levy M.M., Carlet J.M., Bion J., Parker M.M., Jaeschke R. et al (2008). Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. Critical care medicine 36: 296-327.

72 Deurenberg R.H., Stobberingh E.E. (2008). The evolution of Staphylococcus aureus. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 8: 747-763.

73 Devasagayam T.P., Tilak J.C., Boloor K.K., Sane K.S., Ghaskadbi S.S., Lele R.D. (2004). Free radicals and antioxidants in human health: current status and future prospects. The Journal of the Association of Physicians of India 52: 794-804.

74 Diep B.A., Chan L., Tattevin P., Kajikawa O., Martin T.R., Basuino L. et al (2010). Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. Proceedings of the National Academy of Sciences of the United States of America 107: 5587-5592.

75 Djaldetti M., Salman H., Bergman M., Djaldetti R., Bessler H. (2002). Phagocytosis--the mighty weapon of the silent warriors. Microscopy research and technique 57: 421-431.

76 Dobrowsky R.T., Hannun Y.A. (1993). Ceramide-activated protein phosphatase: partial purification and relationship to protein phosphatase 2A. Advances in lipid research 25: 91-104.

77 Dombrovskiy V.Y., Martin A.A., Sunderram J., Paz H.L. (2007). Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. Critical care medicine 35: 1244-1250.

78 Dumitru C.A., Gulbins E. (2006). TRAIL activates acid sphingomyelinase via a redox mechanism and releases ceramide to trigger apoptosis. Oncogene 25: 5612-5625.

79 Eggeling C., Ringemann C., Medda R., Schwarzmann G., Sandhoff K., Polyakova S. et al (2009). Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature 457: 1159-1162.

80 El Atrouni W.I., Knoll B.M., Lahr B.D., Eckel-Passow J.E., Sia I.G., Baddour L.M. (2009). Temporal trends in the incidence of Staphylococcus aureus bacteremia in Olmsted County, Minnesota, 1998 to 2005: a population-based study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 49: e130-138.

81 Emmelot P., Van Hoeven R.P. (1975). Phospholipid unsaturation and plasma membrane organization. Chemistry and physics of lipids 14: 236-246.

82 Enright M.C., Day N.P., Davies C.E., Peacock S.J., Spratt B.G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. Journal of clinical microbiology 38: 1008-1015.

83 Enright M.C. (2006). Genome of an epidemic community-acquired MRSA. Lancet 367: 705-706.

84 Eriksen K.R. (1961). ["Celbenin"-resistant staphylococci]. Ugeskrift for laeger 123: 384-386.

85 Esen M., Schreiner B., Jendrossek V., Lang F., Fassbender K., Grassmé H. et al (2001). Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells. Apoptosis : an international journal on programmed cell death 6: 431-439.

86 Falcone S., Perrotta C., De Palma C., Pisconti A., Sciorati C., Capobianco A. et al (2004). Activation of acid sphingomyelinase and its inhibition by the nitric oxide/cyclic guanosine 3',5'-monophosphate pathway: key events in Escherichia coli-elicited apoptosis of dendritic cells. Journal of immunology 173: 4452-4463.

87 Ferrero M.E. (2004). In vivo vascular leakage assay. Methods in molecular medicine 98: 191-198.

88 Finck S.J., Mashburn J.P., Kottke B.A., Orszulak T.A. (1989). Evaluation of arterialized vein graft permeability with Evans blue dye and iodine 125-labeled albumin. The Annals of thoracic surgery 48: 646-650.

89 Fleming A. (1929). On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenzae. Br J Exp Pathol 10: 226-236.

90 Folkesson H.G., Matthay M.A., Hebert C.A., Broaddus V.C. (1995). Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanisms. The Journal of clinical investigation 96: 107-116.

91 Freedman F.B., Johnson J.A. (1969). Equilibrium and kinetic properties of the Evans blue-albumin system. The American journal of physiology 216: 675-681.

92 Frenay H.M., Bunschoten A.E., Schouls L.M., van Leeuwen W.J., Vandenbroucke-Grauls C.M., Verhoef J. et al (1996). Molecular typing of methicillin-resistant Staphylococcus aureus on the basis of protein A gene polymorphism. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 15: 60-64.

93 Futerman A.H., Stieger B., Hubbard A.L., Pagano R.E. (1990). Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. The Journal of biological chemistry 265: 8650-8657.

94 Gandhirajan R.K., Meng S., Chandramoorthy H.C., Mallilankaraman K., Mancarella S., Gao H. et al (2013). Blockade of NOX2 and STIM1 signaling limits lipopolysaccharide-induced vascular inflammation. The Journal of clinical investigation 123: 887-902.

95 Ganter M.T., Roux J., Miyazawa B., Howard M., Frank J.A., Su G. et al (2008). Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. Circulation research 102: 804-812.

96 Gao Smith F., Perkins G.D., Gates S., Young D., McAuley D.F., Tunnicliffe W. et al (2012). Effect of intravenous beta-2 agonist treatment on clinical outcomes in acute respiratory distress syndrome (BALTI-2): a multicentre, randomised controlled trial. Lancet 379: 229-235.

97 Garattini S., Barbui C., Saraceno B. (1998). Antidepressant agents: from tricyclics to serotonin uptake inhibitors. Psychological medicine 28: 1169-1178.

98 Geerts W.H., Bergqvist D., Pineo G.F., Heit J.A., Samama C.M., Lassen M.R. et al (2008). Prevention of venous thromboembolism: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133: 381S-453S.

99 Geiszt M., Kopp J.B., Varnai P., Leto T.L. (2000). Identification of renox, an NAD(P)H oxidase in kidney. Proceedings of the National Academy of Sciences of the United States of America 97: 8010-8014.

100 Gibson J.G., Evans W.A. (1937). Clinical Studies of the Blood Volume. I. Clinical Application of a Method Employing the Azo Dye "Evans Blue" and the Spectrophotometer. The Journal of clinical investigation 16: 301-316.

101 Goggel R., Winoto-Morbach S., Vielhaber G., Imai Y., Lindner K., Brade L. et al (2004). PAF-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide. Nature medicine 10: 155-160.

102 Gonzalez M.R., Bischofberger M., Pernot L., van der Goot F.G., Freche B. (2008). Bacterial pore-forming toxins: the (w)hole story? Cellular and molecular life sciences : CMLS 65: 493-507.

103 Gorwitz R.J., Kruszon-Moran D., McAllister S.K., McQuillan G., McDougal L.K., Fosheim G.E. et al (2008). Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. The Journal of infectious diseases 197: 1226-1234.

104 Grassmé H., Gulbins E., Brenner B., Ferlinz K., Sandhoff K., Harzer K. et al (1997). Acidic sphingomyelinase mediates entry of N. gonorrhoeae into nonphagocytic cells. Cell 91: 605-615.

105 Grassmé H., Kirschnek S., Riethmueller J., Riehle A., von Kurthy G., Lang F. et al (2000). CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. Science 290: 527-530.

106 Grassmé H., Jekle A., Riehle A., Schwarz H., Berger J., Sandhoff K. et al (2001a). CD95 signaling via ceramide-rich membrane rafts. The Journal of biological chemistry 276: 20589-20596.

107 Grassmé H., Schwarz H., Gulbins E. (2001b). Molecular mechanisms of ceramide-mediated CD95 clustering. Biochemical and biophysical research communications 284: 1016-1030.

108 Grassmé H., Bock J., Kun J., Gulbins E. (2002a). Clustering of CD40 ligand is required to form a functional contact with CD40. The Journal of biological chemistry 277: 30289-30299.

109 Grassmé H., Jendrossek V., Bock J., Riehle A., Gulbins E. (2002b). Ceramide-rich membrane rafts mediate CD40 clustering. Journal of immunology 168: 298-307.

110 Grassmé H., Cremesti A., Kolesnick R., Gulbins E. (2003a). Ceramide-mediated clustering is required for CD95-DISC formation. Oncogene 22: 5457-5470.

111 Grassmé H., Jendrossek V., Riehle A., von Kurthy G., Berger J., Schwarz H. et al (2003b). Host defense against Pseudomonas aeruginosa requires ceramide-rich membrane rafts. Nature medicine 9: 322-330.

112 Grassmé H., Riehle A., Wilker B., Gulbins E. (2005). Rhinoviruses infect human epithelial cells via ceramide-enriched membrane platforms. The Journal of biological chemistry 280: 26256-26262.
113 Grassmé H., Riethmuller J., Gulbins E. (2007). Biological aspects of ceramide-enriched membrane domains. Progress in lipid research 46: 161-170.
114 Grassmé H., Becker K.A., Zhang Y., Gulbins E. (2008). Ceramide in bacterial infections and cystic fibrosis. Biological chemistry 389: 1371-1379.
115 Grommes J., Soehnlein O. (2011). Contribution of neutrophils to acute lung injury. Molecular medicine 17: 293-307.
116 Gu M., Kerwin J.L., Watts J.D., Aebersold R. (1997). Ceramide profiling of complex lipid mixtures by electrospray ionization mass spectrometry. Analytical biochemistry 244: 347-356.
117 Gu Y., Dee C.M., Shen J. (2011). Interaction of free radicals, matrix metalloproteinases and caveolin-1 impacts blood-brain barrier permeability. Frontiers in bioscience 3: 1216-1231.
118 Gulbins E., Szabo I., Baltzer K., Lang F. (1997). Ceramide-induced inhibition of T lymphocyte voltage-gated potassium channel is mediated by tyrosine kinases. Proceedings of the National Academy of Sciences of the United States of America 94: 7661-7666.
119 Gulbins E., Kolesnick R. (2003). Raft ceramide in molecular medicine. Oncogene 22: 7070-7077.
120 Guo R.F., Riedemann N.C., Laudes I.J., Sarma V.J., Kunkel R.G., Dilley K.A. et al (2002). Altered neutrophil trafficking during sepsis. Journal of immunology 169: 307-314.
121 Hakomori S. (1983). *Chemistry of glycosphingolipids*. Plenum Press: New York.
122 Hampton M.B., Kettle A.J., Winterbourn C.C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92: 3007-3017.
123 Hannun Y.A. (1996). Functions of ceramide in coordinating cellular responses to stress. Science 274: 1855-1859.
124 Hannun Y.A., Luberto C. (2000). Ceramide in the eukaryotic stress response. Trends in cell biology 10: 73-80.
125 Hannun Y.A., Obeid L.M. (2002). The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. The Journal of biological chemistry 277: 25847-25850.
126 Hannun Y.A., Obeid L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nature reviews Molecular cell biology 9: 139-150.
127 Hatanaka Y., Fujii J., Fukutomi T., Watanabe T., Che W., Sanada Y. et al (1998). Reactive oxygen species enhances the induction of inducible nitric oxide synthase by sphingomyelinase in RAW264.7 cells. Biochimica et biophysica acta 1393: 203-210.
128 Hauck C.R., Grassmé H., Bock J., Jendrossek V., Ferlinz K., Meyer T.F. et al (2000). Acid sphingomyelinase is involved in CEACAM receptor-mediated phagocytosis of Neisseria gonorrhoeae. FEBS letters 478: 260-266.
129 Hawkins B.T., Davis T.P. (2005). The blood-brain barrier/neurovascular unit in health and disease. Pharmacological reviews 57: 173-185.

130 Hayward A., Knott F., Petersen I., Livermore D.M., Duckworth G., Islam A. et al (2008). Increasing hospitalizations and general practice prescriptions for community-onset staphylococcal disease, England. Emerging infectious diseases 14: 720-726.

131 Heinrich M., Wickel M., Schneider-Brachert W., Sandberg C., Gahr J., Schwandner R. et al (1999). Cathepsin D targeted by acid sphingomyelinase-derived ceramide. The EMBO journal 18: 5252-5263.

132 Herridge M.S., Angus D.C. (2005). Acute lung injury--affecting many lives. The New England journal of medicine 353: 1736-1738.

133 Herz J., Pardo J., Kashkar H., Schramm M., Kuzmenkina E., Bos E. et al (2009). Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes. Nature immunology 10: 761-768.

134 Hocke A.C., Temmesfeld-Wollbrueck B., Schmeck B., Berger K., Frisch E.M., Witzenrath M. et al (2006). Perturbation of endothelial junction proteins by Staphylococcus aureus alpha-toxin: inhibition of endothelial gap formation by adrenomedullin. Histochemistry and cell biology 126: 305-316.

135 Hokin L.E., Hokin M.R. (1959). Diglyceride phosphokinase: an enzyme which catalyzes the synthesis of phosphatidic acid. Biochimica et biophysica acta 31: 285-287.

136 Holopainen J.M., Subramanian M., Kinnunen P.K. (1998). Sphingomyelinase induces lipid microdomain formation in a fluid phosphatidylcholine/sphingomyelin membrane. Biochemistry 37: 17562-17570.

137 Horinouchi K., Erlich S., Perl D.P., Ferlinz K., Bisgaier C.L., Sandhoff K. et al (1995). Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. Nature genetics 10: 288-293.

138 Huang H.W., Goldberg E.M., Zidovetzki R. (1999). Ceramides modulate protein kinase C activity and perturb the structure of Phosphatidylcholine/Phosphatidylserine bilayers. Biophysical journal 77: 1489-1497.

139 Huwiler A., Johansen B., Skarstad A., Pfeilschifter J. (2001). Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15: 7-9.

140 Inoshima I., Inoshima N., Wilke G.A., Powers M.E., Frank K.M., Wang Y. et al (2011). A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nature medicine 17: 1310-1314.

141 Ira, Johnston L.J. (2008). Sphingomyelinase generation of ceramide promotes clustering of nanoscale domains in supported bilayer membranes. Biochimica et biophysica acta 1778: 185-197.

142 Ishibashi Y., Nakasone T., Kiyohara M., Horibata Y., Sakaguchi K., Hijikata A. et al (2007). A novel endoglycoceramidase hydrolyzes oligogalactosylceramides to produce galactooligosaccharides and ceramides. The Journal of biological chemistry 282: 11386-11396.

143 Ito T., Katayama Y., Asada K., Mori N., Tsutsumimoto K., Tiensasitorn C. et al (2001). Structural comparison of three types of staphylococcal cassette chromosome

mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. Antimicrobial agents and chemotherapy 45: 1323-1336.

144 Iwamori M., Costello C., Moser H.W. (1979). Analysis and quantitation of free ceramide containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine by high-performance liquid chromatography. Journal of lipid research 20: 86-96.

145 Janeway C.A., Jr., Medzhitov R. (1998). Introduction: the role of innate immunity in the adaptive immune response. Seminars in immunology 10: 349-350.

146 Jang A.S., Concel V.J., Bein K., Brant K.A., Liu S., Pope-Varsalona H. et al (2011). Endothelial dysfunction and claudin 5 regulation during acrolein-induced lung injury. American journal of respiratory cell and molecular biology 44: 483-490.

147 Jeckel D., Karrenbauer A., Birk R., Schmidt R.R., Wieland F. (1990). Sphingomyelin is synthesized in the cis Golgi. FEBS letters 261: 155-157.

148 Jin S., Yi F., Li P.L. (2007). Contribution of lysosomal vesicles to the formation of lipid raft redox signaling platforms in endothelial cells. Antioxidants & redox signaling 9: 1417-1426.

149 Jin S., Zhang Y., Yi F., Li P.L. (2008). Critical role of lipid raft redox signaling platforms in endostatin-induced coronary endothelial dysfunction. Arteriosclerosis, thrombosis, and vascular biology 28: 485-490.

150 Jones D.P. (2006). Redefining oxidative stress. Antioxidants & redox signaling 8: 1865-1879.

151 Kahl B.C., Mellmann A., Deiwick S., Peters G., Harmsen D. (2005). Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in Staphylococcus aureus. Journal of clinical microbiology 43: 502-505.

152 Kalhorn T., Zager R.A. (1999). Renal cortical ceramide patterns during ischemic and toxic injury: assessments by HPLC-mass spectrometry. The American journal of physiology 277: F723-733.

153 Kaludercic N., Mialet-Perez J., Paolocci N., Parini A., Di Lisa F. (2014). Monoamine oxidases as sources of oxidants in the heart. Journal of molecular and cellular cardiology 73: 34-42.

154 Kanafani Z.A., Kourany W.M., Fowler V.G., Jr., Levine D.P., Vigliani G.A., Campion M. et al (2009). Clinical characteristics and outcomes of diabetic patients with Staphylococcus aureus bacteremia and endocarditis. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 28: 1477-1482.

155 Kannan S., Audet A., Knittel J., Mullegama S., Gao G.F., Wu M. (2006). Src kinase Lyn is crucial for Pseudomonas aeruginosa internalization into lung cells. European journal of immunology 36: 1739-1752.

156 Kannan S., Audet A., Huang H., Chen L.J., Wu M. (2008). Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during Pseudomonas aeruginosa infection. Journal of immunology 180: 2396-2408.

157 Karlsson A.A., Michelsen P., Odham G. (1998). Molecular species of sphingomyelin: determination by high-performance liquid chromatography/mass spectrometry with electrospray and high-performance liquid chromatography/tandem mass spectrometry

with atmospheric pressure chemical ionization. Journal of mass spectrometry : JMS 33: 1192-1198.

158 Karlsson S., Varpula M., Ruokonen E., Pettila V., Parviainen I., Ala-Kokko T.I. et al (2007). Incidence, treatment, and outcome of severe sepsis in ICU-treated adults in Finland: the Finnsepsis study. Intensive care medicine 33: 435-443.

159 Karupiah G., Hunt N.H., King N.J., Chaudhri G. (2000). NADPH oxidase, Nramp1 and nitric oxide synthase 2 in the host antimicrobial response. Reviews in immunogenetics 2: 387-415.

160 Kawai T., Akira S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11: 373-384.

161 Kebaier C., Chamberland R.R., Allen I.C., Gao X., Broglie P.M., Hall J.D. et al (2012). Staphylococcus aureus alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. The Journal of infectious diseases 205: 807-817.

162 Keller P., Simons K. (1998). Cholesterol is required for surface transport of influenza virus hemagglutinin. The Journal of cell biology 140: 1357-1367.

163 Kennedy A.D., Bubeck Wardenburg J., Gardner D.J., Long D., Whitney A.R., Braughton K.R. et al (2010). Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. The Journal of infectious diseases 202: 1050-1058.

164 Kim J.H., Patra C.R., Arkalgud J.R., Boghossian A.A., Zhang J., Han J.H. et al (2011). Single-molecule detection of H(2)O(2) mediating angiogenic redox signaling on fluorescent single-walled carbon nanotube array. ACS nano 5: 7848-7857.

165 Kirby W.M. (1944). Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. Science 99: 452-453.

166 Klevens R.M., Morrison M.A., Nadle J., Petit S., Gershman K., Ray S. et al (2007). Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA : the journal of the American Medical Association 298: 1763-1771.

167 Kolesnick R.N., Goni F.M., Alonso A. (2000). Compartmentalization of ceramide signaling: physical foundations and biological effects. Journal of cellular physiology 184: 285-300.

168 Kolliputi N., Shaik R.S., Waxman A.B. (2010). The inflammasome mediates hyperoxia-induced alveolar cell permeability. Journal of immunology 184: 5819-5826.

169 Kolliputi N., Galam L., Parthasarathy P.T., Tipparaju S.M., Lockey R.F. (2012). NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability. Journal of cellular physiology 227: 3310-3316.

170 Komarova Y., Malik A.B. (2010). Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annual review of physiology 72: 463-493.

171 Kondo Y., Ito T., Ma X.X., Watanabe S., Kreiswirth B.N., Etienne J. et al (2007). Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. Antimicrobial agents and chemotherapy 51: 264-274.

172 Koo Y.E., Fan W., Hah H., Xu H., Orringer D., Ross B. et al (2007). Photonic explorers based on multifunctional nanoplatforms for biosensing and photodynamic therapy. Applied optics 46: 1924-1930.

173 Kornhuber J., Tripal P., Reichel M., Terfloth L., Bleich S., Wiltfang J. et al (2008). Identification of new functional inhibitors of acid sphingomyelinase using a structure-property-activity relation model. Journal of medicinal chemistry 51: 219-237.

174 Kornhuber J., Tripal P., Reichel M., Muhle C., Rhein C., Muehlbacher M. et al (2010). Functional Inhibitors of Acid Sphingomyelinase (FIASMAs): a novel pharmacological group of drugs with broad clinical applications. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 26: 9-20.

175 Kornhuber J., Muehlbacher M., Trapp S., Pechmann S., Friedl A., Reichel M. et al (2011). Identification of novel functional inhibitors of acid sphingomyelinase. PloS one 6: e23852.

176 Kubes P. (2002). The complexities of leukocyte recruitment. Seminars in immunology 14: 65-72.

177 Kumar P., Shen Q., Pivetti C.D., Lee E.S., Wu M.H., Yuan S.Y. (2009). Molecular mechanisms of endothelial hyperpermeability: implications in inflammation. Expert reviews in molecular medicine 11: e19.

178 Kung H.C., Hoyert D.L., Xu J., Murphy S.L. (2008). Deaths: final data for 2005. National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System 56: 1-120.

179 Lambeth J.D. (2004). NOX enzymes and the biology of reactive oxygen. Nature reviews Immunology 4: 181-189.

180 Landry D.W., Oliver J.A. (2001). The pathogenesis of vasodilatory shock. The New England journal of medicine 345: 588-595.

181 Lang P.A., Schenck M., Nicolay J.P., Becker J.U., Kempe D.S., Lupescu A. et al (2007). Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. Nature medicine 13: 164-170.

182 LaRocca T.J., Stivison E.A., Hod E.A., Spitalnik S.L., Cowan P.J., Randis T.M. et al (2014). Human-specific bacterial pore-forming toxins induce programmed necrosis in erythrocytes. mBio 5: e01251-01214.

183 Laufe M.D., Simon R.H., Flint A., Keller J.B. (1986). Adult respiratory distress syndrome in neutropenic patients. The American journal of medicine 80: 1022-1026.

184 Laupland K.B., Lyytikainen O., Sogaard M., Kennedy K.J., Knudsen J.D., Ostergaard C. et al (2013). The changing epidemiology of Staphylococcus aureus bloodstream infection: a multinational population-based surveillance study. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 19: 465-471.

185 Lautz T.B., Raval M.V., Barsness K.A. (2011). Increasing national burden of hospitalizations for skin and soft tissue infections in children. Journal of pediatric surgery 46: 1935-1941.

186 Lavoie P.M., Thibodeau J., Erard F., Sekaly R.P. (1999). Understanding the mechanism of action of bacterial superantigens from a decade of research. Immunological reviews 168: 257-269.

187 Le V.H., Fishman W.H. (1947). Combination of Evans blue with plasma protein; its significance in capillary permeability studies, blood dye disappearance curves, and its use as a protein tag. The American journal of physiology 151: 26-33.

188 Lecour S., Van der Merwe E., Opie L.H., Sack M.N. (2006). Ceramide attenuates hypoxic cell death via reactive oxygen species signaling. Journal of cardiovascular pharmacology 47: 158-163.

189 Lee C., Xu D.Z., Feketeova E., Kannan K.B., Yun J.K., Deitch E.A. et al (2004). Attenuation of shock-induced acute lung injury by sphingosine kinase inhibition. The Journal of trauma 57: 955-960.

190 Lee Y.E., Smith R., Kopelman R. (2009). Nanoparticle PEBBLE sensors in live cells and in vivo. Annual review of analytical chemistry 2: 57-76.

191 Leeuw T.K., Reith R.M., Simonette R.A., Harden M.E., Cherukuri P., Tsyboulski D.A. et al (2007). Single-walled carbon nanotubes in the intact organism: near-IR imaging and biocompatibility studies in Drosophila. Nano letters 7: 2650-2654.

192 Lepple-Wienhues A., Belka C., Laun T., Jekle A., Walter B., Wieland U. et al (1999). Stimulation of CD95 (Fas) blocks T lymphocyte calcium channels through sphingomyelinase and sphingolipids. Proceedings of the National Academy of Sciences of the United States of America 96: 13795-13800.

193 Lewis R.E., Granger H.J. (1986). Neutrophil-dependent mediation of microvascular permeability. Federation proceedings 45: 109-113.

194 Li P.L., Zhang Y., Yi F. (2007). Lipid raft redox signaling platforms in endothelial dysfunction. Antioxidants & redox signaling 9: 1457-1470.

195 Li P.L., Zhang Y. (2013a). Cross talk between ceramide and redox signaling: implications for endothelial dysfunction and renal disease. Handbook of experimental pharmacology: 171-197.

196 Li X., Han W.Q., Boini K.M., Xia M., Zhang Y., Li P.L. (2013b). TRAIL death receptor 4 signaling via lysosome fusion and membrane raft clustering in coronary arterial endothelial cells: evidence from ASM knockout mice. Journal of molecular medicine 91: 25-36.

197 Liebisch G., Drobnik W., Reil M., Trumbach B., Arnecke R., Olgemoller B. et al (1999). Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Journal of lipid research 40: 1539-1546.

198 Lin P.C., Chang C.H., Hsu P.I., Tseng P.L., Huang Y.B. (2010). The efficacy and safety of proton pump inhibitors vs histamine-2 receptor antagonists for stress ulcer bleeding prophylaxis among critical care patients: a meta-analysis. Critical care medicine 38: 1197-1205.

199 Lin T., Genestier L., Pinkoski M.J., Castro A., Nicholas S., Mogil R. et al (2000). Role of acidic sphingomyelinase in Fas/CD95-mediated cell death. The Journal of biological chemistry 275: 8657-8663.

200 Looney M.R., Su X., Van Ziffle J.A., Lowell C.A., Matthay M.A. (2006). Neutrophils and their Fc gamma receptors are essential in a mouse model of transfusion-related acute lung injury. The Journal of clinical investigation 116: 1615-1623.

201 Lovat P.E., Di Sano F., Corazzari M., Fazi B., Donnorso R.P., Pearson A.D. et al (2004). Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. Journal of the National Cancer Institute 96: 1288-1299.

202 Lowy F.D. (1998). Staphylococcus aureus infections. The New England journal of medicine 339: 520-532.

203 Lowy F.D. (2003). Antimicrobial resistance: the example of Staphylococcus aureus. The Journal of clinical investigation 111: 1265-1273.

204 Lozano J., Morales A., Cremesti A., Fuks Z., Tilly J.L., Schuchman E. et al (2001). Niemann-Pick Disease versus acid sphingomyelinase deficiency. Cell death and differentiation 8: 100-103.

205 Lush C.W., Kvietys P.R. (2000). Microvascular dysfunction in sepsis. Microcirculation 7: 83-101.

206 Majcherczyk P.A., Langen H., Heumann D., Fountoulakis M., Glauser M.P., Moreillon P. (1999). Digestion of Streptococcus pneumoniae cell walls with its major peptidoglycan hydrolase releases branched stem peptides carrying proinflammatory activity. The Journal of biological chemistry 274: 12537-12543.

207 Manago A., Becker K.A., Carpinteiro A., Wilker B., Soddemann M., Seitz A.P. et al (2015). Pseudomonas aeruginosa pyocyanin induces neutrophil death via mitochondrial reactive oxygen species and mitochondrial acid sphingomyelinase. Antioxidants & redox signaling 22: 1097-1110.

208 Mano N., Oda Y., Yamada K., Asakawa N., Katayama K. (1997). Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. Analytical biochemistry 244: 291-300.

209 Mariathasan S., Weiss D.S., Newton K., McBride J., O'Rourke K., Roose-Girma M. et al (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228-232.

210 Martin G.S., Mannino D.M., Eaton S., Moss M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. The New England journal of medicine 348: 1546-1554.

211 Matthay M.A., Eschenbacher W.L., Goetzl E.J. (1984). Elevated concentrations of leukotriene D4 in pulmonary edema fluid of patients with the adult respiratory distress syndrome. Journal of clinical immunology 4: 479-483.

212 Matthay M.A. (2003). *Acute respiratory distress syndrome*. M. Dekker: New York.

213 Matthay M.A., Zimmerman G.A., Esmon C., Bhattacharya J., Coller B., Doerschuk C.M. et al (2003). Future research directions in acute lung injury: summary of a National Heart, Lung, and Blood Institute working group. American journal of respiratory and critical care medicine 167: 1027-1035.

214 Matthay M.A., Zimmerman G.A. (2005). Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. American journal of respiratory cell and molecular biology 33: 319-327.

215 Mattix M.E., Hunt R.E., Wilhelmsen C.L., Johnson A.J., Baze W.B. (1995). Aerosolized staphylococcal enterotoxin B-induced pulmonary lesions in rhesus monkeys (Macaca mulatta). Toxicologic pathology 23: 262-268.

216 Matute-Bello G., Frevert C.W., Martin T.R. (2008). Animal models of acute lung injury. American journal of physiology Lung cellular and molecular physiology 295: L379-399.

217 McCollister B.D., Myers J.T., Jones-Carson J., Voelker D.R., Vazquez-Torres A. (2007). Constitutive acid sphingomyelinase enhances early and late macrophage killing of Salmonella enterica serovar Typhimurium. Infection and immunity 75: 5346-5352.

218 McCord J.M., Fridovich I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). The Journal of biological chemistry 244: 6049-6055.

219 McNabb T.J., Cremesti A.E., Brown P.R., Fischl A.S. (1999). The separation and direct detection of ceramides and sphingoid bases by normal-phase high-performance liquid chromatography and evaporative light-scattering detection. Analytical biochemistry 276: 242-250.

220 Meduri G.U., Headley A.S., Golden E., Carson S.J., Umberger R.A., Kelso T. et al (1998). Effect of prolonged methylprednisolone therapy in unresolving acute respiratory distress syndrome: a randomized controlled trial. JAMA : the journal of the American Medical Association 280: 159-165.

221 Meduri G.U., Golden E., Freire A.X., Taylor E., Zaman M., Carson S.J. et al (2007). Methylprednisolone infusion in early severe ARDS: results of a randomized controlled trial. Chest 131: 954-963.

222 Megha, London E. (2004). Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. The Journal of biological chemistry 279: 9997-10004.

223 Megha, Bakht O., London E. (2006). Cholesterol precursors stabilize ordinary and ceramide-rich ordered lipid domains (lipid rafts) to different degrees. Implications for the Bloch hypothesis and sterol biosynthesis disorders. The Journal of biological chemistry 281: 21903-21913.

224 Mehta D., Malik A.B. (2006). Signaling mechanisms regulating endothelial permeability. Physiological reviews 86: 279-367.

225 Melamed A., Sorvillo F.J. (2009). The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. Critical care 13: R28.

226 Menoret A., Kumar S., Vella A.T. (2012). Cytochrome b5 and cytokeratin 17 are biomarkers in bronchoalveolar fluid signifying onset of acute lung injury. PloS one 7: e40184.

227 Middleton J., Neil S., Wintle J., Clark-Lewis I., Moore H., Lam C. et al (1997). Transcytosis and surface presentation of IL-8 by venular endothelial cells. Cell 91: 385-395.

228 Mitra P., Maceyka M., Payne S.G., Lamour N., Milstien S., Chalfant C.E. et al (2007). Ceramide kinase regulates growth and survival of A549 human lung adenocarcinoma cells. FEBS letters 581: 735-740.

229 Moitra J., Sammani S., Garcia J.G. (2007). Re-evaluation of Evans Blue dye as a marker of albumin clearance in murine models of acute lung injury. Translational research : the journal of laboratory and clinical medicine 150: 253-265.

230 Moodley A., Stegger M., Bagcigil A.F., Baptiste K.E., Loeffler A., Lloyd D.H. et al (2006). spa typing of methicillin-resistant Staphylococcus aureus isolated from domestic animals and veterinary staff in the UK and Ireland. The Journal of antimicrobial chemotherapy 58: 1118-1123.

231 Moore C.L., Lu M., Cheema F., Osaki-Kiyan P., Perri M.B., Donabedian S. et al (2011). Prediction of failure in vancomycin-treated methicillin-resistant Staphylococcus aureus bloodstream infection: a clinically useful risk stratification tool. Antimicrobial agents and chemotherapy 55: 4581-4588.

232 Moore C.L., Osaki-Kiyan P., Haque N.Z., Perri M.B., Donabedian S., Zervos M.J. (2012). Daptomycin versus vancomycin for bloodstream infections due to methicillin-resistant Staphylococcus aureus with a high vancomycin minimum inhibitory concentration: a case-control study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 54: 51-58.

233 Morath S., Geyer A., Hartung T. (2001). Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. The Journal of experimental medicine 193: 393-397.

234 Morita Y., Tilly J.L. (2000). Sphingolipid regulation of female gonadal cell apoptosis. Annals of the New York Academy of Sciences 905: 209-220.

235 Moser M., Leo O. (2010). Key concepts in immunology. Vaccine 28 Suppl 3: C2-13.

236 Motta S., Monti M., Sesana S., Mellesi L., Ghidoni R., Caputo R. (1994). Abnormality of water barrier function in psoriasis. Role of ceramide fractions. Archives of dermatology 130: 452-456.

237 Muller G., Ayoub M., Storz P., Rennecke J., Fabbro D., Pfizenmaier K. (1995). PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. The EMBO journal 14: 1961-1969.

238 Munro S. (2003). Lipid rafts: elusive or illusive? Cell 115: 377-388.

239 Nadel S., Goldstein B., Williams M.D., Dalton H., Peters M., Macias W.L. et al (2007). Drotrecogin alfa (activated) in children with severe sepsis: a multicentre phase III randomised controlled trial. Lancet 369: 836-843.

240 Naik P., Fofaria N., Prasad S., Sajja R.K., Weksler B., Couraud P.O. et al (2014). Oxidative and pro-inflammatory impact of regular and denicotinized cigarettes on blood brain barrier endothelial cells: is smoking reduced or nicotine-free products really safe? BMC neuroscience 15: 51.

241 Nathan C. (2006). Neutrophils and immunity: challenges and opportunities. Nature reviews Immunology 6: 173-182.

242 National Heart L., Blood Institute Acute Respiratory Distress Syndrome Clinical Trials N., Wiedemann H.P., Wheeler A.P., Bernard G.R., Thompson B.T. et al (2006).

Comparison of two fluid-management strategies in acute lung injury. The New England journal of medicine 354: 2564-2575.

243 Neushul P. (1993). Science, government, and the mass production of penicillin. Journal of the history of medicine and allied sciences 48: 371-395.

244 Nishimura K., Nakamura A. (1985). High performance liquid chromatographic analysis of long chain bases in intestinal glycolipids of adult and embryonic Japanese quails. Journal of biochemistry 98: 1247-1254.

245 Nix M., Stoffel W. (2000). Perturbation of membrane microdomains reduces mitogenic signaling and increases susceptibility to apoptosis after T cell receptor stimulation. Cell death and differentiation 7: 413-424.

246 Nurminen T.A., Holopainen J.M., Zhao H., Kinnunen P.K. (2002). Observation of topical catalysis by sphingomyelinase coupled to microspheres. Journal of the American Chemical Society 124: 12129-12134.

247 O'Callaghan R.J., Callegan M.C., Moreau J.M., Green L.C., Foster T.J., Hartford O.M. et al (1997). Specific roles of alpha-toxin and beta-toxin during Staphylococcus aureus corneal infection. Infection and immunity 65: 1571-1578.

248 O'Sullivan C.E., Baker M.G., Zhang J. (2011). Increasing hospitalizations for serious skin infections in New Zealand children, 1990-2007. Epidemiology and infection 139: 1794-1804.

249 Ognibene F.P., Martin S.E., Parker M.M., Schlesinger T., Roach P., Burch C. et al (1986). Adult respiratory distress syndrome in patients with severe neutropenia. The New England journal of medicine 315: 547-551.

250 Ogston A. (1880). Ueber Abscesse. Arch Klin Chir 25: 588-600.

251 Ogston A. (1881). Report upon micro-organisms in surgical diseases. Brit Med J 1: 369-375.

252 Ogston A. (1882a). Micrococcus Poisoning. Journal of anatomy and physiology 16: 526-567.

253 Ogston A. (1882b). Micrococcus Poisoning. Journal of anatomy and physiology 17: 24-58.

254 Okino N., He X., Gatt S., Sandhoff K., Ito M., Schuchman E.H. (2003). The reverse activity of human acid ceramidase. The Journal of biological chemistry 278: 29948-29953.

255 Ostergaard H., Henriksen A., Hansen F.G., Winther J.R. (2001). Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein. The EMBO journal 20: 5853-5862.

256 Otterbach B., Stoffel W. (1995). Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). Cell 81: 1053-1061.

257 Pai A.B., Patel H., Prokopienko A.J., Alsaffar H., Gertzberg N., Neumann P. et al (2012). Lipoteichoic acid from Staphylococcus aureus induces lung endothelial cell barrier dysfunction: role of reactive oxygen and nitrogen species. PloS one 7: e49209.

258 Paine R., 3rd, Standiford T.J., Dechert R.E., Moss M., Martin G.S., Rosenberg A.L. et al (2012). A randomized trial of recombinant human granulocyte-macrophage colony stimulating factor for patients with acute lung injury. Critical care medicine 40: 90-97.

259 Pandey S., Murphy R.F., Agrawal D.K. (2007). Recent advances in the immunobiology of ceramide. Experimental and molecular pathology 82: 298-309.

260 Parsons P.E., Fowler A.A., Hyers T.M., Henson P.M. (1985). Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. The American review of respiratory disease 132: 490-493.

261 Patterson C.E., Rhoades R.A., Garcia J.G. (1992). Evans blue dye as a marker of albumin clearance in cultured endothelial monolayer and isolated lung. J Appl Physiol (1985) 72: 865-873.

262 Peek G.J., Clemens F., Elbourne D., Firmin R., Hardy P., Hibbert C. et al (2006). CESAR: conventional ventilatory support vs extracorporeal membrane oxygenation for severe adult respiratory failure. BMC health services research 6: 163.

263 Pena L.A., Fuks Z., Kolesnick R.N. (2000). Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. Cancer research 60: 321-327.

264 Perez G.I., Knudson C.M., Leykin L., Korsmeyer S.J., Tilly J.L. (1997). Apoptosis-associated signaling pathways are required for chemotherapy-mediated female germ cell destruction. Nature medicine 3: 1228-1232.

265 Perichon B., Courvalin P. (2004). Heterologous expression of the enterococcal vanA operon in methicillin-resistant Staphylococcus aureus. Antimicrobial agents and chemotherapy 48: 4281-4285.

266 Peter J.V., John P., Graham P.L., Moran J.L., George I.A., Bersten A. (2008). Corticosteroids in the prevention and treatment of acute respiratory distress syndrome (ARDS) in adults: meta-analysis. Bmj 336: 1006-1009.

267 Petrucci N., De Feo C. (2013). Lung protective ventilation strategy for the acute respiratory distress syndrome. The Cochrane database of systematic reviews 2: CD003844.

268 Powers M.E., Kim H.K., Wang Y., Bubeck Wardenburg J. (2012). ADAM10 mediates vascular injury induced by Staphylococcus aureus alpha-hemolysin. The Journal of infectious diseases 206: 352-356.

269 Powers S.K., Jackson M.J. (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiological reviews 88: 1243-1276.

270 Previati M., Bertolaso L., Tramarin M., Bertagnolo V., Capitani S. (1996). Low nanogram range quantitation of diglycerides and ceramide by high-performance liquid chromatography. Analytical biochemistry 233: 108-114.

271 Quintern L.E., Schuchman E.H., Levran O., Suchi M., Ferlinz K., Reinke H. et al (1989). Isolation of cDNA clones encoding human acid sphingomyelinase: occurrence of alternatively processed transcripts. The EMBO journal 8: 2469-2473.

272 Rahmani M., Reese E., Dai Y., Bauer C., Payne S.G., Dent P. et al (2005). Coadministration of histone deacetylase inhibitors and perifosine synergistically induces apoptosis in human leukemia cells through Akt and ERK1/2 inactivation and the generation of ceramide and reactive oxygen species. Cancer research 65: 2422-2432.

273 Ranieri V.M., Thompson B.T., Barie P.S., Dhainaut J.F., Douglas I.S., Finfer S. et al (2012). Drotrecogin alfa (activated) in adults with septic shock. The New England journal of medicine 366: 2055-2064.

274 Rawson R.A. (1943). *The binding of T-1824 and structurally related diazo dyes by the plasma proteins*: New York city,.

275 Reinehr R., Becker S., Eberle A., Grether-Beck S., Haussinger D. (2005). Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis. The Journal of biological chemistry 280: 27179-27194.

276 Reinehr R., Becker S., Braun J., Eberle A., Grether-Beck S., Haussinger D. (2006). Endosomal acidification and activation of NADPH oxidase isoforms are upstream events in hyperosmolarity-induced hepatocyte apoptosis. The Journal of biological chemistry 281: 23150-23166.

277 Rice T.W., Wheeler A.P., Thompson B.T., deBoisblanc B.P., Steingrub J., Rock P. et al (2011). Enteral omega-3 fatty acid, gamma-linolenic acid, and antioxidant supplementation in acute lung injury. JAMA : the journal of the American Medical Association 306: 1574-1581.

278 Richards R.L., Habbersett R.C., Scher I., Janoff A.S., Schieren H.P., Mayer L.D. et al (1986). Influence of vesicle size on complement-dependent immune damage to liposomes. Biochimica et biophysica acta 855: 223-230.

279 Robinson D.A., Enright M.C. (2004). Multilocus sequence typing and the evolution of methicillin-resistant Staphylococcus aureus. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 10: 92-97.

280 Rochfort K.D., Collins L.E., Murphy R.P., Cummins P.M. (2014). Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions. PloS one 9: e101815.

281 Rubenfeld G.D., Caldwell E., Peabody E., Weaver J., Martin D.P., Neff M. et al (2005). Incidence and outcomes of acute lung injury. The New England journal of medicine 353: 1685-1693.

282 Russell J.A. (2006). Management of sepsis. The New England journal of medicine 355: 1699-1713.

283 Sallee J.L., Wittchen E.S., Burridge K. (2006). Regulation of cell adhesion by protein-tyrosine phosphatases: II. Cell-cell adhesion. The Journal of biological chemistry 281: 16189-16192.

284 Samapati R., Yang Y., Yin J., Stoerger C., Arenz C., Dietrich A. et al (2012). Lung endothelial Ca2+ and permeability response to platelet-activating factor is mediated by acid sphingomyelinase and transient receptor potential classical 6. American journal of respiratory and critical care medicine 185: 160-170.

285 Samuelsson B., Samuelsson K. (1969). Gas--liquid chromatography-mass spectrometry of synthetic ceramides. Journal of lipid research 10: 41-46.

286 Santana P., Pena L.A., Haimovitz-Friedman A., Martin S., Green D., McLoughlin M. et al (1996). Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell 86: 189-199.

287 Sanvicens N., Cotter T.G. (2006). Ceramide is the key mediator of oxidative stress-induced apoptosis in retinal photoreceptor cells. Journal of neurochemistry 98: 1432-1444.

288 Saria A., Lundberg J.M. (1983). Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. Journal of neuroscience methods 8: 41-49.

289 Scheel-Toellner D., Wang K., Assi L.K., Webb P.R., Craddock R.M., Salmon M. et al (2004). Clustering of death receptors in lipid rafts initiates neutrophil spontaneous apoptosis. Biochemical Society transactions 32: 679-681.

290 Schissel S.L., Schuchman E.H., Williams K.J., Tabas I. (1996). Zn2+-stimulated sphingomyelinase is secreted by many cell types and is a product of the acid sphingomyelinase gene. The Journal of biological chemistry 271: 18431-18436.

291 Schissel S.L., Jiang X., Tweedie-Hardman J., Jeong T., Camejo E.H., Najib J. et al (1998a). Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. The Journal of biological chemistry 273: 2738-2746.

292 Schissel S.L., Keesler G.A., Schuchman E.H., Williams K.J., Tabas I. (1998b). The cellular trafficking and zinc dependence of secretory and lysosomal sphingomyelinase, two products of the acid sphingomyelinase gene. The Journal of biological chemistry 273: 18250-18259.

293 Schneider-Brachert W., Tchikov V., Neumeyer J., Jakob M., Winoto-Morbach S., Held-Feindt J. et al (2004). Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 21: 415-428.

294 Schneider E.G., Kennedy E.P. (1973). Phosphorylation of ceramide by diglyceride kinase preparations from Escherichia coli. The Journal of biological chemistry 248: 3739-3741.

295 Schramm M., Herz J., Haas A., Kronke M., Utermohlen O. (2008). Acid sphingomyelinase is required for efficient phago-lysosomal fusion. Cellular microbiology 10: 1839-1853.

296 Schroder K., Tschopp J. (2010). The inflammasomes. Cell 140: 821-832.

297 Schwarzlander M., Logan D.C., Fricker M.D., Sweetlove L.J. (2011). The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide 'flashes'. The Biochemical journal 437: 381-387.

298 Seeger W., Birkemeyer R.G., Ermert L., Suttorp N., Bhakdi S., Duncker H.R. (1990). Staphylococcal alpha-toxin-induced vascular leakage in isolated perfused rabbit lungs. Laboratory investigation; a journal of technical methods and pathology 63: 341-349.

299 Senchenkov A., Litvak D.A., Cabot M.C. (2001). Targeting ceramide metabolism--a strategy for overcoming drug resistance. Journal of the National Cancer Institute 93: 347-357.

300 Shen Q., Wu M.H., Yuan S.Y. (2009). Endothelial contractile cytoskeleton and microvascular permeability. Cell health and cytoskeleton 2009: 43-50.

301 Shopsin B., Gomez M., Montgomery S.O., Smith D.H., Waddington M., Dodge D.E. et al (1999). Evaluation of protein A gene polymorphic region DNA sequencing for

typing of Staphylococcus aureus strains. Journal of clinical microbiology 37: 3556-3563.

302 Sies H., Cadenas E. (1985). Oxidative stress: damage to intact cells and organs. Philosophical transactions of the Royal Society of London Series B, Biological sciences 311: 617-631.

303 Sies H. (1997). Oxidative stress: oxidants and antioxidants. Experimental physiology 82: 291-295.

304 Simons K., Ikonen E. (1997). Functional rafts in cell membranes. Nature 387: 569-572.

305 Singer S.J., Nicolson G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science 175: 720-731.

306 Siskind L.J., Colombini M. (2000). The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. The Journal of biological chemistry 275: 38640-38644.

307 Siskind L.J., Kolesnick R.N., Colombini M. (2006). Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations. Mitochondrion 6: 118-125.

308 Sivan Y., Mor C., al-Jundi S., Newth C.J. (1990). Adult respiratory distress syndrome in severely neutropenic children. Pediatric pulmonology 8: 104-108.

309 Skinner D., Keefer C.S. (1941). Significance of bacteremia caused by staphylococcus aureus: A study of one hundred and twenty-two cases and a review of the literature concerned with experimental infection in animals. Archives of Internal Medicine 68: 851-875.

310 Smith E.R., Merrill A.H., Jr. (1995). Differential roles of de novo sphingolipid biosynthesis and turnover in the "burst" of free sphingosine and sphinganine, and their 1-phosphates and N-acyl-derivatives, that occurs upon changing the medium of cells in culture. The Journal of biological chemistry 270: 18749-18758.

311 Soehnlein O., Oehmcke S., Ma X., Rothfuchs A.G., Frithiof R., van Rooijen N. et al (2008). Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. The European respiratory journal 32: 405-412.

312 Soehnlein O. (2009). Direct and alternative antimicrobial mechanisms of neutrophil-derived granule proteins. Journal of molecular medicine 87: 1157-1164.

313 Spiegel S., Milstien S. (2002). Sphingosine 1-phosphate, a key cell signaling molecule. The Journal of biological chemistry 277: 25851-25854.

314 Steinberg K.P., Milberg J.A., Martin T.R., Maunder R.J., Cockrill B.A., Hudson L.D. (1994). Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. American journal of respiratory and critical care medicine 150: 113-122.

315 Steinberg K.P., Hudson L.D., Goodman R.B., Hough C.L., Lanken P.N., Hyzy R. et al (2006). Efficacy and safety of corticosteroids for persistent acute respiratory distress syndrome. The New England journal of medicine 354: 1671-1684.

316 Steinberg S.F. (2013). Oxidative stress and sarcomeric proteins. Circulation research 112: 393-405.

317 Stutz A., Golenbock D.T., Latz E. (2009). Inflammasomes: too big to miss. The Journal of clinical investigation 119: 3502-3511.

318 Suh Y.A., Arnold R.S., Lassegue B., Shi J., Xu X., Sorescu D. et al (1999). Cell transformation by the superoxide-generating oxidase Mox1. Nature 401: 79-82.

319 Szabo I., Gulbins E., Apfel H., Zhang X., Barth P., Busch A.E. et al (1996). Tyrosine phosphorylation-dependent suppression of a voltage-gated K+ channel in T lymphocytes upon Fas stimulation. The Journal of biological chemistry 271: 20465-20469.

320 Takimoto E., Kass D.A. (2007). Role of oxidative stress in cardiac hypertrophy and remodeling. Hypertension 49: 241-248.

321 Taylor R.W., Zimmerman J.L., Dellinger R.P., Straube R.C., Criner G.J., Davis K., Jr. et al (2004). Low-dose inhaled nitric oxide in patients with acute lung injury: a randomized controlled trial. JAMA : the journal of the American Medical Association 291: 1603-1609.

322 Teichgraber V., Ulrich M., Endlich N., Riethmuller J., Wilker B., De Oliveira-Munding C.C. et al (2008). Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nature medicine 14: 382-391.

323 ten Grotenhuis E., Demel R.A., Ponec M., Boer D.R., van Miltenburg J.C., Bouwstra J.A. (1996). Phase behavior of stratum corneum lipids in mixed Langmuir-Blodgett monolayers. Biophysical journal 71: 1389-1399.

324 Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H. et al (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Journal of clinical microbiology 33: 2233-2239.

325 Tepper A.D., Van Blitterswijk W.J. (2000). Ceramide mass analysis by normal-phase high-performance liquid chromatography. Methods in enzymology 312: 16-22.

326 Tom S., Galbraith J.C., Valiquette L., Jacobsson G., Collignon P., Schonheyder H.C. et al (2014). Case fatality ratio and mortality rate trends of community-onset Staphylococcus aureus bacteraemia. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases.

327 Tomita T., Kamio Y. (1997). Molecular biology of the pore-forming cytolysins from Staphylococcus aureus, alpha- and gamma-hemolysins and leukocidin. Bioscience, biotechnology, and biochemistry 61: 565-572.

328 Turnage R.H., Kadesky K.M., Bartula L., Myers S.I. (1995). Intestinal reperfusion up-regulates inducible nitric oxide synthase activity within the lung. Surgery 118: 288-293.

329 Utermohlen O., Karow U., Lohler J., Kronke M. (2003). Severe impairment in early host defense against Listeria monocytogenes in mice deficient in acid sphingomyelinase. Journal of immunology 170: 2621-2628.

330 Utermohlen O., Herz J., Schramm M., Kronke M. (2008). Fusogenicity of membranes: the impact of acid sphingomyelinase on innate immune responses. Immunobiology 213: 307-314.

331 van Hal S.J., Jensen S.O., Vaska V.L., Espedido B.A., Paterson D.L., Gosbell I.B. (2012). Predictors of mortality in Staphylococcus aureus Bacteremia. Clinical microbiology reviews 25: 362-386.

332 Van Veldhoven P.P., Bishop W.R., Yurivich D.A., Bell R.M. (1995). Ceramide quantitation: evaluation of a mixed micellar assay using E. coli diacylglycerol kinase. Biochemistry and molecular biology international 36: 21-30.

333 Vaska V.L., Nimmo G.R., Jones M., Grimwood K., Paterson D.L. (2012). Increases in Australian cutaneous abscess hospitalisations: 1999-2008. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 31: 93-96.

334 Veiga M.P., Arrondo J.L., Goni F.M., Alonso A. (1999). Ceramides in phospholipid membranes: effects on bilayer stability and transition to nonlamellar phases. Biophysical journal 76: 342-350.

335 Vincent P.A., Xiao K., Buckley K.M., Kowalczyk A.P. (2004). VE-cadherin: adhesion at arm's length. American journal of physiology Cell physiology 286: C987-997.

336 von Eiff C., Becker K., Machka K., Stammer H., Peters G. (2001). Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. The New England journal of medicine 344: 11-16.

337 Ware L.B., Matthay M.A. (2000). The acute respiratory distress syndrome. The New England journal of medicine 342: 1334-1349.

338 Watts J.D., Aebersold R., Polverino A.J., Patterson S.D., Gu M. (1999). Ceramide second messengers and ceramide assays. Trends in biochemical sciences 24: 228.

339 Weigel L.M., Donlan R.M., Shin D.H., Jensen B., Clark N.C., McDougal L.K. et al (2007). High-level vancomycin-resistant Staphylococcus aureus isolates associated with a polymicrobial biofilm. Antimicrobial agents and chemotherapy 51: 231-238.

340 Wheeler A.P., Bernard G.R. (2007). Acute lung injury and the acute respiratory distress syndrome: a clinical review. Lancet 369: 1553-1564.

341 Wilke G.A., Bubeck Wardenburg J. (2010). Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. Proceedings of the National Academy of Sciences of the United States of America 107: 13473-13478.

342 Williams R.E., Jevons M.P., Shooter R.A., Hunter C.J., Girling J.A., Griffiths J.D. et al (1959). Nasal staphylococci and sepsis in hospital patients. British medical journal 2: 658-662.

343 Williamson D.A., Ritchie S.R., Lennon D., Roberts S.A., Stewart J., Thomas M.G. et al (2013). Increasing incidence and sociodemographic variation in community-onset Staphylococcus aureus skin and soft tissue infections in New Zealand children. The Pediatric infectious disease journal 32: 923-925.

344 Williamson D.A., Zhang J., Ritchie S.R., Roberts S.A., Fraser J.D., Baker M.G. (2014). Staphylococcus aureus Infections in New Zealand, 2000-2011. Emerging infectious diseases 20: 1157-1162.

345 Windsor A.C., Mullen P.G., Fowler A.A., Sugerman H.J. (1993). Role of the neutrophil in adult respiratory distress syndrome. The British journal of surgery 80: 10-17.

346 Wyllie D.H., Walker A.S., Miller R., Moore C., Williamson S.R., Schlackow I. et al (2011). Decline of meticillin-resistant Staphylococcus aureus in Oxfordshire hospitals is strain-specific and preceded infection-control intensification. BMJ open 1: e000160.

347 Xia M., Zhang C., Boini K.M., Thacker A.M., Li P.L. (2011). Membrane raft-lysosome redox signalling platforms in coronary endothelial dysfunction induced by adipokine visfatin. Cardiovascular research 89: 401-409.

348 Xu M., Li X.X., Ritter J.K., Abais J.M., Zhang Y., Li P.L. (2013). Contribution of NADPH oxidase to membrane CD38 internalization and activation in coronary arterial myocytes. PloS one 8: e71212.

349 Xu X., Bittman R., Duportail G., Heissler D., Vilcheze C., London E. (2001). Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. The Journal of biological chemistry 276: 33540-33546.

350 Yang Y., Yin J., Baumgartner W., Samapati R., Solymosi E.A., Reppien E. et al (2010). Platelet-activating factor reduces endothelial nitric oxide production: role of acid sphingomyelinase. The European respiratory journal 36: 417-427.

351 Yano M., Kishida E., Muneyuki Y., Masuzawa Y. (1998). Quantitative analysis of ceramide molecular species by high performance liquid chromatography. Journal of lipid research 39: 2091-2098.

352 Yao B., Zhang Y., Delikat S., Mathias S., Basu S., Kolesnick R. (1995). Phosphorylation of Raf by ceramide-activated protein kinase. Nature 378: 307-310.

353 Yi F., Chen Q.Z., Jin S., Li P.L. (2007). Mechanism of homocysteine-induced Rac1/NADPH oxidase activation in mesangial cells: role of guanine nucleotide exchange factor Vav2. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 20: 909-918.

354 Youn J.Y., Zhang J., Zhang Y., Chen H., Liu D., Ping P. et al (2013). Oxidative stress in atrial fibrillation: an emerging role of NADPH oxidase. Journal of molecular and cellular cardiology 62: 72-79.

355 Yuan S.Y., Rigor R.R. (2010). *Regulation of Endothelial Barrier Function*: San Rafael (CA).

356 Zemann B., Urtz N., Reuschel R., Mechtcheriakova D., Bornancin F., Badegruber R. et al (2007). Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. Immunology letters 109: 56-63.

357 Zhang A.Y., Yi F., Zhang G., Gulbins E., Li P.L. (2006). Lipid raft clustering and redox signaling platform formation in coronary arterial endothelial cells. Hypertension 47: 74-80.

358 Zhang A.Y., Yi F., Jin S., Xia M., Chen Q.Z., Gulbins E. et al (2007). Acid sphingomyelinase and its redox amplification in formation of lipid raft redox signaling platforms in endothelial cells. Antioxidants & redox signaling 9: 817-828.

359 Zhang Y., Yao B., Delikat S., Bayoumy S., Lin X.H., Basu S. et al (1997). Kinase suppressor of Ras is ceramide-activated protein kinase. Cell 89: 63-72.

360 Zhang Y., Li X., Carpinteiro A., Gulbins E. (2008). Acid sphingomyelinase amplifies redox signaling in Pseudomonas aeruginosa-induced macrophage apoptosis. Journal of immunology 181: 4247-4254.

361 Zielonka J., Kalyanaraman B. (2010). Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free radical biology & medicine 48: 983-1001.

362 Zundel W., Giaccia A. (1998). Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes & development 12: 1941-1946.

363 Zundel W., Swiersz L.M., Giaccia A. (2000). Caveolin 1-mediated regulation of receptor tyrosine kinase-associated phosphatidylinositol 3-kinase activity by ceramide. Molecular and cellular biology 20: 1507-1514.

8. PUBLICATIONS

1. **Huiming Peng**, Cao Li, Stephanie Kadow, Brian D. Henry, Jörg Steinmann, Katrin Anne Becker, Andrea Riehle, Natalie Beckmann, Barbara Wilker, Pin-Lan Li, Timothy Pritts, Michael J. Edwards, Yang Zhang, Erich Gulbins, Heike Grassmé. Acid sphingomyelinase inhibition protects mice from lung edema and lethal Staphylococcus aureus sepsis. J Mol Med (2015) 93:675–689

2. Rolf Hilker, Antje Munder, Jens Klockgether, Patricia Moran Losada, Philippe Chouvarine, Nina Cramer, Colin F. Davenport, Sarah Dethlefsen, Sebastian Fischer, **Huiming Peng**, Torben Schönfelder, Oliver Türk, Lutz Wiehlmann, Florian Wölbeling, Erich Gulbins, Alexander Goesmann and Burkhard Tümmler. Interclonal gradient of virulence in the *Pseudomonas aeruginosa* pangenome from disease and environment. Environmental Microbiology (2015) 17(1), 29–46

3. Cao Li, **Huiming Peng**, Lukasz Japtok, Aaron Seitz,Andrea Riehle, Barbara Wilker, Matthias Soddemann, Burkard Kleuser, Michael Edwards, David Lammas, Pin Lan Li, Krishna M. Boini, Yang Zhang, Erich Gulbins, Heike Grassmé. Neutral sphingomyelinase reduction protects mice against systemic tuberculosis through autophagy stimulation via ROS-downregulation. Front Biosci (Elite Ed). (2016) 1(8), 311-25.

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