Sphingolipids in gonococcal infection

Sphingolipide in der Gonokokkeninfektion

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ABSTRACT

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, has the potential to spread in the human host and cause a severe complication called disseminated gonococcal infection (DGI). The expression of the major outer membrane porin PorB$_{IA}$ is a characteristic of most gonococci associated with DGI. PorB$_{IA}$ binds to the scavenger receptor expressed on endothelial cells (SREC-I), which mediates the so-called low phosphate-dependent invasion (LPDI). This uptake mechanism enables *N. gonorrhoeae* to rapidly invade epithelial and endothelial cells in a phosphate-sensitive manner.

We recently demonstrated that the neutral sphingomyelinase, which catalyses the hydrolysis of sphingomyelin to ceramide and phosphorylcholine, is required for the LPDI of gonococci in non-phagocytic cells. Neutral sphingomyelinase 2 (NSM2) plays a key role in the early PorB$_{IA}$ signaling by recruiting the PI3 kinase to caveolin. The following activation of the PI3 kinase-dependent downstream signaling leads to the engulfment of the bacteria. As a part of this work, I could confirm the involvement of the NSM2. The role of the enzyme was further elucidated by the generation of antibodies directed against NSM2 and the construction of an epithelium-based NSM2 knockout cell line using CRISPR/Cas9. The knockout of the NSM2 strongly inhibits the LPDI. The invasion could be, however, restored by the complementation of the knockout using an NSM2-GFP construct. However, the results could not be reproduced.

In this work, I could show the involvement of further members of the sphingolipid pathway in the PorB$_{IA}$-mediated invasion. Lipidome analysis revealed an increase of the bioactive molecules ceramide and sphingosine due to gonococcal infection. Both molecules do not only affect the host cell, but seem to influence the bacteria as well: while ceramide seems to be incorporated by the gonococci, sphingosine is toxic for the bacteria. Furthermore, the sphingosine kinase 2 (SPHK2) plays an important role in invasion, since the inhibition and knockdown of the enzyme revealed a negative effect on gonococcal invasion. To elucidate the role of the sphingosine kinases in invasion in more detail, an activity assay was established in this study. Additionally, the impact of the sphingosine-1-phosphate lyase (S1PL) on invasion was investigated. Inhibitor studies and infection experiments conducted with a CRISPR/Cas9 HeLa S1PL knockout cell line revealed a role of the enzyme not only in the PorB$_{IA}$-mediated invasion, but also in the Opa$_{50}$/HSPG-mediated gonococcal invasion.

The signaling experiments allowed the categorization of the SPHK and S1PL activation in the context of infection. Like the NSM2, both enzymes play a role in the early PorB$_{IA}$ signaling events leading to the uptake of the bacteria. All those findings indicate an important role of sphingolipids in the invasion and survival of *N. gonorrhoeae*.

In the last part of this work, the role of the NSM2 in the inhibition of apoptosis in neutrophils due to gonococcal infection was investigated. It could be demonstrated that the delayed onset of apoptosis is independent of neisserial porin and Opa proteins. Furthermore, the influence of neisserial peptidoglycan on PMN apoptosis was analysed using mutant strains, but no connection could be determined. Since the NSM2 is the most prominent sphingomyelinase in PMNs, fulfils manifold cell physiological functions and has already been connected to apoptosis, the impact of the enzyme on apoptosis inhibition due to gonococcal infection was investigated using inhibitors, with no positive results.

ZUSAMMENFASSUNG

Neisseria gonorrhoeae, der Auslöser der sexuell übertragbaren Krankheit Gonorrhö, hat das Potenzial sich im menschlichen Wirt auszubreiten und eine schwere Komplikation, die disseminierende Gonokokkeninfektion (DGI), hervorzurufen. Die Expression des Porins $PorB_{IA}$, das eines der häufigsten Proteine der äußeren Membran ist, stellt ein Charakteristikum der mit DGI assoziierten Gonokokken dar. $PorB_{IA}$ bindet an SREC-I (scavenger receptor expressed on endothelial cells), der die phosphatabhängige Invasion (low phosphate-dependent invasion LPDI) vermittelt. Dieser Aufnahmemechanismus erlaubt es *N. gonorrhoeae* Epithel- sowie Endothelzellen, schnell zu invadieren.

Wir haben kürzlich gezeigt, dass die neutrale Sphingomyelinase 2 (NSM2), welche die Hydrolyse von Sphingomyelin zu Ceramid und Phosphorylcholin katalysiert, für die LPDI der Gonokokken in nicht-phagozytische Zellen benötigt wird. Dabei spielt die neutrale Sphingomyelinase 2 eine Schlüsselrolle in der frühen $PorB_{IA}$ Signalübertragung, indem sie die PI3 Kinase zu Caveolin rekrutiert. Die darauffolgende Aktivierung von nachgeschalteten Signalwegen, die von der PI3 Kinase abhängig sind, führt zur Aufnahme der Bakterien. Als Teil dieser Arbeit konnte ich die Beteiligung der NSM2 bestätigen. Die Rolle des Enzyms sollte durch die Herstellung von NSM2-spezifischen Antikörpern und einer auf Epithelzellen basierenden NSM2 knockout Zelllinie, die mit Hilfe des CRISPR/Cas9 Systems hergestellt wurde, aufgeklärt werden. Der knockout der NSM2 führte zu einer starken Inhibition der LPDI. Die Invasion konnte jedoch durch die Komplementation mit Hilfe eines NSM2-GFP Konstruktes wiederhergestellt werden. Wobei die Ergebnisse jedoch nicht reproduziert werden konnten.

In dieser Arbeit konnte ich die Beteiligung weiterer Mitglieder des Sphingolipid Signalwegs an der $PorB_{IA}$-vermittelten Invasion zeigen. Die Lipidomanalysen zeigten einen Anstieg der bioaktiven Moleküle Ceramide und Sphingosin aufgrund der Gonokokkeninfektion. Beide Moleküle beeinflussen nicht nur die Wirtszelle, sondern schienen auch Auswirkungen auf die Bakterien selbst zu haben: während Ceramid anscheinend von den Gonokokken aufgenommen wird, ist Sphingosin für die Bakterien toxisch. Weiterhin spielt die Sphingosinkinase 2 (SPHK2) eine wichtige Rolle in der Invasion, da die Inhibierung und der Knockdown des Enzyms die Gonokokkeninfektion negativ beeinflussen. Um die Rolle der Sphingosinkinasen in der Invasion im Detail zu erforschen, wurde in dieser Arbeit ein

Aktivitätsassay etabliert. Außerdem wurde der Einfluss der Sphingosin-1-phosphat Lyase (S1PL) auf die Invasion erforscht. Inhibitorstudien und Infektionsexperimente, die mit einer CRISPR/Cas9 HeLa S1PL knockout Zelllinie durchgeführt wurden, zeigten, dass das Enzym nicht nur eine Rolle in der PorB$_{IA}$-vermittelten, sondern auch in der Opa$_{50}$/HSPG-vermittelten Gonokokkeninfektion spielt. Die Experimente, die bezüglich der zugrundeliegenden Signalwege durchgeführt wurden, erlaubten die Einordnung der Aktivierung der SPHK und der S1PL im Kontext der Invasion. Wie auch die NSM2, spielen beide Enzyme in der frühen PorB$_{IA}$ Signalübertragung eine Rolle, die schließlich zur Aufnahme der Bakterien führt. Alle diese Ergebnisse weisen auf eine wichtige Rolle der Sphingolipide für die Invasion und das Überleben von *N. gonorrhoeae* hin.

Im letzten Teil dieser Arbeit, wurde die Inhibierung der Apoptose von Neutrophilen aufgrund der Gonokokkeninfektion untersucht. Es konnte gezeigt werden, dass das verspätete Einsetzen der Apoptose von neisseriellen Porinen und Opa Proteinen unabhängig ist. Weiterhin wurde der Einfluss von neisseriellem Peptidoglycan auf die Apoptose der Neutrophilen mit Hilfe von Mutanten untersucht, wobei eine Verbindung nicht bestätigt werden konnte. Da die NSM2 die bedeutendste Sphingomyelinase in Neutrophilen darstellt, sowie vielfältige zellphysiologische Funktionen erfüllt und im Vorfeld schon mit der Apoptose in Verbindung gebracht wurde, wurde der Einfluss des Enzymes auf die Inhibierung der Apoptose durch die Gonokokkeninfektion mit Hilfe von Inhibitoren überprüft.

1 INTRODUCTION

1.1 *Neisseria gonorrhoeae*

The genus *Neisseria* belongs to the Neisseriaceae family within the class of β-proteobacteria and was first discovered by Albert Neisser in 1879. There are several commensal species which preferentially colonize the human mucosal surface of the nasopharynx. In rare cases some of these species can develop pathogenic potential and act as opportunistic pathogens (Johnson, 1983). *Neisseria gonorrhoeae*, besides *Neisseria meningitides*, is one of the two pathogenic species in the genus *Neisseria*. *N. gonorrhoeae* (gonococci) is a Gram-negative, aerobic or facultative anaerobic diplococcus with a diameter of 0.6 to 1 μm (Knapp & Clark, 1984).

1.1.1 Pathogenesis

N. gonorrhoeae is the causative agent of the sexual transmitted disease gonorrhea. The bacterium infects humans for thousands of years and is first mentioned in the Old Testament. Currently, gonorrhoea is the second most prevalent bacterial sexual transmitted disease worldwide with over 78 million new infections in 2012 (Unemo & Shafer, 2011, WHO, 2012). The infection manifests as urethritis is men and cervicitis in woman and can, if untreated, lead to infertility in both men and women. Although *N. gonorrhoeae*'s favored location for infection is the mucosal surface of the male urethra and the female cervix, it can also infect the conjunctiva of the eye, the throat and the rectum, rectal and pharyngeal infections being to a large extent asymptomatic in both sexes. While men suffer mostly from urethritis, epididymitis, and prostatitis about half of the infected women remain asymptomatic but are infectious and can transmit the bacterium. The consequences of an unrecognized and untreated infection can be serious complications like pelvic inflammatory disease (PID), ectopic pregnancy and infertility (WHO, 2016, Ndowa *et al.*, 2012). Typical symptoms in women are vaginal discharge, pelvic pain, dysuria and abnormal uterine bleeding, which can lead to ascending gonococcal infection. *N. gonorrhoeae* has the ability to spread within the human body, enter the bloodstream and cause disseminated gonococcal infection (DGI) if the infection is left untreated. Although with an incidence of 1–

3% DGI is a rare event, it may lead to severe conditions such as meningitis, arthritis and endocarditis (Kerle *et al.*, 1992).

The bacterium is transferred via contact with secretions from infected mucosal surfaces (Sarwal *et al.*, 2003). The most common transmission path is the sexual transmission. Besides, mother-to-child transmission can occur during birth and can lead to gonococcal conjunctivitis of the neonate which may cause blindness (Handsfield *et al.*, 1973, Ndowa *et al.*, 2012). In 10-30% of the cases patients suffering from *N. gonorrhoeae* show a co-infection with *Chlamydia trachomatis* (Lyss *et al.*, 2003). Furthermore, studies revealed that sexual transmitted diseases facilitate the transmission of HIV-1. Gonococci in particular increase the infectiousness of men on the one hand and enhance the acquisition of the virus on the other hand (Cohen *et al.*, 1997, Fleming & Wasserheit, 1999).

1.1.2 Diagnosis and therapy

The diagnosis of *N. gonorrhoeae* can be performed using two different methods: microbiological cultures and/or nucleic acid amplification tests (NAATs). The advantages of microbiological cultures are its low costs combined with a relatively high sensitivity of 85 – 95%. Additionally, the cultures provide information on antimicrobial susceptibility. The disadvantage of the method refers to the level of experience that is needed for the optimal isolation and handling of the bacteria. Compared to culture tests, NAATs show a higher sensitivity (over 90%). The extra benefit of this specific diagnostic test is the wide range of samples which can be tested. The method works with urine, vulvovaginal and cervical as well as urethral swabs (WHO, 2016, Cook *et al.*, 2005). In countries lacking laboratory diagnostic tests, diagnosis is often made clinically. In these cases, the presence of typical symptoms such as vaginal and urethral discharge is used as the basis for the diagnosis (WHO, 2016).

The disease is treated with antibiotics. The WHO recommends either a dual or a single therapy for the treatment of gonococcal infections. Due to the emerging resistances of *N. gonorrhoeae* against several antibiotics, it is useful to choose the treatment depending on the antimicrobial susceptibility (WHO, 2016). Unfortunately, a previous infection with Ngo does not protect the patient from getting reinfected. Even a reinfection with the same *Neisseria* strain that was responsible for the first infection is possible (Schmidt *et al.*, 2001).

The low immunity to reinfection after an uncomplicated infection could have different reasons. One possibility is the antigenic variation of the bacterium and its assumed capacity to avoid the effects of an immune response. Additionally, *N. gonorrhoeae* does not induce a strong humoral immune response during the first genital infection. In fact, uncomplicated infections do not seem to elicit the immunological memory at all (Hedges *et al.*, 1999). Together with the suppression or redirection of the immune response this could be a second reason for the low level of immunity to reinfection (Zhu *et al.*, 2011).

Vaccine development

Throughout the years, plenty of different attempts have been undertaken to obtain a functional vaccine. Already in 1975 field trials of a gonococcal whole cell vaccine started. The results indicated the vaccine as compatible with humans. Although antibodies were produced, the study lacked the evidence of the vaccine's ability to prevent gonorrhea (Greenberg, 1975). The pili of the Ngo appeared to be a good vaccine candidate. Even though there were promising animal studies and a small number of promising studies in humans, the vaccine showed poor immunogenicity and failed to initiate a sufficient antibody production (Tramont & Boslego, 1985). Another gonococcal pilus vaccine tested a few years later in a double-blind trial revealed to be safe and to have no influence on the clinical expression of the disease. Unfortunately, the vaccine did not succeed in protecting men against urethritis caused by gonococci (Boslego *et al.*, 1991).

The major outer membrane protein PorB has also been considered as an appropriate vaccine candidate. A DNA vaccine encoding PorB was used to immunize BALB/C mice either by intramuscular needle injection or by epidermal gene gun bombardment. The treated mice produced detectable specific antibodies independent of the delivery route used. The number of antibodies could be increased and the immune response prolonged via the combination of DNA priming with a protein or viral replicon particles (VRP) boost (Zhu *et al.*, 2004). Several research groups investigated the transferrin receptor system regarding its antigen potential. One group found that gonococcal TbpB (transferrin binding protein B) expressed form Venezuelan equine encephalitis viral replicon particles lead to the production of specific antibodies in mice. A requirement for the successful immunization was the expression with an eukaryotic secretion signal or boosting with recombinant protein

(Thomas *et al.*, 2006). Another team of researchers used the crystal structure of TbpA (transferrin binding protein A) to gain insights into its function and vaccine potential (Cash *et al.*, 2015). Meanwhile new methods like *in silico* hierarchical approaches are established to identify potential universal vaccine candidates (Jain *et al.*, 2016). Such quantitative proteomics-driven methods can be combined with two-dimensional liquid chromatography and mass spectrometry to gain knowledge about the bacterium and discover potential vaccine targets (Zielke *et al.*, 2016, Baarda & Sikora, 2015).

Unfortunately, until now, there is no successful vaccine against *N. gonorrhoeae*. The reasons for this are manifold, ranging from the variability of the antigens of the pathogen and a lack of proper animal models to the absent knowledge about what immune response induces a long lasting protection (Edwards *et al.*, 2016).

The development of a vaccine gets more urgent as untreatable gonococcal infections arise due to increasing antimicrobial resistances (Zielke *et al.*, 2016, Zhu *et al.*, 2011).

Antibiotic resistance

The first antibiotic used for the treatment of gonorrhea was penicillin. It has been successfully introduced for the therapy of sulfonamide-resistant gonorrhea in men (Van Slyke *et al.*, 1943). But after 10 to 15 years, the bacteria became resistant. In 1976, the first strains resistant against penicillin, ampicillin, and cephaloridine due to the production of ß-lactamase were verified (Phillips, 1976). Since then, *N. gonorrhoeae* has developed antimicrobial resistances to mainly all antibiotics introduced so far, such as aminoglycosides, macrolides, tetracyclines and fluoroquinolones. The mechanisms of those resistances are manifold. For example, the bacteria can decrease the uptake or increase the export of the antibiotics. They are able to inactivate antimicrobials or change the antimicrobial targets (Unemo, 2015). Today, treatment failures concerning third-generation cephalosporins have been reported. There are ceftriaxone resistant strains in Japan and bacteria, which are no longer susceptible to cefixime, in Europe (Ohnishi *et al.*, 2011, Unemo *et al.*, 2011). The superbug *N. gonorrhoeae* and, therefore, the disease gonorrhea remains a major global public health problem, which could become untreatable in the near future (Unemo *et al.*, 2016).

1.1.3 Virulence factors

Bacterial virulence factors mediate the colonization as well as the damage of host cell tissue. Those factors are necessary for the invasion of the host, the promotion of survival, immune evasion and transmission to a new host. *N. gonorrhoeae* expresses different virulence factors. The most important are reviewed below and include Type IV pili, which conduct the initial attachment of the bacteria to host cells. Opacity-associated (Opa) proteins represent another virulence factor, which enables intimate binding and engulfment. PorB$_{IA}$, a subtype of the major outer membrane protein PorB mediates the invasion into non-professional phagocytes. Other cellular factors that are important for the pathogenic potential of the bacteria are lipooligosaccharide (LOS), the IgA1 protease and the factor H binding protein.

Type IV Pili

Pili are long filamentous polymers on the surface of bacteria. Pathogenic *Neisseria* produce type IV pili. Those dynamic structures are normally up to several micrometers long and about 6 nm in diameter. They participate in many different functions, which are all based on fundamental mechanisms: fiber assembly and extension, fiber adhesion, fiber disassembly and retraction. The pili contribute to bacterial aggregation, twitching motility, the initial attachment to host cells and microcolony formation. Besides, the filaments are necessary for the transfer of genetic material. They are involved in transformation, conjugation and phage transduction (Merz & So, 2000, Heckels, 1989, Swanson *et al.*, 1971, Rudel *et al.*, 1995a).

The major component of pili is the subunit PilE, but there are 22 additional pilus-associated proteins, which are involved in pilus composition. *N. gonorrhoeae* possesses several pilus genes. There is one pilin expression locus (*pilE*), which includes a functional promotor, while multiple silent pilin loci (*pilS*) lack a promotor (Meyer *et al.*, 1984). RecA mediated homologous recombination between the expression locus containing the complete pilin gene and several different pilin gene copies (silent loci) results in antigenic variation or in a switch from a pilus positive to a pilus negative phenotype (Koomey *et al.*, 1987). The phenotypical transition from piliated to non-piliated gonococci leads to changes in colony morphology and can be observed using a binocoluar (Swanson *et al.*, 1971). Besides the

described antigenic and phase variation there are two more mechanisms leading to phase variation. One involves the expression of PilC, a protein which is essential for pilus assembly, the other one targets pilus synthesis (Jonsson *et al.*, 1991, Jonsson *et al.*, 1992, Gibbs *et al.*, 1989, Haas *et al.*, 1987).

PilC is not only necessary for pilus assembly, but is also essential for the transformation competence of *N. gonorrhoeae* (Rudel *et al.*, 1995a). Furthermore, PilC is located at the tip of the pilus and has been identified as the pilus adhesin of gonococci (Rudel *et al.*, 1995b). In contrast, there is still no definite agreement about the host cell receptor binding to the pilus. First, human CD46 (membrane cofactor protein MCP) has been described as the neisserial pilus receptor (Kallstrom *et al.*, 1997). But soon, CD46 was discussed controversially, because several studies indicated the proteins inability to serve as an adhesion receptor (Kirchner *et al.*, 2005, Tobiason & Seifert, 2001, Maisner *et al.*, 1996, Teuchert *et al.*, 1999). Until now, the question which receptor binds to the pilus remains unsolved, although there are suggestions that integrins containing an I-domain could bind to pili in urethral epithelial cells (Edwards *et al.*, 2002).

It is without controversy that pili enhance the initial attachment of gonococci to host cells (Swanson, 1973, Punsalang & Sawyer, 1973). But while adherence is mediated by pili, the invasion is actively inhibited. The bacterial uptake is blocked by phosphorylation of caveolin-1, which triggers cytoskeletal rearrangements under gonococcal microcolonies (Böttcher *et al.*, 2010). As soon as the natural pilus phase variation results in the loss of pili the bacteria are able to invade. Then they switch from adherence to invasion (Faulstich *et al.*, 2013).

Opa proteins

Opa proteins (opacity-associated proteins) are located in the membrane of *N. gonorrhoeae* and play an important role in the pathogenesis of the disease gonorrhea due to their contribution to adherence and invasion in human tissues (Bos *et al.*, 1997). Opa proteins can mediate the engulfment of the bacteria in epithelial cells as well as the opsonin-independent phagocytosis by professional phagocytes like neutrophils (Dehio *et al.*, 1998a, Belland *et al.*, 1992, Makino *et al.*, 1991). The name of the protein family derives from the opaque phenotype that gonococcal colonies show on translucent, solid medium. Ngo can express 11 different versions of the integral outer membrane protein (Swanson, 1978). The 11

corresponding genes are located in separate loci, each of them possesses its own promotor and the different alleles were probably evolved by different mechanisms like gene duplication, gene replacement and recombination events (Bhat *et al.*, 1991). All *opa* genes are constituvely transcribed, but the expression of the proteins is controlled on the translational level by a pentameric coding repeat sequence. Therefore, Opa undergoes phase variation. The expression of each single Opa protein can be controlled independently, which results in neisserial colonies composed of individuals expressing different Opa versions (Bhat *et al.*, 1991, Stern *et al.*, 1986).

Opa proteins are grouped based on their host cell receptor binding specificity. There are two different classes (Kupsch *et al.*, 1993): one class consists only of Opa_{50}, which targets heparin sulfate proteoglycans (HSPGs), whereas the other class includes all remaining Opa proteins (Opa_{51-60}). Those Opa proteins bind to carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) (Chen *et al.*, 1997).

Porin

Porin is the predominant protein in the outer membrane of *N. gonorrhoeae* (Johnston *et al.*, 1976). A trimer of the protein forms a voltage-dependent and anion-selective pore (Mauro *et al.*, 1988). The activity of the generated PorB channel is regulated by the interaction with purine nucleoside triphosphates. Cytosolic ATP/GTP leads to a down-regulation of the pore size, which results in changed voltage dependence and ion selectivity (Rudel *et al.*, 1996).

There are two serotypes of porin existing: $PorB_{IA}$ and $PorB_{IB}$. A single bacterium expresses either $PorB_{IA}$ or $PorB_{IB}$. The serotypes are antigenically distinct variants, which differ in the surface-exposed loops of the protein (McKnew *et al.*, 2003). 70 to 80% of all Ngo strains express $PorB_{IB}$, only 20 to 30% express $PorB_{IA}$ (Sun *et al.*, 2010, Il'ina *et al.*, 2003, McKnew *et al.*, 2003). While *N. gonorrhoeae* strains containing $PorB_{IB}$ are associated with local infections, $PorB_{IA}$-containing strains can invade the bloodstream and are linked to disseminating gonococcal infection (DGI) (Gotschlich *et al.*, 1987, Cannon *et al.*, 1983). 80% of all strains causing DGI are expressing $PorB_{IA}$ (Bash *et al.*, 2005). The possible reason is the increased resistance of gonococci to normal human serum due to $PorB_{IA}$ expression compared to gonococci expressing $PorB_{IB}$. $PorB_{IA}$ acts as acceptor molecule for factor H. This protein plays an important role in the down-regulation of the alternative complement

pathway in the human body. The binding of factor H to the 5th loop of PorB$_{IA}$ results in the strong reduction of C3b, which is converted to the inactive form iC3b. Therefore, the complement cascade is interrupted and the chances of the bacteria surviving in the human bloodstream are enhanced (Rice *et al.*, 1994, Ram *et al.*, 1998, Carbonetti *et al.*, 1990).

Another feature of the major outer membrane protein from Ngo is its capability to incorporate in foreign membranes. Thereby, it is not crucial if the targeted membrane is of natural or artificial origin (Young *et al.*, 1983, Weel & van Putten, 1991, Lynch *et al.*, 1984). The translocation of porin in host cell membranes can influence diverse cellular functions. For example, the phagosome maturation in macrophages is arrested due to incorporation of neisserial porin (Mosleh *et al.*, 1998). The impact of translocated porin on apoptosis is discussed controversially. On the one hand, different studies indicate a pro-survival effect on cells due to porin. The neisserial protein enhances the expression of host antiapoptotic factors in human urethral epithelial cells and translocated to mitochondria, the meningococcal PorB also guards cells from apoptosis (Binnicker *et al.*, 2004, Massari *et al.*, 2000). On the other hand, Müller *et al.* identified gonococcal PorB which interacts with mitochondria, to be responsible for the breakdown of the membrane potential and the release of cytochrome *c*, which finally leads to apoptosis (Muller *et al.*, 2000).

PorB$_{IA}$ has the ability to mediate adherence to and invasion into cells under low phosphate conditions. Those conditions also exist in the human bloodstream and could explain the connection to DGI. Structural and functional analysis of PorB$_{IA}$ revealed the importance of arginine on position 92 for phosphate binding and host-cell invasion. The residue is located at the extracellular exit of the channel and is considered to be responsible for the distinction of invasion-promoting porins compared to other neisserial porins (Zeth *et al.*, 2013). The host cell receptors involved in adherence and invasion under low phosphate conditions were identified as Gp96, a heat shock glycoprotein, and the scavenger receptor SREC-I (Rechner *et al.*, 2007).

LOS -lipooligosaccharide

Lipooligosaccharides are the major glycolipids of the neisserial outer-membrane (Griffiss *et al.*, 1988). The molecule is the analog to lipopolysaccharides (LPS), which is expressed by other Gram-negative families. LPS is highly conserved and composed of lipid A, the core

oligosaccharides and the O-antigen subunit. The lipid A structure of LPS and LOS are similar, but in contrast to LPS, LOS lacks the O-antigen subunit and the oligosaccharide structures contain at the most 10 saccharide subunits (Preston *et al.*, 1996). Neisserial LOS can be modified by antigenic variation resulting in evasion of the humoral immune response. Additionally, it can cause severe tissue damage (Danaher *et al.*, 1995). LOS is also able to bind to human receptors like asialoglyoprotein receptors (ASP-R) and can act as adhesin and promote invasion (Preston *et al.*, 1996, Harvey *et al.*, 2001, Porat *et al.*, 1995). Therefore, this molecule plays an important role in the pathogenesis of *Neisseria*.

IgA1 protease

N. gonorrhoeae produces a protease, which cleaves specifically human immunoglobulin IgA1. The secretion of the serin-protease is an important characteristic for the distinction of non-pathogenic versus pathogenic *Neisseria* (Pohlner *et al.*, 1987). Besides its main function to protect the bacteria against the human immune system by destruction of mucosal immunoglobulins, the enzyme has an additional task: it plays a role in the trafficking through epithelial barriers (Hopper *et al.*, 2000, Hagen, 2013). LAMP1 is a highly conserved major integral glycoprotein on the membranes of late endosomes and lysosomes, which possesses an IgA1 protease cleavage site. The neisserial protease cleaves LAMP1 and therefore supports intracellular survival, which also enables the transcytosis of the bacteria (Hauck & Meyer, 1997, Lin *et al.*, 1997).

1.2 Host pathogen interaction

A host and its specialized pathogen share a long evolutionary history, defined as arms race. The pathogen constantly tries to colonize the host, whereas the host tries to defend itself. Hence, humans developed a complicated system to identify and destroy pathogens, the immune system. On the other side, pathogens found various ways to evade the immune system using virulence factors. The arms race between the pathogen and the humans resulted in complex interactions that reach the molecular level. In the case of *N. gonorrhoeae* and the human host, neisserial virulence factors serve as ligand for host cell receptors and these interactions finally result in the engulfment of the bacteria by human cells.

1.2.1 HSPG-triggered uptake into epithelial cells

Heparin sulfate proteoglycans HSPGs are glycoproteins covalently bound to one or more heparan sulfate chains, which are expressed at the cell surface and in the extracellular matrix. They are able to interact with a broad range of ligands like growth factors, cytokines, chemokines, enzymes and extracellular matrix proteins (Sarrazin *et al.*, 2011, Esko *et al.*, 2009). The neisserial Opa_{50} proteins bind to HSPGs to enter epithelial cells (van Putten & Paul, 1995). However, different signaling pathways are triggered by the binding of Opa_{50} to the receptor according to the cell line. The Opa_{50}-mediated internalization of Ngo in Chang cells involves the activation of the acid sphingomyelinase and the phosphatidylcholine-specific phospholipase C. The activation of the enzymes leads to the release of diacylglycerol and ceramide and is critical for cytoskeletal rearrangements and the uptake of the gonococci (Grassmé *et al.*, 1997). In contrast, the Opa_{50}-mediated invasion in other cell lines like HEp-2, HeLa or CHO (Chinese hamster ovary) cells is dependent on bridging molecules. For example, the serum glycoprotein fibronectin binds to Opa_{50} and acts as a molecular bridge between the Opa-proteoglycan complex and host cell integrin receptors, facilitating bacterial uptake (van Putten *et al.*, 1998). Vitronectin serves as another bridging molecule, mediating the co-ligation of HSPG and integrin receptors and enabling the invasion involving protein kinase C (Dehio *et al.*, 1998b, Duensing & Putten, 1998).

1.2.2 Opa-mediated invasion via cellular CEACAM receptors

In contrast to Opa_{50}, all other Opa proteins (Opa_{51-60}) bind to carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) to trigger the uptake of *N. gonorrhoeae*. The twelve existing human CEACAMs are members of the CD66 antigen family (Chen *et al.*, 1997, Dehio *et al.*, 1998a), but not all of them are involved in neisserial invasion. Only CEACAM1, 3, CEA and CEACAM6 act as receptors for Opa proteins (Popp *et al.*, 1999, Bos *et al.*, 1997, Gray-Owen *et al.*, 1997). Thereby, the interaction of Opa proteins with epithelial receptors CEACAM1, 6 and CEA results in a different uptake mechanism than the interaction with CEACAM3, which is only expressed on human granulocytes (Voges *et al.*, 2012). The uptake by epithelial CEACAMs requires functional cholesterol-rich membrane microdomains and involves PI3K. In contrast, the opsonin-independent phagocytosis by human granulocytes

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through CEACAM3 is dependent on the activation of the Src family protein tyrosine kinase (PTK) and independent of cholesterol-rich membrane microdomains (Voges *et al.*, 2012, Schmitter *et al.*, 2007, Hauck *et al.*, 1998). Besides invasion into different cell types, the binding of Opa proteins to CEACAMs can trigger several cellular functions. For example, the Opa-CEACAM interaction can mediate the adherence and the traversal of gonococci across a polarized epithelial cell monolayer (Wang *et al.*, 1998). Additionally, it results in the suppression of the activation and proliferation of CD4$^+$ T lymphocytes (Boulton & Gray-Owen, 2002).

1.2.3 SREC-I/PorB$_{IA}$-mediated invasion

PorB$_{IA}$ mediates invasion into a variety of human cells including epithelial and endothelial cells under phosphate free conditions. The invasion is mediated by the binding of PorB$_{IA}$ to SREC-I (scavenger receptor expressed on endothelial cells), a type F scavenger receptor (Berwin *et al.*, 2004, Rechner *et al.*, 2007). SREC-I belongs to the group of scavenger receptors, a heterogeneous group of endocytic membrane receptors, which are able to bind a broad spectrum of different ligands, so called pattern recognition receptors (PRRs) (Krieger, 1997). The major task of the transmembrane receptor in the human body is the selective uptake of acetylated and oxidized LDL (low density lipoprotein) in endothelial cells (Adachi & Tsujimoto, 2002, Adachi *et al.*, 1997). Besides, SREC-I serves as a receptor for further substances like calreticulin, heat shock protein 90, zymogen granule protein 2 (GP2), protein phosphatase 1α and advillin (Berwin *et al.*, 2004, Murshid *et al.*, 2010, Holzl *et al.*, 2011, Ishii *et al.*, 2007, Shibata *et al.*, 2004). *N. gonorrhoeae* is not the only pathogen using SREC-I as key to human cells: *Klebsiella pneumoniae* binds to SREC-I with its outer membrane protein A (OmpA), and the NS3 protein of hepatitis C virus also uses SREC-I as endocytic receptor (Beauvillain *et al.*, 2010, Jeannin *et al.*, 2005). Neisserial PorB$_{IA}$ binds to SREC-I as well as to Gp96, a heat shock glycoprotein. But while SREC-I mediates the highly efficient uptake of PorB$_{IA}$-expressing gonococci in a phosphate dependent manner, Gp96 seems to be an anti-invasion factor, which strongly enhances adherence (Rechner *et al.*, 2007).

Although the signaling mechanisms involved in low-phosphate-dependent PorB$_{IA}$-mediated invasion were not resolved completely in 2006, investigators already claimed the pathway to be a general way for the bacteria to enter host cells distinct from Opa-mediated invasion.

The authors came to this conclusion due to the missing overlap in the signaling molecules involved in the different invasion pathways. While the formation of clathrin-coated pits and the activation of actin and Rho GTPases was necessary for PorB$_{IA}$-mediated invasion, proteins required for Opa-mediated invasion such as acid sphingomyelinase, microtubules, phosphatidylinositol 3-kinase, myosin light chain kinase and Src kinases are not involved in PorB$_{IA}$-mediated invasion (Kühlewein *et al.*, 2006). Meanwhile, the signaling cascade leading to the engulfment of PorB$_{IA}$-expressing *Neisseria* has been uncovered. The invasion is dependent on membrane rafts and the phosphorylation of caveolin-1-Y14. The binding of the gonococci to SREC-I results in the translocation of the receptor in membrane rafts and caveolin-1 is phosphorylated. Thereby, PI3K-p85 is recruited as a function of SREC-I and PLCγ. The activation of PI3K triggers a signaling pathway leading to the activation of PKD1 and Rac1 which finally initiates cytoskeletal rearrangements, membrane ruffling and the uptake of the bacteria (Faulstich *et al.*, 2013).
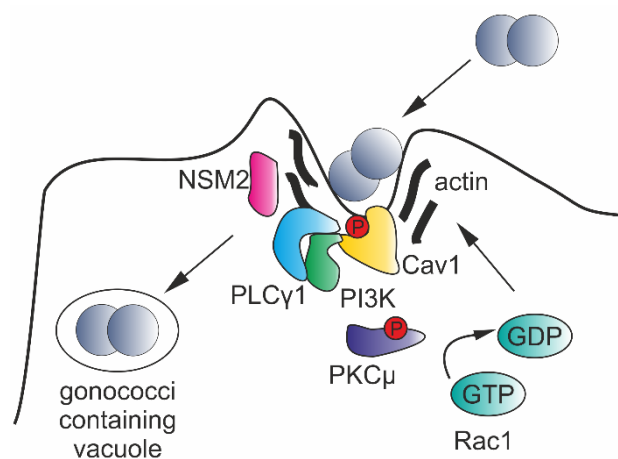
Figure 1.1: Model of the molecular events underlying the SREC-I/PorB$_{IA}$-mediated invasion pathway. Modified from (Faulstich *et al.*, 2013).

1.3 Neutrophils in neisserial infection

Neutrophils, which are also called polymorphonuclear leukocytes (PMNs), represent the largest group of white blood cells in humans and are generated in the bone marrow. The professional phagocytes are an important part of the human innate immune system because they build the first line of defense against infections. Hence, PMNs are recruited to sites of

infection and inflammation where they ingest invading microbes in order to kill and degrade them.

In the case of *N. gonorrhoeae*, the infected mucosa releases chemokines such as interleukin-8 (IL-8), which leads to the recruitment of PMNs to the side of infection and to their activation (Chen & Seifert, 2011, Ramsey *et al.*, 1995). Activated PMNs possess various mechanisms to kill bacteria including cytoplasmic granules containing antimicrobial enzymes, peptides and reactive chemicals like ROS (reactive oxygen species). The microorganisms are either phagocytosed and degraded intracellular by fusing the phagolysosome with the granules or killed extracellular by DNA-rich neutrophil extracellular traps (NETs) or granule exocytosis (Criss & Seifert, 2012, Urban *et al.*, 2006). On the other side, *Neisseria* evolved several defense mechanisms to survive the attacks of PMNs. Therefore, Ngo possess a Mn(II) uptake system (MntABC) which allows the bacteria to use Mn as a chemical quenching agent. The system is crucial for the resistance to oxidative stress induced by PMN ROS generation. Additionally, the bacteria express a catalase for detoxification and can even survive NET formation (Tseng *et al.*, 2001, Johnson *et al.*, 1993, Gunderson & Seifert, 2015). *In vitro*, more than 80% of the extracellular Ngo survive the incubation with neutrophils (Criss *et al.*, 2009, Criss & Seifert, 2012). Altogether, neutrophils are not able to clear initial neisserial infections by killing extracellular bacteria.

To prevent intracellular killing, the gonococci avoid phagocytosis by neutrophils. Therefore, they use antigenic variation to evade humoral immunity and limit the attachment of opsonic antibody or complement (Criss & Seifert, 2012). But the bacteria cannot prevent the binding to and phagocytosis by neutrophils completely. For example, the neisserial Opa protein binds to CEACAM receptors expressed on the surface of PMNs. Particularly CEACAM3 can mediate the efficient, opsonin-independent phagocytosis of *Neisseria* binding to the receptor. Therefore, gonococci expressing Opa proteins are at higher risk of being internalized and eliminated by PMNs compared to Opa-negative bacteria (Schmitter *et al.*, 2004, Pils *et al.*, 2008, Billker *et al.*, 2002).

The bacteria are ingested by the PMNs and stimulate the cells to produce a respiratory burst. But instead of getting killed by the NADPH oxidase activation after phagocytosis, some *Neisseria* survive and can even replicate within PMN phagosomes (Simons *et al.*, 2005, Casey *et al.*, 1986).

A characteristic feature of neutrophils is their short life span. They survive less than 24 hours in the bloodstream before they undergo apoptosis (McCracken & Allen, 2014, Nauseef, 2007). However, PMNs infected with gonococci show a delayed onset of apoptosis, eventually to gain time for replication (Simons *et al.*, 2006, Chen & Seifert, 2011). Taken together, *Neisseria* may benefit from the PMN recruitment in terms of disease-causing potential. The neutrophils could provide additional nutrients, create a protective niche and mediate the transmission of the bacteria in tissues deeper in the human body (Criss & Seifert, 2012).

1.4 Sphingolipids and associated enzymes

Sphingolipids are structural components, which are essential for and abundant in all eukaryotic cell membranes. Sphingolipids are composed of a sphingoid long-chain base backbone, which is linked to a fatty acid molecule through an amine bond (Takabe *et al.*, 2008, Haberkant *et al.*, 2016). For a long time, sphingolipids were thought to be inactive and stable, but meanwhile some of them proved to be biologically active molecules. Those active molecules include ceramide, sphingosine and sphingosine-1-phosphate, which all are crucial for the regulation of different cellular processes (Haberkant *et al.*, 2016, Garcia-Gil *et al.*, 2017). They are involved in cell proliferation, apoptosis, senescence, and intracellular trafficking, basically in central cell-signaling pathways (Hannun & Obeid, 2008, Haberkant *et al.*, 2016). The balance of the levels of the three biologically active sphingolipids was named "sphingolipid rheostat". This fine-tuned balance is able to determine cell fate and alterations of the sphingolipid profile can lead to the development of severe pathologies (Neubauer & Pitson, 2013, Garcia-Gil *et al.*, 2017, Spiegel & Milstien, 2003). Therefore, the bioactive sphingolipids as well as key enzymes involved in the synthesis of those molecules and important for this study are reviewed below.
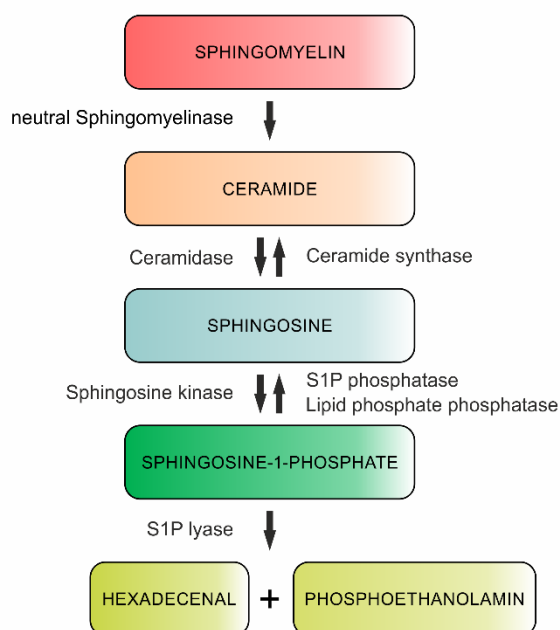
Figure 1.2: Sphingolipid pathway. Sphingolipids are shown with the key enzymes that catalyse their synthesis and degradation. Modified from (Neubauer & Pitson, 2013).

1.4.1 Sphingomyelinases

Sphingomyelinases (SMases) catalyze the hydrolysis of sphingomyelin (SM) to ceramide and phosphorylcholine. SMases are grouped in three major classes: acid, neutral, and alkaline SMases according to their pH optimum. Besides the pH optimum, the SMases can be discriminated due to the cofactors used, the tissue distribution, the mechanism for regulation and the cellular processes they contribute to (Liu *et al.*, 1997). The alkaline SMase is suggested to play a role in the breakdown of dietary sphingomyelin because it is secreted in the gut. Acid SMase (ASM) can be secreted too, but it is additionally a lysosomal enzyme whose absence or deficient activity results in Niemann-Pick disease (Clarke *et al.*, 2011). While activation of ASM is crucial for the entry of *N. gonorrhoeae* in non-phagocytic human cells by HSPG receptors (Grassmé *et al.*, 1997), the neutral sphingomyelinase (NSM), more precise NSM2 is required for the PorB$_{IA}$-mediated invasion of gonococci into host cells (Faulstich *et al.*, 2015). NSM2 is suggested to play a key role in stress-induced ceramide production, the activation of the "sphingomyelin cycle" in a regulated manner and in cell signalling (Tomiuk *et al.*, 1998, Clarke *et al.*, 2011).

The starting substance for the reaction catalysed by SMases is sphingomyelin. The phospholipid is abundant in plasma membranes and related organelles of mammalian cells.

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The amid linkage between the fatty acid moieties and sphingosine is symptomatic for SM (Barnholz *et al.*, 1966). Disturbances in sphingomyelin metabolism are connected to different diseases like atherosclerosis, cancer and genetically transmitted disease (Tomiuk *et al.*, 1998, Merrill & Jones, 1990). The NSM2 itself is linked to inflammation and cell death as well as further diseases like Alzheimer's, heart failure and ischemia/reperfusion damage (Philipp *et al.*, 2010).

Neutral sphingomyelinases represent a subset of the DNaseI superfamily with closely related isoforms. A major characteristic of the family are conserved catalytic core residues, which indicate a common mechanism for SM hydrolysis. Aside from the catalytic core residues, the individual NSMs differ strongly, but have hydrophobic domains in common. Another shared feature is the dependency on divalent ions, mostly Mg^{2+} and a neutral pH around 7.4 (Clarke *et al.*, 2011). Until now, four different neutral SMs have been identified in mammals. However, the NSM2 seems to play the most important role in cellular systems, physiologies and pathologies (Shamseddine *et al.*, 2015).

NSM2 is localized in the inner leaflet of the plasma membrane, the Golgi and recycling compartments (Milhas *et al.*, 2010a, Tani & Hannun, 2007a). The enzyme can traffic from the Golgi to the plasma membrane due to signalling stimuli and afterwards recycles back to the Golgi. This recycling step is associated with the catalytic regulation of the enzyme (Milhas *et al.*, 2010b). The localisation of the enzyme at the plasma membrane as well as its stability is dependent on the palmitoylation of the protein by thioester bonds at multiple cysteine residues (Tani & Hannun, 2007b). Another post-translational modification that plays a role for NSM2 is phosphorylation. The phosphorylation is mediated by the phosphatase calcineurin, which directly interacts with NSM2 and is suggested to act as a negative regulator of phosphorylation (Filosto *et al.*, 2010). Besides calcineurin, the protein EED (embryonic ectodermal development) binds to the NSM2 and regulates its activation. EED interacts with NSM2 as well as the TNF (tumor-necrosis factor) receptor in response to TNF binding. Therefore, the protein bridges the activation of the TNF receptor and the increased activity of the NSM2 (Philipp *et al.*, 2010). Furthermore, a peptide corresponding to the scaffolding domain of caveolin is suggested to directly interact with NSM resulting in the inhibition of the enzyme activity (Veldman *et al.*, 2001).

But the most important factor for the regulation of NSMs as far as it is known are anionic phospholipids (APL). All mammalian NSMs are regulated by APLs. The activation of NSM2 due to APLs *in vitro* is dose-dependent. The binding of APLs to NSM2 occurs at the N-terminus of the protein at two positively charged sites (Clarke *et al.*, 2011, Marchesini *et al.*, 2003, Wu *et al.*, 2011).

1.4.2 Ceramide

Ceramide is an important part of the membrane of mammalian cells and the precursor of all complex sphingolipids. The molecule consists of a sphingosine molecule which is linked to a fatty acid through an amide bond. It is formed by three major pathways: *de novo* synthesis, sphingomyelin hydrolysis, and the salvage pathway (Bikman & Summers, 2011, Mencarelli & Martinez-Martinez, 2013).

The molecule is an important structural component of the membrane, but it is also a biologically active molecule, which serves as a second messenger. Ceramide is involved in the regulation of cell fate, signal transduction, angiogenesis, inflammation and excitability. Generally, the molecule plays an important role in stress response, including cell-cycle arrest, cell senescence and apoptosis (Garcia-Gil *et al.*, 2017, Hannun & Obeid, 2011, Hannun & Luberto, 2000).

Increased concentration of ceramide due to TNFα and Fas ligand signalling result in DNA fragmentation and morphological changes that are characteristics of apoptosis (Cuvillier *et al.*, 1996). In general, ceramide is suggested to foster growth arrest and cell death (Garcia-Gil *et al.*, 2017). However, more and more studies reveal a far more complicated and differentiated function of ceramide in human cells. For example, different ceramide species defined by their acyl chain lengths seem to have different functions (Garcia-Gil *et al.*, 2017, Ben-David & Futerman, 2010). Hannun hypothesizes ceramide as a family of closely related molecules, which possess specific structural modifications. The highly complex ceramide metabolism involves different ceramide species. The regulation of those species, which conduct different functions is carried out by distinct subcellular compartments (Hannun & Obeid, 2011). Furthermore, ceramide can control the activity of multiple enzymes such as cathepsin D, phospholipase A2, PKC isoforms and other more (Garcia-Gil *et al.*, 2017).

Ceramide plays an essential role in different diseases such as diabetes/metabolic syndrome, neurodegeneration and cancer (Hannun & Obeid, 2011). Due to its ability to modify intracellular signalling pathways, ceramide can slow down the human anabolism. This links the molecule to diseases connected with obesity like cardiovascular disease and diabetes (Bikman & Summers, 2011). Additionally, ceramide is involved in several neurodegenerative diseases like HIV-induced dementia and Alzheimer's disease. Further, the neutral sphingomyelinase-ceramide pathway could play a role in the development of multiple sclerosis and an unusual high level of ceramide and other sphingolipids was reported in an amyotrophic lateral sclerosis (ALS) mouse model. Furthermore, there is evidence of a potential role of ceramide in depression pathophysiology (Dinoff *et al.*, 2017). Besides, a correlation between C24-sphingolipids and aging was observed (Ben-David & Futerman, 2010).

Ceramide is also of great importance for several pathogens in terms of infection and host-pathogen interaction. In most cases, the ASM is involved as producer of ceramide. Examples of pathogens are *N. gonorrhoeae*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Mycobacteria* and *Pseudomonas aeruginosa* (Grassme *et al.*, 2008). But the production of ceramide does not have the same effect on or consequences for all bacteria. In Ngo, the production of ceramide as a result of ASM or NSM2 activation is essential for the uptake of the bacteria in human epithelial cells (Grassme *et al.*, 1997, Faulstich *et al.*, 2015). Also in *Mycobacteria*, the ASM is crucial for a successful infection in an ASM-deficient mouse model (Utermohlen *et al.*, 2008). In contrast, ASM-deficient mice and macrophages facilitate and enhance infection with *Listeria monocytogenes* (Grassme *et al.*, 2008).

There is evidence that ceramide-enriched membrane domains are built due to ceramide production in biological membranes. Those membrane domains may contribute to the organisation of receptors and signalling molecules (Gulbins *et al.*, 2004). The uptake of *Pseudomonas aeruginosa* is dependent on the establishment of those large signalling platforms resulting from ASM activity and ceramide release (Grassmé *et al.*, 2003). Additionally, the connection between membrane rafts and infection has been described for gonococci expressing PorB$_{IA}$. There, the membrane raft signalling platforms are suggested to be stabilized by ceramide, which is produced by NSM2 activity (Faulstich, Dissertation 2013).

1.4.3 Sphingosine

Sphingosine is a part of the cell membrane, a biological active molecule and represents the backbone component for all sphingolipids (LaBauve & Wargo, 2014, Hannun & Bell, 1989a). It is produced by the cleavage of fatty acid from ceramide. The reaction is executed by ceramidases. Until now, five different human ceramidases were identified. The classification of the enzymes is conducted due to their optimal pH for catalytic activity (Coant *et al.*, 2017). Sphingosine can be generated in different compartment, such as lysosomes and the plasma membrane as well as in the Golgi apparatus and the endoplasmic reticulum (ER) (Bernardo *et al.*, 1995, Hwang *et al.*, 2005, Mao *et al.*, 2001).

Sphingosine belongs to the group of sphingolipids and fatty acids, which are necessary to guarantee the functionality of several skin functions including the physical barrier, permeability barrier and immunological barrier (Cameron *et al.*, 2007, Jungersted *et al.*, 2008). The molecule is not only an important antimicrobial substance on the human skin, but it also fulfils tasks in cell physiology and pathology. Sphingosine mediates cell growth arrest and promotes apoptosis by multiple mechanisms acting in concert (Cuvillier, 2002, Suzuki *et al.*, 2004).

For example, sphingosine can modulate and inhibit protein kinase C, a key enzyme in signal transduction and cell regulation (Hannun & Bell, 1989b, Smith *et al.*, 1997). Besides, it inhibits several calmodulin-dependent enzymes, therefore, sphingosine is an inhibitor of the phosphatidylinositol signalling pathway (Jefferson & Schulman, 1988). In relation to its potential of mediating apoptosis, sphingosine regulates stress- and mitogen-activated protein kinases (SAPK and MAPK) and can inhibit AKT kinase (Jarvis *et al.*, 1997, Chang *et al.*, 2001). Furthermore, intracellular sphingosine regulates the release of calcium from acidic stores like lysosomes in a positive manner, which promotes autophagy (Hoglinger *et al.*, 2015).

Sphingosine is also associated with different diseases. It plays a role in the pathophysiology of the Niemann-Pick disease type C (NPC). The progressive neurodegenerative disease is caused by a lysosomal storage disorder, which results in the accumulation of sphingosine and other lipids (Hoglinger *et al.*, 2015, te Vruchte *et al.*, 2004). In contrast, the sphingosine

levels in patients suffering from cystic fibrosis were significantly reduced, resulting in a higher susceptibility to infections by *Pseudomonas aeruginosa*. Hence, pulmonary *P. aeruginosa* infections are prevented by luminal sphingosine in healthy individuals (Pewzner-Jung *et al.*, 2014, Seitz *et al.*, 2015).

1.4.4 Sphingosine kinases

Sphingosine kinases (SPHK) catalyze the phosphorylation of sphingosine, producing sphingosine-1-phosphate. The first human SPHK was purified and characterized in the year 2000 (Pitson *et al.*, 2000). Meanwhile, two different sphingosine kinases were identified in mammals: SPHK1 and SPHK2 (Alemany *et al.*, 2007). The two enzymes belong to the diacylglycerol kinase family and contain five conserved domains, which mediate substrate binding and catalytic activity (Strub *et al.*, 2010, Liu *et al.*, 2002). The two isoforms are to a large extent homologous, but SPHK2 possesses about 240 additional amino acids in its amino terminal and central region (Liu *et al.*, 2000). The catalytic domain of SPHKs is unique and includes a consensus sequence of an ATP-binding site (Spiegel & Milstien, 2003, Pitson *et al.*, 2002).

The isozymes show differences in several areas. For example, they differ in their developmental and tissue expression patterns in mammals and have different kinetic properties (Spiegel & Milstien, 2003). They also are differently distributed in the tissue and show diverse subcellular locations. While SPHK1 is mainly located in the cytosol, SPHK2 resides primarily in intracellular compartments like the nucleus, endoplasmic reticulum, and mitochondria, apart from its cytosolic localization (Johnson *et al.*, 2002, Igarashi *et al.*, 2003). Furthermore, the two enzymes prefer different substrates and conditions. While SPHK1 preferably catalyzes D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine, SPHK2 also favours phytosphingosine, DL-*threo*-dihydrosphingosine and FTY720 as starting material (Liu *et al.*, 2000, Billich *et al.*, 2003). Also salt and detergents have an opposed effect on the activity of the enzymes (Liu *et al.*, 2000). All the mentioned differences indicate different responsibilities of the two isozymes SPHK1 and SPHK2 concerning cellular function, which might also concern the regulation of the enzymes (Spiegel & Milstien, 2003).

In general, SPHKs can be activated by G-protein coupled receptors, receptor tyrosine kinases, immunoglobulin receptors, cytokines and other stimuli (Alemany *et al.*, 2007). Until now, the regulation of the SPHK1 is investigated in more detail compared to that of SPHK2. SPHK1 can be activated by various growth factors including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor, steroid hormone (estradiol), cytokines like TNFα, GPCR ligand (acetylcholine), lysophosphatidic acid, and many other factors, as well as by its own product S1P (Takabe *et al.*, 2008). The activation can occur by several mechanisms including the interaction with acidic phospholipids or the association with other proteins. Another important mechanism is the phosphorylation of the enzyme and its translocation to the plasma membrane. The translocation brings the enzyme in close proximity to its substrate sphingosine and is mediated by protein kinase C (PKC). Thereby, the affinity of the enzyme to the membrane as well as the plasma membrane selectivity and its activity is enhanced by the phosphorylation of Ser^{225} (Takabe *et al.*, 2008, Johnson *et al.*, 2002, Stahelin *et al.*, 2005, Pitson *et al.*, 2003).

In contrast, the knowledge about the regulation of SPHK2 is comparatively small. Like SPHK1, SPHK2 can be activated by phosphorylation. In this case, the phosphorylation is triggered by the epidermal growth factor (EGF) as well as phorbol ester, which activates protein kinase C. The actual process is performed by ERK1 at Ser^{351} and Thr^{578} and results in increased enzyme activity (Hait *et al.*, 2007). Other studies revealed protein kinase D (PKD) as the enzyme mediating SPHK2 phosphorylation. The post-translational modification regulates the localization of the enzyme, resulting in its nuclear export for subsequent cellular signalling (Ding *et al.*, 2007).

Two different SPHKs are functioning in an opposing way in the sphingolipid metabolism. In general, SPHK1 promotes cell growth and survival, while SPHK2 inhibits growth and enhances apoptosis (Maceyka *et al.*, 2005). The promotion of cell growth and survival connects SPHK1 to several aspects of cancer development and progression, including proliferation, migration, invasion and angiogenesis (Liu *et al.*, 2013, Gault & Obeid, 2011). The functional role of SPHK2 and its connection to cancer is still discussed controversially, but evidence increases that SPHK2 is able to physiologically perform pro- as well as anti-survival functions in cells. The regulation still has to be investigated, but the subcellular

localization of SPHK2 is assumed to have a great impact (Neubauer & Pitson, 2013). Besides cancer related studies, recent investigations revealed a potential role of the nucleic enzyme in histone acetylation, gene expression, and cancer pathology by the binding of sphingosine-1-phosphate (S1P) to HDAC (Liu *et al.*, 2013, Hait *et al.*, 2009). Furthermore, SPHK2 is important for the respiratory function of mitochondria (Strub *et al.*, 2011). The apoptosis-inducing properties of SPHK2 involve its catalytic activity as well as its localization to the ER during stress (Maceyka *et al.*, 2005).

SPHKs balance the levels of pro-apoptotic ceramides and mitogenic S1P. Therefore, they are investigated as therapeutic targets for cancer using inhibitor studies (Gao *et al.*, 2012). SPHK1 is already known to play a role in oncogenesis. The expression of the enzyme is upregulated in many human solid cancers and haematological malignancies and the increased expression shows a correlation with severity of malignancy, chemotherapeutic resistance and shorter patient survival (Pitman *et al.*, 2016). New studies also reveal the involvement of SPHK2 in tumor diseases. The enzyme is suggested to play a role in T-cell and B-cell acute lymphoblastic leukemia (Pyne *et al.*, 2016a). Besides cancer, both isozymes are potentially connected to the central nervous system, the cardiovascular system, inflammation and diabetes (Pyne *et al.*, 2016b).

Many different pathogens including virus, bacteria, protozoa and fungi manipulate SPHKs during infection resulting in increased or decreased sphingosine-1-phosphate production. Thereby, the pathogens either inhibit the enzyme or manipulate it to increase or decrease the enzymatic activity in order to enhance their infection (Arish *et al.*, 2015).

1.4.5 Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid, which is exclusively produced from the precursor sphingosine by sphingosine kinases. It plays an important role in intracellular signalling as a second messenger, but it is also secreted. It can bind to S1P receptors and induce both autocrine and paracrine signalling (Takabe *et al.*, 2008, Spiegel & Milstien, 2003). Thereby, the secretion of S1P is probably mediated by ABC transporters, a large family of transporters characterized by their ATP-binding cassette. The balance between synthesis and degradation of the sphingolipid is the key for the regulation of the

S1P levels (Takabe *et al.*, 2008). The molecule is catabolized by either sphingosine-1-phosphate phosphatase or sphingosine-1-phosphate lyase. Together with SPHKs, these enzymes control the intracellular level of S1P (Bourquin *et al.*, 2010).

The level of S1P in the human body differ. Whereas the concentration in tissue is low, between 0.5 and 75 pmol/mg (Edsall & Spiegel, 1999, Schwab *et al.*, 2005), the concentration in fluids like plasma and serum is much higher. In plasma the concentration ranges from 0.2 to 0.9 µM and in serum even from 0.4 to 1.1 µM (Murata *et al.*, 2000, Caligan *et al.*, 2000, Berdyshev *et al.*, 2005).

S1P is linked to various important cellular processes including invasion, angiogenesis, and vascular maturation, as well as cytoskeletal rearrangements and cell motility (Takabe *et al.*, 2008). Additionally, it plays a role in the migration in connection to the trafficking of immune cells (Spiegel & Milstien, 2003, Cyster, 2005, Rosen & Goetzl, 2005). The major tasks of S1P which were discovered first, include the regulation of the cell growth (Olivera & Spiegel, 1993, Zhang *et al.*, 1991) and the suppression of apoptosis (Cuvillier *et al.*, 1996).

As mentioned above, S1P binds to a family of G protein-coupled receptors (GPCR). Five different receptors are known, which are termed $S1P_1$ to $S1P_5$. The specific expression pattern in different cell types as well as the differential coupling of the S1P receptors to several heterotrimeric G-proteins allows the specific regulation of the many physiological processes in which S1P is involved (Takabe *et al.*, 2008, Strub *et al.*, 2010). The small G-proteins to which S1P receptors are coupled can activate various downstream signalling molecules. Those signalling molecules include protein kinase C (PKC), phospholipase C (PLC), extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) (Arish *et al.*, 2015, Xiang *et al.*, 2013). Besides the autocrine and paracrine signalling mediated by S1P receptors, S1P can act as an intracellular second messenger. However, in some cases, it is unclear, whether the effect of S1P can be traced back on intracellular S1P or if internalized G protein coupled receptors are involved (Blom *et al.*, 2010).

S1P is associated with different diseases in humans, including insulin resistance, hyperglycaemia, obesity, dyslipidaemia and hypertension. There is also evidence of an involvement in the so called metabolic syndrome, but the role of S1P in the syndrome is discussed controversially (Chen *et al.*, 2016). Recent studies revealed a connection between S1P and sphingosine kinases with breast cancer and inflammation (Pyne *et al.*, 2016a, Pyne

et al., 2016b). In other types of cancer, S1P is involved in the proliferation, survival, movement and invasion of cancer cells. Besides cancer, the sphingolipid is linked to allergic response, atherosclerosis and autoimmune disease like multiple sclerosis. Because the molecule and its signalling is involved in so many serious medical conditions, S1P signalling is investigated as a therapeutic target (Takabe *et al.*, 2008).

There is increasing evidence suggesting a dysregulation of the S1P metabolism and signalling during infectious diseases. The pathogens seem to impair the S1P metabolism by activating or deactivating SPHKs and therefore increasing or decreasing the S1P production. Pathogens changing the S1P signalling include viruses, bacteria, protozoa and fungi. In general, the microbial agents target the S1P signalling pathway to promote the survival of infected cells in order to gain time for replication. However, some pathogens impair the S1P pathway including the downstream signalling to evade the immune response. Bacterial infections which influence the S1P metabolism are for example caused by *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Yersinia pestis*, *Clostridium perfringens*, and *Pseudomonas aeruginosa* (Arish *et al.*, 2015).

1.4.6 Sphingosine-1-phosphate lyase

The sphingosine-1-phosphate lyase (S1PL), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyzes the last step in sphingolipid breakdown by cleaving S1P at the C2-C3 carbon bond. The irreversible reaction results in the formation of ethanolamine phosphate and hexadecanal. Besides its function as a regulator of the S1P level, the enzyme represents a gatekeeper in the lipid metabolic flow, because it is the only exit point for sphingolipid intermediates (Aguilar & Saba, 2012). The S1PL gene was first cloned in the yeast *Saccharomyces cerevisiae*. A few years later, also the human S1PL was cloned (Gerl *et al.*, 2016, Saba *et al.*, 1997, Van Veldhoven *et al.*, 2000, McLean *et al.*, 2017). In mammals, the enzyme is located at the endoplasmic reticulum. Thereby, the hydrophilic domain of the integral membrane protein contains the active site and faces the cytosol (Van Veldhoven & Mannaerts, 1991, Ikeda *et al.*, 2004).

S1P is associated with many different diseases in humans. Therefore, the enzymes involved in biosynthesis and degradation of the sphingolipid receive more and more attention as

potential drug targets (Fyrst & Saba, 2010). For example, Novartis recently introduced an active-site inhibitor of S1PL for the treatment of multiple sclerosis (Weiler *et al.*, 2014). Additionally, autoimmune disease could be treated with S1PL inhibitors for achieving immunosuppression, because S1PL plays a crucial role in the regulation of the immune system (Aguilar & Saba, 2012, Bourquin *et al.*, 2010). Furthermore, the S1PL is suggested to play a role in the response to tissue injury. Ischemia, radiation and chemical injury in other tissues results in the upregulation and activation of the enzyme in mice (Fyrst & Saba, 2010). Finally, the enzyme might play a role in cancer surveillance and tumor-suppressor pathways (Bourquin *et al.*, 2010, Oskouian *et al.*, 2006).

There are some bacteria known, which possess their own S1PL homolog, including *Burkholderia pseudomallei* and *Legionella pneumophila*. Recent studies try to elucidate the role of the enzymes in bacterial metabolism as well as in the host-microbe interaction. The S1PL homolog of *B. pseudomallei*, an intracellular Gram-negative pathogen, is suggested to degrade host sphingolipids (McLean *et al.*, 2017). *L. pneumophila*, another intracellular pathogen, uses its S1PL homolog as a virulence factor which targets the host cell mitochondria. Additionally, the effector protein disrupts the host sphingolipid biosynthesis in order to inhibit autophagy (Degtyar *et al.*, 2009, Rolando *et al.*, 2016).

1.5 Aim of the work

PorB$_{IA}$-expressing *N. gonorrhoeae* strains are associated with the development of DGIs, which can cause severe health problems (Morello & Bohnhoff, 1989, Kerle *et al.*, 1992). The phosphate-sensitive invasion of these strains through an interaction with the cellular receptor SREC-I is dependent on NSM2 (Rechner *et al.*, 2007, Faulstich *et al.*, 2015). The present study aims to define the impact of NSM2 and investigate the role of other members of the sphingolipid pathway on PorB$_{IA}$-mediated invasion.

Therefore, the involvement of the NSM2 was analyzed by inhibitor studies. In order to obtain suitable tools for the elucidation of the role of NSM2 in gonococcal invasion, antibodies directed against NSM2 were produced and a NSM2 knockout cell line was generated using the CRISPR/Cas9 system.

In the last years, the awareness of the importance of sphingolipids as bioactive molecules and the whole "sphingolipid rheostat" for human cells rose dramatically (Hannun & Obeid, 2008). As NSM2 is a part of the sphingolipid pathway, the question if other members of this pathway, both enzymes and sphingolipids, play a role in SREC-I/PorB$_{IA}$-mediated invasion were addressed in this study. For this purpose, different approaches were applied including inhibitor as well as siRNA studies. Furthermore, different knockout cell lines were used and lipidome analysis were conducted to address the research question and identify components of the sphingolipid pathway involved in gonococcal invasion.

Former studies revealed the inhibitory effect of gonococci on apoptosis in neutrophils (Simons *et al.*, 2006). Until now, the exact neisserial factor resulting in the delay of apoptosis is unknown. Thus, I tested different peptidoglycan mutants for their ability to inhibit apoptosis. Additionally, I aimed to elucidate the role of NSM2 in this context using inhibitor studies.

2 MATERIAL & METHODS

2.1 Material

2.1.1 Bacterial strains

Table 2.1: Bacterial strains

strain	phenotype	Genotype/Plasmid	Source
DG 005	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, Δ $amiC$	(Garcia & Dillard, 2006)
DG 122	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, comp. $amiC$	(Garcia & Dillard, 2006)
DG 132	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, Δ $ampG$	(Garcia & Dillard, 2008)
DG 133	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, comp. $ampG$	(Garcia & Dillard, 2008)
KH 560	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, Δ $ltgA$ $ltgD$	(Cloud-Hansen et $al.$, 2008)
KH 567	PorB$_{IB}$, Pili$^+$, Opa$^-$	MS11, comp. $ltgA$ $ltgD$	(Cloud-Hansen et $al.$, 2008)
MS11	PorB$_{IB}$, Pili$^+$, Opa$^-$	$porB$	Our lab
N138	PorB$_{IB}$, Pili$^+$, Opa$^-$		(Haas et $al.$, 1987)
N924	PorB$_{IB}$, Pili$^-$, Opa$^-$	ΔPilE1/2, cat<porB$_{IA}$< >ermC	Bauer, Dissertation 1997
N927	PorB$_{IA}$, Pili$^-$, Opa$^-$	ΔPilE1/2, cat<porB$_{IA}$< >ermC	Bauer, Dissertation 1997
N931	PorB$_{IB}$, Pili$^-$, Opa^{50}	ΔPilE1/2, cat<porB$_{IB}$< >ermC pTH6a (opa50)	Bauer, Dissertation 1997

Arrowheads indicate 5' end (> or <) and 5' to 3' orientation (>) of genes.

E. coli strains DH5α, BL21 and XL1 Blue were used for amplification of plasmids.

2.1.2 Cell lines

Table 2.2: Cell lines

Cell line	properties	media	source
Chang	Human conjunctiva epithelial cells	RPMI 1640, 10% FCS	ATCC CCL20.0
CRISPR/Cas9 NSM2 KO clones 4, 7, 11	Chang cells, CRISPR/Cas9 knockout of NSM2	RPMI 1640, 10% FCS	This study
End1/E6E7	Human epithelial cervix cells	Defined K-SFM, Supplement	ATCC CRL-2615
HEK 293T	Human kidney epithelial cells	DMEM, 10% FCS	ATCC CRL-11268
HeLa ΔS1PL	HeLa (ATTC), CRISPR/Cas9 knockout of S1PL	RPMI 1640, 10% FCS	Britta Brügger (Gerl et $al.$, 2016)
HeLa 2000	Human epithelial cervical carcinoma cells	RPMI 1640, 10% FCS	
HeLa 229	Human epithelial cervical carcinoma cells	RPMI 1640, 10% FCS	ATCC CCL-227

HeLa WT	HeLa (ACCT), CRISPR/Cas9 control	RPMI 1640, 10% FCS	Britta Brügger (Gerl *et al.*, 2016)
MEF SK1-/-	Mouse embryonic fibroblasts, SK1 knockout	DMEM, 20% FCS	Andrea Huwiler (Schwalm *et al.*, 2015)
MEF SK1+/+	Mouse embryonic fibroblasts, control	DMEM, 20% FCS	Andrea Huwiler (Schwalm *et al.*, 2015)
MEF SK2-/-	Mouse embryonic fibroblasts, SK2 knockout	DMEM, 20% FCS	Andrea Huwiler (Schwalm *et al.*, 2015)
shNSM2-GFP clones 3, 10, 7.4, 7.12, 11.1, 11.9	CRISPR/Cas9 NSM2 knockout, complementation of NSM2-GFP using shRNA	RPMI 1640, 10% FCS	This study

2.1.3 Plasmids

Table 2.3: Plasmids

Plasmid	Properties	Source
16 CRISPR/Cas9: NSM2 guideRNA3 Klon1	pSpCas9(BB)-2A-GFP (PX458) with guideRNA3 insert	This study
17 CRISPR/Cas9: NSM2 guideRNA4 Klon1	pSpCas9(BB)-2A-GFP (PX458) with guideRNA 4 insert	This study
20 SK1-GFP	Sphingosine kinase 1 coupled to GFP in pcDNA3	Dagmar Meyer zu Heringdorf (Pitson *et al.*, 2003)
21 SK2- YFP	Sphingosine kinase 2 coupled to YFP in pEYFP-C1	Dagmar Meyer zu Heringdorf (Alemany *et al.*, 2007)
277 pcDNA3-GFP-S	pcDNA3 vecctor containing GFP (C-terminus)	Elke Maier
464 NSM2-GFP pLVTHM	shRNA expression vector (Addgene plasmid 12247) containing NSM2-GFP	Elke Maier
5 NSM2-GFP	Human NSM2 in pcDNA3-GFP-S	This study
pCMV-VSV-G	Viral envelope vector, Addgene plasmid 8454	(Stewart *et al.*, 2003)
psPAX	Viral packaging vector, Addgene plasmid 12260	(Wiznerowicz & Trono, 2003)
pSpCas9(BB)-2A-GFP (PX458)	CRISPR/Cas9 plasmid, Addgene plasmid 48138	(Ran *et al.*, 2013)

2.1.4 Oligonucleotides

Table 2.4: Oligos for siRNA knockdown

Oligo name	Manufacturer/Sequence
Accell Human SPHK2 siRNA – SMART pool	Dharmacon
On-TARGET plus Human SPHK1 siRNA – SMART pool	Dharmacon

Table 2.5: Primer qRT-PCR

Oligo name	Sequence
58 NSM1-qRT for	CTCTACCCACGGACCAGCAGAG
59 NSM1-qRT rev	CCTCCTCCAGCCGCCAGGACAC
60 NSM3-qRT for	TCAACCACAGATTTGCAGGACAG
61 NSM3-qRT rev	AGCGCAGGCTGAGCCTGGGGC
62 SPHK1-qRT for	CATTATGCTGGCTATGAGCAGG
63 SPHK1-qRT rev	CTAGGTCCACATCAGCAATGAAG
64 SPHK2-qRT for	GCACGGGGGCTGTATCC
65 SPHK2-qRT rev	GCTGGCAATGAGACCACGA
66 GAPDH-qRT for	GAAATCCCATCACCATCTTCCAGG
67 GAPDH-qRT rev	GAGCCCCAGCCTTCTCCATG
NSM2-qRT2forw	GCCTCCTCTTTGCCAGCCGCTA
NSM2-qRT2rev	CATGCAGGTGTGTGCAGGCGAT

Table 2.6: Oligonucleotides for CRISPR/Cas9 guide RNA

Oligo name	Sequence
guideRNA3 oligo1 NSM2	CACCGGAGTACATCCTGTACGACGT
guideRNA3 oligo2 NSM2	AAACACGTCGTACAGGATGTACTCC
guideRNA4 oligo1 NSM2	CACCGCATCTACCTGGCCCTCCTGG
guideRNA4 oligo2 NSM2	AAACCCAGGAGGGCCAGGTAGATGC

2.1.5 Antibodies

Table 1.7: Primary antibodies for flow cytometry (FACS), immunoblotting (IB) and immunofluorescence (IF)

Antibody	Origin	Application/Dilution	Manufacturer
Actin-β (AC-15)	Monoclonal mouse IgG1	IB 1:3000	Sigma Aldrich A5441
AKT	Monoclonal rabbit IgG	IB 1:1000	Cell signaling 4685
C16/C24 Ceramide	Rabbit IgG	IF 5 µg/ml (dSTORM)	Bieberich, E. (Krishnamurthy et al., 2007)
Ceramide (MID 15B4)	Monoclonal mouse IgM	IF 1:1000, FACS 1:50	ENZO ALX-804-196
GFP	Polyclonal rabbit IgG	IB 1:2000	Gene Tex GTX 113617
His-probe (H-3)	Monoclonal mouse IgG$_1$	IB 1:3000	Santa Cruz sc-8036
IgM isotype control	Mouse IgM	FACS 1:50	Invitrogen
κ Isotype control, purified mouse IgM	Monoclonal mouse IgM	IF 1:100, FACS 1:50	BD Pharmingen™

N. gonorrhoeae	Polyclonal rabbit	IF 1:200	US Biological N0600-02
NSM2 peptide antibody	Polyclonal rabbit	IB 1:100	Davids Biotechnology (self-made)
NSM2-1	Polyclonal rabbit	IB 1:100	Davids Biotechnology (self-made)
pAKT (Thr308)	Monoclonal rabbit IgG	IB 1:1000	Cell signaling 2965
SPHK1	Polyclonal mouse	IB 1:1000	Abnova
SPHK1	Polyclonal rabbit	IB 1:1000	Abcam ab71700
SPHK2	Polyclonal rabbit	IB 1:1000	Abcam ab37977
SPHK2	Polyclonal rabbit	IB 1:1000	Santa Cruz sc-366378
SREC-I/SCARF1	Polyclonal goat IgG	IB 1:1000	R&D

Table 2.8: Secondary antibodies

Antibody	Source	Application/Dilution	Manufacturer
Alexa Fluor® 594 Goat Anti-mouse IgM (µ chain)	goat	IF 1:100	Life technologies
Anti-rabbit Cy2™ linked	goat	IF 1:100	Dianova
Anti-rabbit Cy3™ linked	goat	IF 1:100	Dianova
Anti-rabbit Cy5™ linked	goat	IF 1:100	Dianova
Anti-rabbit F(ab')2 Alexa Fluor 647	goat	IF 10 µg/ml (dSTORM)	Life Technologies
ECL™ anti-goat IgG HRP linked	donkey	IB 1:3000	Santa Cruz sc2020
ECL™ anti-mouse IgG HRP linked	goat	IB 1:3000	Santa Cruz sc2005
ECL™ anti-rabbit IgG HRP linked	goat	IB 1:3000	Santa Cruz sc2004

2.1.6 Kits

Table 2.9: Commercial Kits

Kit	Manufacturer
AxyPrep™ Plasmid Miniprep Kit	Axygen Biosciences
GeneJET™ Gel Extraction Kit	Fermentas
miRNeasy Micro Kit	Quiagen
NucleoBond®PC100	Machery Nagel
NucleoSpin® Tissue	Machery-Nagel
pGEM®-T and pGEM®-T Easy Vector Systems	Promega
RevertAid™ Premium First Strand cDNA Synthesis Kit	Fermentas
RNase-Free DNase Set	Quiagen

2.1.7 Buffers, solutions and media

Table 2.10: Media and solutions for cell culture

Medium/Chemicals	Manufacturer
Cell stocking medium	FCS, 10% DMSO (v/v)
Defined K-SFM	GIBCO
DMEM	Sigma-Aldrich
DPBS	GIBCO
Fetal calf serum (FCS)	PAA
Opti-MEM®	GIBCO
RPMI 1640	GIBCO
TrypLETM Express	GIBCO

Table 2.11: Buffers for SDS-PAGE and Immunoblotting

Buffer	Ingredients
10% SDS lower gel solution	for 10 ml: 3.3 ml acrylamide, 2.5 ml lower buffer, 4.1 ml H_2O, 100 µl 10% (w/v) APS, 10 µl TEMED
10x SDS Electrophoresis buffer (1 l)	30.25 g Tris, 144 g glycine, 10 g SDS
10x Semi Dry buffer (1 l)	24 g Tris, 113 g glycine, 2 g SDS
10x TBS-T (1 l)	48.5 g Tris, 175 g NaCl, 10 ml Tween 20, adjust to pH 7.5 with Hcl
3% SDS upper gel solution	for 3.3 ml: 330 µl 30% acrylamide, 825 µl upper buffer, 2.1 ml H_2O, 40 µl 10% (w/v) APS, 4 µl TEMED
4x SDS lower buffer (1 l)	1.5 M Tris/HCl (pH 8.8), 0.4% (w/v) SDS
4x SDS upper buffer (1 l)	0.5 M Tris/HCl (pH 6.8), 0.4% (w/v) SDS
Blocking solution	TBS-T, 3% (w/v) serum bovine albumin Fraction V
Coomassie destaining	7% acetic acid, 30% methanol
Coomassie Staining	0.5 g Coomassie R250 in 100 ml H_2O, mix, add 20 ml acetic acid
Laemmli buffer (4x)	2% (w/v) SDS, 50 mM Tris/HCl (pH 6.8), 0.2 mg/ml bromphenol blue, 0.1 M DTT, 10% (w/v) glycerol
Semi Dry transfer buffer	1x semi dry buffer, 20% (v/v) methanol
Stripping buffer	Manufacturer: Thermo Fisher

Table 2.12: Buffers for Immunofluorescence

Buffer	Ingredients
4% PFA	1x PBS, 4% (w/v) PFA, 4% (w/v) sucrose, adjust to pH 7.4
Blocking solution	1x PBS, 1% (w/v) serum bovine albumin Fraction V
Mowiol mounting medium	2.4 g Mowiol 4-88, 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris/HCl pH 8.5
Permeabilization solution	1x PBS, 0.1% (v/v) Triton-X-100

Table 2.13: cell, infection and bacterial culture media

Medium	Ingredients
GC agar	36.23 g GC Agar base (Oxoid) in 1 l dd H_2O, add 1 % vitamin mix after autoclaving
HEPES medium (phosphate free medium)	50 ml solution I, 10 ml solution II, 200 µl solution III, 3 ml solution IV/V, 5 ml solution VI, 50 ml solution VII, 50 ml solution VIII, in distilled water up to 500 ml, pH 7.3, sterilize by filtration
HEPES solution I	0.1% (w/v) L-alanine, 0.15% (w/v) L-arginine, 0.0025% (w/v) L-asparagine, 0.025% (w/v) glycine, 0.018% (w/v) L-histidine, 0.05% (w/v) L-lysine, 0.015% (w/v) L-methionine, 0.05% (w/v) proline, 0.05% (w/v) L-serine, 0.05% (w/v) L-threonine, 0.061% (w/v)L-cysteine, 0.036% (w/v) L-cystine, 0.05% (w/v) L-glutamine, 0.046% (w/v)GSH, 0.0032 (w/v) hypoxanthine, 0.008% (w/v) uracil, 0.004% (w/v) D-biotin, in 18% 1 N NaOH and 82 % ddH_2O, pH 7.2
HEPES solution II	37.5% (w/v) glucose
HEPES solution III	1% (w/v) $Fe(NO_3)_3$ x $9H_2O$
HEPES solution IV/V	0.33% (w/v) NAD, 0.33% (w/v) cocarboxylase, 0.33% (w/v) thiamine, 0.33% (w/v) calcium pantothenate, 0.188% (w/v) $CaCl_2$ x 2 H_2O, 4.17% (w/v) sodium lactate, 15.33% (w/v) glycerol, 3.33% (w/v) ocaloacetate
HEPES solution VI	5% (w/v) $MgCl_2$ x 7 H_2O
HEPES solution VII	5% (w/v) NaCl, 3.4% (w/v) sodium acetate
HEPES solution VIII	2.38% (w/v) Hepes
LB agar (1 l)	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar
LB medium (1 l)	10 g tryptone, 5 g yeast extract, 10 g NaCl
***Neisseria* growth medium**	PPM, 1% vitamin mix, 0.5 $NaHCO_3$ (8.4%)
Proteose Peptone Medium (PPM 1 l)	15 g proteose peptone No. 5, 5 g NaCl, 0.5 g soluble starch, 1 g KH_2PO_4, 4 g K_2HPO_4, adjust to pH 7.2, sterilize by sterile filtration
Stocking medium *E. coli*	LB-medium, 26% (v/v) glycerol
Stocking medium *N. gonorrhoeae*	PPM-Medium, 26% (v/v) glycerol
Vitamin mix	Combine solution I and II, add H_2O to 2 l and sterile filter

Vitamin mix solution I	200 g D(+)-glucose, 20 g L-glutamine, 52 g L-cysteine-hydrochloride-monohydrate, 0.2 g cocarboxylase, 0.04 g iron-(III)-nitrate-nonahydrate, 0.006 g thiamine hydrochloride (vitamin B1), 0.026 g 4-aminobenzoic acid, 0.5 g NAD, 0.02 g vitamin B12, add 1 l H_2O
Vitamin mix solution II	2.2 g L-cystine, 2 g adenine-hemisulfate, 0.06 g guanine-hydrochloride, 0.3 g L-arginine-monohydrochloride, 1 g uracil, add 600 ml dest. H_2O, 30 ml 32% HCl

Table 2.14: Buffers for Flow Cytometry, ECL detection

Buffer	Ingredients
FACS buffer	1x PBS, 5% FCS
ECL detection sol ution I	2.5 mM Luminol, 0.4 mM p-coumaric acid
ECL detection solution II	100 mM Tris HCl pH 8.5, 0.02% H_2O_2
	Solution I and II were mixed together in ration 1:1 immediately before usage

Table 2.15: Buffer for shRNA cell line/ cloning

Buffer	Ingredients
Annealing buffer	100 mM CH_3CO_2K, 30 mM HEPES KOH pH 7.4, 2 mM $Mg(CH_3COO)_2$
Calcium phosphate transfection buffer 2 x HBS	50 mM HEPES pH 7.05, 140 mM NaCl, 1.5 mM Na_2HPO_4

Table 2.16: Buffer for NSM2 activity assay

Buffer	Ingredients
HMU-PC substrate buffer	1.35 mM in 0.25 M Na-acetate, 30 µM Na-taurocholate, pH 7.4
Lysis buffer	20 mM Hepes pH 7.4, 2 mM EDTA, 5 mM EGTA, 5 mM DTT, 1x protease inhibitor cocktail, 1 mM Na-orto-canadate, 10 mM β-glycerolphosphate
Resuspension buffer	20 mM Hepes pH 7.4, 15 mM $MgCl_2$. 10 mM β-glycerolphosphate, 1x protease inhibitor cocktail
Stop buffer	0.2 M glycine, 0.25% tritonX-100, pH 10.7

Table 2.17: Buffer for SPHK activity assay

Buffer	Ingredients
Kinase buffer	50 mM Hepes pH 7.4, 250 mM NaCl, 30 mM $MgCl_2$
Running buffer	$CHCl_3$: CH_3OH : Acetic acid : H_2O
	90 l : 90 ml : 15 ml : 5 ml

2.1.8 Enzymes

Table 2.18: Enzymes

Enzymes	Manufacturer
DNase I	Thermo Scientific
MolTaq DNA Polymerase	Molzym
Phusion Polymerase	Thermo scientific
recombinant mouse Sphingosine Kinase 1/SPHK1	R&D Systems
Restriction enzymes	Thermo Scientific, New England BioLabs (NEB)
T4 DNA Ligase	Fermentas
T4 DNA Polymerase	Fermentas
Taq DNA Polymerase	Genaxxon

2.1.9 Inhibitors

Table 2.19: Inhibitors

Inhibitor	Target	Manufacturer/Source
5C	Sphingosine kinase 1	Santa Cruz
ABC294640	Sphingosine kinase 2	Active Biochemicals Co., Limited
C11AG	Neutral sphingomyelinase 2	Christoph Arenz, unpublished
Compound A	S1P-lyase	Burkard Kleuser, unpublished
Eso48	Neutral sphingomyelinase 2	Christoph Arenz, unpublished
GW4869	Neutral sphingomyelinase 2	Sigma
K145	Sphingosine kinase 2	Sigma Aldrich
SKI-II	Sphingosine kinase 1 + 2	Sigma Aldrich

2.1.10 Chemicals

Table 2.20: Fine chemicals, inductors

Compound	Manufacturer
[gamma-P32]-ATP	Hartmann Analytic
Acrylamid Rotiphorese Gel 30 (37,5:1)	Roth
Albumin Fraktion V (BSA)	Roth
Alexa Fluor® 555 Phalloidin	Invitrogen
APC-Annexin V	BD Pharming
ATP (adenosine triphosphate)	Carl Roth
Bacto™ Proteose Peptone No. 3	BD
bSMase	Sigma
C16 Ceramide	Santa Cruz
Complete protease inhibitor EDTA free	Roche
D-erythro-Sphingosine	Santa Cruz
Dexamethason	Serva Electrophoresis

Dimethylsulfoxid (DMSO)	Carl Roth
GC Agar Base	Oxoid
GeneRuler™ 1 kb DNA ladder	Thermo Scientific
Gentamicin	Sigma
H_2O_2	Sigma
HDGreen	Intas
HiPerFect® Transfection Reagent	Quiagen
HMU-PC	Moscerdam
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Roth
Lipofectamine® LTX	Invitrogen
Lipofectamine™ 2000	Invitrogen
Loading dye 6x	Thermo Scientific
Methanol LC-MS Chromasolve®	Fluka Analytical
PageRuler™ Prestained Protein Ladder	Thermo Scientific
PerfeCTa™ SYBR® Green FastMix™, ROX	Quanta Bioscience
Phorbol 12-myristate 13-acetate (PMA)	Sigma
PhosSTOP phosphatase inhibitor cocktail	Roche
PMA	Cell Signaling
Poly-L-lysin	Sigma Aldrich
Propidium Iodide	Immunochemistry Technologies
Restore™ Plus Western Blot Stripping Buffer	Thermo Scientific
Saponin	Sigma
Soluble starch	Riedel-deHaen
Sphingosine	Avanti Polar Lipids
Sphingosine-1-phosphate	PD Dr. Andreas Weigert (Institute of Biochemistry I, Faculty of Medicine, Goethe University Frankfurt, Germany)
TNFα	Cell Signaling
X-gal	Sigma-Aldrich

All chemicals used, which are not listed here, were obtained from Merck Chemicals, Roth, Serva or Sigma Aldrich if not stated otherwise.

2.1.11 Technical equipment

Table 2.21: technical equipment

Equipment	Manufacturer
2100 Bioanalyzer	Agilent Technologies
Autoclave	WEBECO, Sytec VX 150
Binocular SMZ-168	Motic
Chemiluminescence camera system	Intas
cold centrifuge CT15RE	Himac
Diaphragm Vacuum Pump MZ 2C	Vacuumbrand
DMIL light microscope	Leica
Douncer RW16basic	IKA Labortechnik

electric balance ABS-80-4	Kern
FACSaria III	BD
Hera Cell 240i incubator	Thermo
Hera Safe sterile bench	Thermo
Megafuge 1.0R centrifuge	Heraeus
MultiTemp III	Pharmacia Biotech
NanoDrop 1000 spectrophotometer	Peqlab Biotechnology
Operetta LCS^{TM}	Perkin Elmer
Optima$_{TM}$ L-80-XP Ultracentrifuge	Beckman Coulter
PerfectBlue$_{TM}$ Dual Gel Twin PAGE chambers	Peqlab Biotechnology
PerfectBlue™ 'Semi-Dry'-Elektroblotter	Peqlab Biotechnology
pH Electrode SenTix	WTW series inolab
Plate reader infinite 200	TECAN
RW 16 basic drill-fitted Dounce homogenizer	IKA Labortechnik
Scanjet G4010	HP
Shaker TR125	Infors HT
Sonifier 250	Branson
Spectrophotometer Ultrospec 3100 pro	Amersham Bioscience
Speed Vac Concentrator	SAVANT
Step One Plus RT PCR system	Applied Biosystems
TCS SPE confocal microscope	Leica
Thermal cycler 2720	Applied Biosystems
Thermo mixer comfort	Eppendorf
TLC tank	Desaga
transmission electron microscope JEOL JEM-2001	Carl Zeiss
Typhoon 9200 variable mode imager	Amersham Pharmacia Biotech
Vortex Genie 2	Bender & Hobein AG

2.1.12 Special materials

Table 2.22: special materials

Materials	**Manufacturer**
µ-Plate 24 well	Ibidi
Amicon® Ultra Centrifugal Filters	Merck Millipore
Lab-Tek® Chamber Slide^{TM} system	Sigma Aldrich
Microflour black flat bottom Microtiter Plate	Thermo
S-MONOVETTE®	Sarstedt
TLC Silica gel 60	Merck

2.1.13 Software

Table 2.23: Software

Software	Company/Source
Abi StepOne v2.1	Life Technologies
Argus x1 version 7.6.17	Biostep® GmbH
ClustalW2	http://www.ebi.ac.uk/Tools/psa/
Codon Code Aligner 3.7.1	CodonCode Corporation
CorelDRAW X8	Corel Corporation
Endnote X7	Thomson Reuters
Harmony® High Content Imaging and Analysis Software	Perkin Elmer
ImageJ	W. Rasnband, National Institutes of Health
ImageScanner III Labscan^{TM} 6.0	GE Healthcare
LabImage Chemostar	Intas
Leica LAS AF confocal microscopy software	Leica Microsystems
NCBI blast	http://blast.ncbi.nlm.nih.gov
ND-100 V3.7.1	NanoDrop Technologies, Inc. Wilmington
Office 2010	Microsoft
Serial Cloner 2.6.1	Franck Perez Serial Basics
SnapGene Viewer 2.7.1	SnapGene
Tecan i-Control	Tecan
Windows 10	Microsoft

2.2 Methods

2.2.1 Bacterial culture techniques

Growth of *E. coli*

The bacteria were either cultured on LB agar plates at 37° C or in liquid medium at 37° C and 190 rpm each over night. The LB agar plates respectively the LB medium were supplemented with the appropriate antibiotics needed for selection.

Stocking of bacteria

E. coli were suspended in LB medium, which was supplemented with the respective antibiotics if necessary, and transferred to a cryotube vial. Glycerol was added to a final concentration of 26% (v/v) and the bacteria were stored at – 80° C.

N. gonorrhoeae were collected from GC agar plates and suspended in PPM medium. The mixture was transferred to a cryotube vial and glycerol was added to a final concentration of 26% (v/v) and the bacteria were stored at – 80° C.

Transformation of chemo-competent *E. coli* by heat shock

The chemo-competent *E. coli* were thawed on ice for 10 min. The recombinant vector DNA was added to the bacteria and they were incubated additional 30 min on ice. The following heat shock was performed in a water bath at 42° C for 90 sec. Immediately after the heat shock, the bacteria were incubated on ice for 2 min before 1 ml LB medium was added. The mixture was incubated for 30 – 60 min at 37° C at 190 rpm. Finally, the bacteria were plated on LB agar plates, supplemented with the appropriate antibiotics, and incubated at 37° C over night.

Solid growth of *N. gonorrhoeae*

Gonococci were grown on GC agar plates supplemented with 1% vitamin mix at 37° C and 5% CO_2. After a maximum of 24 h the bacteria were streaked on a new GC agar plate. To prevent autolysis, the bacteria were used 14 – 18 h after streaking for infection experiments or stocking. The selection for phenotypes like Opa or Pili was carried out with the help of a stereo microscope.

Liquid growth of *N. gonorrhoeae*

Liquid growth of Gonococci was only used to equalize the growth stages of different cultures before growth curve experiments. Therefore, the bacteria were collected from GC agar plates and suspended in neisserial growth medium. The medium was pre-warmed at 37° C and inoculated to an optical density (OD_{550nm}) of 0.15. The bacteria were then incubated at 37° C and 190 rpm. To prevent autolysis, the OD should not exceed a value of 0.5 – 0.6.

Growth curve experiments with *N. gonorrhoeae*

N. gonorrhoeae were grown in liquid culture as previous described until an OD_{550nm} of about 0.5. The growth curve experiment was either performed in bulbs or in 48-well plates. In both cases, fresh neisserial growth medium was inoculated with the pre-culture to an OD of 0.1. There was used a volume of 10 – 20 ml in the bulbs. The bacteria were treated with the chemicals or inhibitors of interest and the optical density was measured every hour. When the growth curve was performed in 48-well plates, there was used a volume of 400 µl of the inoculated neisserial growth medium per well. The bacteria were also treated with the respective concentrations of chemicals or inhibitors, but the measurements were performed by an Elisa Reader infinite 200, which measured the OD every 10 min over 5 h. Additionally, the experiments were executed in triplicates.

Sphingosine testing

N927 grown on GC agar plates for 14-18 h were harvested in *Neisseria* growth medium. The optical density (OD_{550nm}) was adjusted to 0.15 and the *Neisseria* were incubated at 190 rpm at 37° C for 2 h. Sphingosine was diluted 1:20 in DMSO and sonicated for 5 min at 37° C. The

OD was used to calculate the number of *Neisseria*. $1x10^3$ *Neisseria* were laid in 1 ml PBS, Hepes-medium or *Neisseria* growth medium and sphingosine was added to a final concentration of 5 µM. The bacteria were incubated at 37° C and 5% CO_2 for 2 h. Folllowing, the bacteria were either plated on GC agar plates in different dilutions, incubated at 37° C and 5% CO_2 over night and counted the next day, or prepared for electron microscopy.

2.2.2 Cell culture methods

Cultivation

Cell lines used in this study were cultured in flasks reaching from 25 to 175 cm^2 at 37° C and 5% CO_2 in a humidified atmosphere. The cells were passaged every 1 – 4 days and were kept in culture no longer than 4 – 6 weeks in total. During this time, the confluence should never reach 100%. Therefore. the cells were split. The cells were first washed once with PBS, afterwards they were incubated with trypsin at 37° C and 5% CO_2 for 5 to 10 minutes. The detached cells were collected in cell culture medium supplemented with 10% FCS and either split in a new flask for further cultivation or seeded in multi-well plates or cell culture dishes to conduct experiments. The cell lines used in this study as well as the corresponding media and supplements are listed in table 2.2 and 2.10.

Long time storage of cell lines/ cryo stocking of cell lines

Adherent cells were grown in a 75 cm^2 flask to a confluence of 80 – 90%. The cells were washed once with PBS to remove the remaining FCS and were then trypsinized. The detached cells were collected in medium supplemented with 10% FCS and centrifuged at 800 g for 5 min at RT. The cell pellet was resuspended in 5 ml stocking medium and the suspension was transferred to cryo tubes. The tubes were gradually colled to – 80° C in isopropanol immediately. After a few days, the cryo tubes were transferred to a liquid nitrogen tank for long term storage.

Inhibitor treatments

After the shift into HEPES medium, the cells were incubated with inhibitors for 2 h before infection was performed. The inhibitors were dissolved in DMSO and stored at 4° C or -20° C according to manufacturer's protocol. GW4869 was used in a final concentration of 1.6 µM. Different concentrations reaching from 1 up to 40 µM were tested for the inhibitors SKI-II, 5C, K145 and NVP-CHY007.

Karyogram

The experiment was performed by Julia Flunkert and Anna Maierhofer from the department of human genetics (Institut für Humanngenetik, Biozentrum, Am Hubland, 97074 Würzburg).

Chang cells were seeded in a T25 flask and grown to 70 – 80% confluence. 10 µg/ml colcimid was added to the medium and the cells were incubated for 3 h at 37° C. The cells were trypsinised and collected. After a centrifugation step (1400 g, 8 min) the cell pellet was resolved in 14 ml 0.075 M KCl and incubated for 21 min at 37° C. The cells were pelleted again (1400 g, 8 min) and afterwards resolved in 14 ml fixative (methanol : glacial ethanolic acid, 3 : 1) and stored at over night at – 20° C. The next day, the cells were centrifuged (1400 g, 8 min), resuspended in 2 – 3 ml fixative and dropped on an object slide for microscopical analysis.

Isolation of primary human PMNs

The Ficoll-Hypaque method used here is based on a protocol published in 2001 (Hattar *et al.*, 2001).

Fresh venous blood from healthy donors was collected with S-monovette Lithium heparin tubes. 15 ml Ficoll reagent was provided in a 50 ml tube and 10 ml of the heparinized blood was layered on top of the Ficoll. As the step is critical it should be performed carefully. The tubes were centrifuged at 1000 rpm for 30 min at 20° C (without brakes). The lower granulocyte layer was transferred to a new tube and mixed 1:3 with 1% polyvinyl alcohol in 0.85% saline and was then incubated for 45 min at RT. The upper layer was transferred to a new tube, too, and was centrifuged at 1000 rpm for 5 min. The pellet was resolved in 16 ml H_2O and incubated for exact 30 sec at RT before 4 ml 5X PBS was added to the mixture.

Finally, the neutrophiles were collected via centrifugation at 1000 rpm for 5 min and resuspended in 1X HBSS and stored at RT.

2.2.3 Transfection protocols

Transfection of plasmid DNA

Transfection of cells with plasmid DNA was performed using either Lipofectamine® LTX or Lipofectamine™ 2000. Cells were grown in 12-wells to 60 – 80% confluence. The medium of the cells was shifted to 400 µl Opti-MEM® per well prior to transfection.

The transfection with Lipofectamine® LTX was performed by mixing 50 µl Opti-MEM® with 1 µg DNA and 1 µl PlusReagent. A second solution mixing 50 µl Opti-MEM® with 1 – 4 µl Lipofectamine® LTX was prepared. Subsequently both solutions were combined and incubated for 10 min at RT before the mixture was dropped on the cells. After 4 – 6 h incubation at 37° C the medium was changed to normal cell culture medium.

The transfection with Lipofectamine™ 2000 was performed by mixing 50 µl Opti-MEM® with 1 µg DNA and preparing a second solution mixing 50 µl Opti-MEM® with 1 – 4 µl Lipofectamine™ 2000. The solutions were incubated at RT for 5 min before they were combined and incubated additionally 25 min at RT. Finally, the mixture was dropped on the cells and after 4 – 6 h incubation at 37° C the medium was changed to normal cell culture medium.

Transfection of siRNA

Chang cells were grown in a 12-well to 60 – 70% confluence for siRNA transfection. The medium of the cells was shifted to 400 µl Opti-MEM® per well prior to transfection. 100 µl of Opti-MEM® were mixed with siRNA to a final concentration of 25 nM. After dissolving the siRNA, 3 µl HiPerFect® was added and mixed by vortexing. The mixture was incubated for 10 min at RT and finally added dropwise to the cells. After 4 – 6 h normal medium was added to the cells. The cells were sub-cultured 27 h after transfection and were either used for

analysis or infection experiments 72 h after transfection or the cells were transfected once again after 72 h and finally used after 6 days.

2.2.4 Generation of stable knockdown and overexpressing cell lines

CRISPR/Cas9 NSM2 knockout cell lines

The Nature Protocols paper "Genome engineering using the CRISPR-Cas9 system" was used as a guideline for the experimental set up for the production of a neutral sphingomyelinase 2 knockout cell line (Ran *et al.*, 2013).

The first step was to design guide RNAs for the gene of interest, using a webtool recommended in the paper. The corresponding oligonucleotides were ordered and the 20 nucleotide long fragment was produced by oligonucleotide annealing. The DNA fragment as well as the plasmid pSp cas9 (BB)-2A-GFP were cut using the restrictionenzym BbsI. The vector and the guide RNA were ligated and transformed in competent *E. coli*. Positive clones were identified by colony PCR, their plasmids were isolated and the insertion of the guide RNA into the vector was verified by sequencing. Next, the verified plasmids were transfected into Hela and Chang cells as described in 2.2.3. 48 h after transfection, the cells were checked for green fluorescence and the green cells, the one's which incorporated the plasmid, were sorted as single cells into a 96-well plate. Subsequently, the single cell clones were cultured for about 2 weeks in RPMI containing 10% FCS and P/S. One part of the cells was used for cryo stocking, another part was used for DNA isolation and a third part was further cultured and used for infection experiments. The DNA of the cell lines was isolated and a PCR was performed. The amplified DNA fragment was covering the area around the guide RNA binding sites of the gene of interest. The DNA fragment was cloned using the pGEM®-T Easy Vector System and the plasmid of 10 positive clones were sent for sequencing. Afterwards alignments of the sequences were prepared to verify the knockout of the gene of interest.

shRNA Zelllinie – complementation of CRISPR/Cas9 NSM2 knockout clones using lenti virus

Recombinant lentivirus was produced by transient transfection of 293T cells. 293T cells were grown in 6-wells until 80% confluence and transfected with 1 µg psPAX, 1 µg pCMV-VSV-G and 2 µg NSM2-GFP pLVTHM by calciumphosphate precipitation. Therefore 2 µl chloroquine (25 mM stock solution) were added to the cells before transfection. Meanwhile 4 µg of plasmid were mixed with 50 µl 2.5 M $CaCl_2$ and 200 µl H_2O. 205 µl 2X HBS were slowly added to the mixture while making bubbles. After 1 min incubation at RT, the precipitate was added dropwise to the cells, while shaking the plate. The medium was changed after 6 – 8 h. For harvesting the virus, the cell culture supernatant was filtered through a 0.45 mm filter 48 h post transfection. In the meantime, Chang cells as well as CRISPR/Cas9 NSM2 knockout clones were grown to 70 – 80% confluence in a 25 cm^2 flask. The viral supernatant was used to infect the cells in the presence of 0.5 µg/ml polybrene. 24 h post infection the medium was changed. The lentiviral cell pools were cultivated and used for western blotting and infection experiments to verify the expression of the transfected construct NSM2-GFP as well as for single cell sorting via the FACS AriaIII. The single cell clones were cultivated for about 2 weeks and then also used for western blotting and infection experiments.

2.2.5 Infection experiments

Infection at low phosphate concentrations

Cells were grown to 70 – 80% confluence. Prior to infection, the cells were washed once with HEPES medium to remove phosphate and were incubated in HEPES medium for 30 min. Meanwhile the *Neisseria* were collected from GC agar plates, washed once with HEPES medium and finally resuspended in HEPES medium. The optical density was determined to calculate the number of bacteria for the respective MOI. The bacteria were added to the cells and the cells were centrifuged at 600 g for 3 min at RT to synchronize the infection. The infected cells were incubated for at least 15 min up to 3 h at 37° C and 5% CO_2. The infection was stopped by washing the cells three times with HEPES medium.

Gentamicin protection assay

The assay is used to quantify bacterial adherence to and entry into the host cell. Cells were seeded in 24-wells and grown to 70 – 80% confluence. Cells were infected with *Neisseria* at MOI 50 for the indicated duration, afterwards the infection was stopped by washing. The adherent bacteria were determined by lysing the cells with 1% saponin in HEPES medium for 10 min. The complete lysis was verified by microscopy and 900 µl HEPES medium were added. After forceful pipetting, a serial dilution was generated and suitable dilutions (10^{-1} to 10^{-3}) were plated on GC agar plates. The invasive viable bacteria were determined by killing all extracellular *Neisseria* using 50 µg/ml gentamicin in HEPES medium for 2 h. Afterwards the cells were washed trice with HEPES medium to remove the remaining gentamicin. The cells were lysed in 1% saponin for 10 min and 400 µl HEPES medium was added, a serial dilution was generated and suitable dilutions (10^0 to 10^{-2}) were plated on GC agar plates. The plates were incubated at 37° C and 5% CO_2 for 24 h and the CFU was determined. The experiment was performed in duplicates and sometimes in triplicates.

Differential Immunoflourescence Staining (Infectivity Assay)

The staining method is used to distinguish intracellular from extracellular bacteria. Cells were seeded on 12 mm^2 cover slips in 12-wells and grown to 70 – 80% confluence. The cells were infected with *Neisseria* at MOI 10 as previous described. After the infection was stopped, the cells were fixed with 4% PFA for 15 min in the dark at RT. The cells were washed three times with PBS and subsequently blocked with 1% BSA in PBS for 45 min at RT in the dark. All following incubation steps were performed at RT and in the dark. After blocking, the cells were incubated in a 1:200 dilution of the primary antibody, polyclonal rabbit anti-*Neisseria gonorrhoeae* in 1% bovine serum albumin in PBS, for 1 h to detect the extracellular bacteria. The cells were washed twice with PBS and once with 1% BSA in PBS before they were incubated with the Cy5 conjugated secondary anti-rabbit antibody (1:100) for 1 h. Afterwards the cells were washed three times with PBS and were incubated in 0.1% Triton-X-100 in PBS for 15 min for permeabilization. After another washing step, the cells were again blocked in 1% BSA in PBS for 45 min. Next, the extra- and intracellular bacteria were stained using first the anti-*Neisseria gonorrhoeae* primary antibody (1:200) followed by washing steps and then the Cy2 conjugated secondary anti-rabbit antibody (1:100). The actin

cytoskeleton was stained using Alexa Fluor® 555 Phalloidin (1:100 in 1% BSA in PBS) and the DNA was stained using Dapi (1:3000 1% BSA in PBS). The cells were incubated with Phalloidin and Dapi at the same time for 30 min. Finally, after a last washing step, the cover slips were embedded in mowiol on object slides, dried over night and analyzed by confocal microscopy (63x oil immersion objective).

Infection and Staining of PMNs

$1x10^6$ PMNs were laid in 500 µl RPMI 1640 without any supplements and infected with different *Neisseria* strains at a MOI of 100. Therefore, the bacteria were taken from a GC agar plate and dissolved in RPMI 1640, washed once (5000 rpm, 5 min) and the number of bacteria was determined using a photometer for the detection of the optical density and a standard growth curve. The respective volume of bacteria suspension was added to the PMNs. The infected cells were incubated at 37° C on a rotary shaker for 24 h. The staining was performed by adding 1 µl $CaCl_2$ and 5 µl APC-Annexin V to the infected cells, followed by 10 min of incubation in the dark at room temperature. The cells were kept on ice until they were analysed with the FACS Aria III. The results were gated to obtain single cells and to exclude cell fragments, bacteria and cells sticking to each other. In general, 10.000 events were recorded per sample.

2.2.5 Flow Cytometry

Cytotoxicity Assay

Chang cells ($1x10^5$) were seeded in 12-wells and grown to 80 – 90% confluence. The medium was shifted to HEPES medium and the cells were treated with the indicated concentrations of inhibitors or DMSO as control for 2 h. Subsequently the cells were trypsinised and collected. After a centrifugation step (800 g, 5 min, 4° C) the cell pellet was resolved in 500 µl PBS and 1 µl Propidium Iodide (250 µg/ml) was added. The cells were incubated for 10 min in the dark. Afterwards the cells were put on ice and one sample was treated with 0.1% Triton as a negative control shortly before analysis. The analysis was performed using FACS AriaIII.

Flow Cytometry – Ceramide staining (total ceramide amount)

Chang cells were seeded in 6-wells and grown to 80- 90% confluence. The cells were either infected under low phosphate conditions or treated with stimuli like bSMase (bacterial sphingomyelinase) or inbihitors. The cells were then trypsinized, collected in RPMI with 10% FCS and pelleted by centrifugation at 800 g for 5 min at 4° C. The cells were washed once with PBS before they were fixed with 4% PFA in PBS for 15 min on ice. Subsequently the cells were washed once and then permeabilized with 0.1 Triton in PBS for 15 min on ice. Alternative the cells were permeabilized by adding 0.33% saponin to the FACS buffer while incubating the cells in the primary antibody. The cells were washed once with FACS buffer before they were blocked in the FACS buffer for 30 min on ice. Next, the cells were incubated with a monoclonal anti-ceramide IgM antibody (1:100 in FACS buffer) for 90 min on ice. After one washing step, the cells were incubated in a Cy^{TM} conjugated anti-mouse antibody solution (1:100 in FACS buffer) for another 90 min on ice. The cells were washed twice and were then resuspended in 300 μl PBS and analyzed using FACS AriaIII.

2.2.6 DNA methods

DNA isolation

DNA from eukaryotic cells was isolated using the NucleoSpin® Tissue kit. The isolation was performed according to the manufacturer's protocol.

PCR

PCR reactions with Phusion polymerase were performed in a total volume of 50 μl. The reaction mix consisted of 5 μl 10X polymerase buffer, 1 μl of 0.5 mM dNTP mix, 1 μl DMSO, 1 μl of 10 mM forward and reverse primer, 100 ng template DNA, 1 U Phusion polymerase and ddH$_2$O. The PCR program started with an initiation step of 10 min at 98° C, followed by 30 cycles including a denaturation step at 98°C for 10 s, the primer annealing 55 – 60° C (depending on the melting temperature of the primers) for 20 s, and the elongation at 72° C for 30 s to 2 min (depending on the length of the DNA fragment, 30 s/kb). The final

elongation took 10 min at 72° C. Afterwards, the product was cooled to 4° C, analyzed via an agarose gel, purified using a PCR purification kit and stored at – 20° C.

Colony PCR: colony PCR was used to verify a successful ligation of the vector with the insert and a successful transformation into *E. coli*. Therefore 10 clones were picked, streaked on a LB agar plate and dissolved in 50 µl ddH$_2$O. The suspension was boiled at 95° C for 5 min and incubated on ice for additional 5 min, before centrifuged at 12.000 g for 3 min at RT. 2 µl of the supernatant were then used as template in a PCR reaction. The PCR reaction was performed using Taq polymerase. The reaction mix consisted of 2.5 µl 10X polymerase buffer, 1 µl of 0.5 mM dNTP mix, 1 µl of 10 mM forward and reverse primer, 2 µl bacterial supernatant, 1 U Taq polymerase and ddH$_2$O to a final volume of 25 µl. The PCR program started with an initiation step of 2 min at 94° C, followed by 30 cycles including a denaturation step at 94°C for 20 s, the primer annealing 55 – 60° C (depending on the melting temperature of the primers) for 30 s, and the elongation at 72° C for 30 s to 3 min (depending on the length of the DNA fragment, 1 min/kb). The final elongation took 7 min at 72° C. Afterwards, the product was cooled to 4° C, analyzed via an agarose gel and stored at – 20° C.

Agarose gel electorphoresis

Agarose gels were used to analyze PCR products and DNA fragments in generally. Therefore 1% agarose (w/v) was dissolved in 0.5% TBE buffer and boiled. 5 µl HD green per 100 ml agarose gel was added and the gel was hardening at RT. The DNA samples were mixed with 6X loading dye before application on the gel and GeneRulerTM 1 kb DNA Ladder was used as a marker. The electrophoresis was performed in TBE buffer at 120 V and the DNA fragments were visualized under UV-light.

Restriction

Restriction reaction was performed by following the manufacturer's protocol (Fermentas). 500 ng to 10 µg were used for the reaction, 3 µl 10X buffer, 2 µl restriction enzyme and ddH$_2$O were added to a final volume of 60 µl. The mixture was incubated for 3 h at 37° C.

Afterwards, the DNA was purified using GeneJET Gel Extraction Kit and if necessary, the reaction was repeated with a second restriction enzyme.

Ligation of insert in vector

Ligation reaction was performed by following the manufacturer's protocol (Fermentas). The vector to insert ration was set 3:1. The DNA was mixed with 2 µl 10x T4 ligase buffer 1 µl T4 DNA-ligase as well as ddH$_2$O to obtain a total volume of 20 µl. The ligation reaction was conducted in a thermo cycler at 16° C over night.

Oligonucleotide Annealing

Oligonucleotides were diluted in ddH$_2$O to a concentration of 1 mM. The reaction was set by mixing 1 µl sense oligonucleotide with 1 µl antisense oligonucleotide and 48 µl annealing buffer. The mixture was incubated at 94° C for 4 min followed by a temperature step at 70° C for 10 min. Then it was allowed to cool down slowly at RT for about 1 h before it was diluted 1:100. The concentration was measured the resulting DNA fragment was used as insert in a ligation reaction.

TA-cloning: pGEM®-T Easy Vector Systems

pGEM®-T Easy Vector Systems are suitable systems for the cloning of PCR products. The vectors used in this system are already cut by restriction enzymes and contain a single 3' –T overhang which prevents recirculation of the vector and improves the efficiency of ligation. Because of the T-overhang of the vector, the insert, which means the PCR fragment, has to gain an A-overhang. This processing is called A-tailing. Therefore 7 µl of PCR fragment were mixed with 1 µl Taq DNA buffer, dATP in a final concentration of 0.2 mM, 1 µl Taq DNA polymerase (2 u) and 0.8 µl H$_2$O. The reaction was running at 70° C for 15 – 30 min.

The next step was the ligation of the insert into the pGEM®-T Easy Vector. Therefore 5 µl ligation buffer were mixed with 1 µl pGEM®-T Easy Vector, 2 µl A-tailing reaction, 1 µl T4 DNA ligase and 1 µl H$_2$O to a final volume of 10 µl. The reaction was running at 16° C over night. The transformation was performed as described in 2.2.1. The bacteria were plated on

LB agar plates supplemented with the respective antibiotics as well as with IPTG and X-gal. This indicator plates in combination with the features of the vector system allow the direct identification of recombinant clones by blue/white screening.

Sequenzierung

All recombinant constructs were verified by sequencing. Sequencing was executed by Seqlab in Göttingen.

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RNA isolation

RNA was isolated following the manufacturer's instructions for the miRNeasy Micro Kit. Cells were seeded in 6-wells and grown to 70 – 80% confluence. Cells were trypsinized and centrifuged at 800 g for 5 min at 4° C. The cell pellet was resolved in 700 μl QIAzol Lysis Reagent and incubated for 5 min at RT. 140 μl chloroform was added and the tubes were shaked vigorously for 15 s, followed by an incubation step at RT for 2 – 3 min. The lysed cells were centrifuged at 12.000 g for 15 min at 4° C to obtain phase separation. The upper aqueous phase was transferred to a new collection tube and mix with 525 μl 100% ethanol. The mixture was pipetted into an RNeasy MinElute spin column in a 2 ml collection tube. After centrifugation at 12.000 g for 15 s at RT the flow-through was discarded. All following centrifugation steps were performed at RT. DNA digestion was performed by incubating the column with 80 μl DNase mixture (DNase 1:8 RTT buffer) for 15 min at RT. The column was washed with 500 μl RPE (12.000 g for 1 min), the flow-through was discarded. Next the column was washed with 500 μl 80% ethanol (12.000 g for 2 min). Afterwards the column was placed in a new collection tube and centrifuged at 12.000 g for 5 min to dry the membrane. To finally elute the RNA, the column was placed in a new collection tube, 20 μl RNase-free water were added directly on the membrane and incubated for 5 min at RT before centrifugation at 12.00 g for 2 min. The RNA was stored at –80° C.

cDNA Synthesis

cDNA synthesis was performed using the RevertAid^{TM} Premium First Strand cDNA Synthesis Kit. 1 µg RNA was mixed with 1 µl random hexamer primers and water was added to a final volume of 12 µl. Next, a mastermix consisting of 4 µl 5X reaction buffer, 1 µl RiboLock RNase Inhibitor, 20 µl 10 mM dNTP Mix and 1 µl Reverse Transkriptase was added to the RNA/Primer/H$_2$O mix, resulting in a final volume of 20 µl. The reaction was executed at 42° C for 1 h followed by 5 min at 70° C. The generated cDNA was not measured or purified, but diluted 1:10 and directly used for qRT-PCR or stored at -20° C.

Quantitative reverse transcription polymerase chain reaction

qRT-PCR was performed to quantify specific mRNA populations and therefore verify a siRNA-mediated knockdown of target mRNA. The reaction was performed in a Step One Plus RT-PCR system using PerfeCta^{TM} SYBR Green FastMix. Therefore 5 µl of the 1:10 diluted cDNA was mixed with 10 µl SYBR Green FastMix, 100 nM forward and reverse primer and H$_2$O to a final volume of 20 µl. Each sample was analyzed in triplicates. The house-keeping gene GAPDH was used for normalization.

2.2.7 SDS-PAGE and Western Blotting

SDS-PAGE

Samples subjected to SDS-PAGE were collected in 2X Laemmlie buffer and denatured by heating at 95° C for 10 min. Proteins were separated by electrophoresis in 10% polyacrylamide gels under denaturing conditions with sodium dodecyl sulphate. The separation was performed at 120 V for about 1 – 2 h.

Coomassie staining

After electrophoresis was performed, the gel was incubated with coomassie stain solution for 30 – 60 min at RT. The staining solution was removed and the gel was washed once with destaining solution before it was incubated in destainer until the background staining of the

gel was gone and only the protein bands were visible. Finally, the stained gel was scanned for documentation.

Western Blot

PVFD membranes were incubated in methanol for 1 min for activation and then washed shortly with transfer buffer. Gels were blotted on the activated membranes using a semi-dry blotting chamber for 2 h at 1 mA/cm^2. The membranes were blocked in blocking solution (3% BSA in TBS-T) for 45 min before they were incubated with the primary antibody dissolved in blocking solution over night at 4°C on a rotary shaker. The blots were washed three times for 10 min in TBS-T before they were incubated in the diluted secondary antibody (HRP-conjugated, 1:3000 in 5% milk in TBS-T) for 1h at RT. Subsequently, the membranes were washed three times for 10 min in TBS-T and ECL reagent was added. Chemiluminescence was detected using and IntasImager digital system and proteins were quantified by ImageJ software.

Stripping of Western Blots

PVFD membranes were incubated in methanol for 1 min to activate the membranes. Then the membranes were washed twice for 5 min in TBS-T before they were incubated in stripping buffer for 15 min at RT. Subsequently the blots were washed three times for 10 min with TBS-T. The stripped blots were blocked again and afterwards incubated in primary and secondary antibody.

2.2.8 Purification of native and recombinant proteins and antibodies

Expression of recombinant proteins

IPTG inducible vectors, which encoded for the desired recombinant proteins were used for the expression of those recombinant proteins. The vector was transformed into *E. coli* BL21. An overnight culture was inoculated with positive transformants in LB medium supplemented with the respective antibiotics and incubated at 37° C and 190 rpm. The next

day, a pre-culture was inoculated using a 1:20 dilution of the overnight culture in fresh medium supplemented with antibiotics. The bacterial culture was incubated at 37° C and 190 rpm until an OD_{600} of 0.4 – 0.6 was reached. Subsequently, the pre-culture was used inoculate the main culture using a 1:10 dilution in fresh LB medium supplemented with antibiotics. IPTG was added to a final concentration of 1 mM to induce protein expression. The culture was incubated at 37° C and 190 rpm for up to 4 h. During this time, 1 ml of the bacterial suspension was collected each hour. The samples were pelleted and mixed with 50 µl 2X Laemmli buffer, denatured at 95° C for 10 min and stored at – 20° C before they were used for SDS-PAGE. The complete main culture was collected after 1 – 4 h and centrifuged at 5000 g for 15 min at 4°C. Afterwards the pellet was washed once with 10 ml dH_2O and stored at – 20° C for further use.

Determination of protein solubility

To determine the solubility of recombinant proteins, the proteins were first expressed as previous described. The bacterial pellet was thawed on ice and resuspended in 5 ml lysis buffer. The suspension was incubated on ice for 30 min after 1 mg/ml lysozyme was added. Subsequently, the suspension was sonicated with 6 pulses of 10 seconds each at 200 – 300 W on ice. The lysate was centrifuged at 10.000 g for 30 min at 4° C. The supernatant was then collected and the pellet was dissolved in 2 ml lysis buffer. 50 µl of the pellet and 50 µl of the supernatant were both mixed with 50 µl 2X Laemmli buffer, denatured at 95° C for 10 min and stored at – 20°C before the samples were used for SDS-PAGE.

Antibody purification

Affinity purification of antibodies using nitrocellulose membrane

The protein used for immunization is also used for the affinity purification of the antibody. Therefore, the protein is concentrated if necessary via a spin column. The protein is solubilized in 4X Laemmli buffer, denatured at 95° C for 10 min and loaded in one single large pocket on a big SDS gel. After the gel was running, the proteins were transferred to a nitrocellulose membrane at 1 mA/cm² for 90 min in a semi-dry blotting chamber and stained with Ponceau S solution for 15 min and destained with water until protein bands were

visible. The band where the protein is localized was cut out and the strips were incubated for 1 h in TBS for destaining. Next, the strips were blocked for 1 h in 3% milk in TBS before they were washed with TBS and incubated in 2 ml antibody serum diluted in 8 ml TBS for 1 h. The strips were washed three times for 5 min with TBS. The elution was performed using 4 ml 100 mM glycine pH 2.5 for 5 min. The strips were then washed with 4.5 ml TBS for 5 min. The eluate and the wash were combined and 5% 1 M Tris pH 8.0 (v/v) were added. The purified antibody solution was stored at − 20° C and tested in Western Blot.

Antibody purification using Cu-Br-activated sepharose

The protein used for immunization was rebuffered in 0.2 M carbonate buffer. First, 0.14 g of Cu-Br-activated sepharose beads was incubated in 1 mM HCl for 30 min. The sepharose was then washed twice with 1 ml carbonate buffer (centrifuge: 600 g for 2 min) and was finally resuspended in 1 ml protein (antigen) in carbonate buffer. There should be added about 100 µg of protein to the activated sepharose. The mixture was incubated over night at 4° C on a rotary shaker. The next day, the coated beads were washed twice with carbonate buffer and were then incubated with 100 mM ethanol amine (0.1 M Tris-HCl pH 8.0) for 1 h at RT. The sepharose was washed three times before it was resolved in 500 mM NaCl in PBS. 7 ml of the antibody serum was incubated with 7 ml activated beads in PBS at 4° C over night on a rotary shaker. The next day, the beads were washed twice with 500 mM NaCl in PBS. The elution was performed in 500 µl steps using a total volume of 10 ml 0.1 M glycine pH 2.5. Immediately after the elution, the eluate was neutralized with 100 µl 1 M Tris-HCl pH 7.5. Next, the eluate was pooled and concentrated, and the purified antibodies were rebuffered in PBS before the solution was mixed with glycerol 1:1 and stored at − 20° C.

2.2.9 Activity Assays

NSM2 Activity Assay

The assay is based on a protocol established by Elita Avota, member of Sibylle Schneider-Schaulies' group (Department of Virology, University of Würzburg).

Cells were seeded in 12-wells and grown to 70 – 80% confluence. The cells were treated occasionally with inhibitors or assumed activators of the neutral sphingomyelinase 2 prior to infection. The infection was performed as described. The cells were washed twice with HEPES medium, 800 µl RPMI were added and the cells were collected using cell scrabber. After a centrifugation step (800 g for 5 min at 4° C), the cell pellet was resuspended in 100 µl lysis buffer. Followed by 5 freezing-thawing cycles in a methanol-dry ice mixture to lyse the cells. After another centrifugation step (1600 rpm for 5 min at 4° C) the supernatant was transferred into a tube suitable for ultracentrifugation and 1 ml PBS was layered on top of the supernatant. Ultracentrifugation was performed at 26.000 g for 1.5 h and 4° C. Subsequently, the pellet was resolved in 42 µl lysis buffer by vortexing. 10 µl of the membrane fraction were transferred into a fresh tube and 10 µl resuspension buffer as well as 10 µl of the substrate HMU-PC and 1.8 µl Tris-HCl (pH 8.0, 1M) were added. The mixture was incubated over night at 37° C. The next day, the reaction was stopped by adding 200 µl stop buffer. 200 µl of the mixture were transferred into a 96-well plate (Corning 96 Flat Bottom Black Polystrol, Thermo Scientific) and the fluorescence was measured using an Elisa Reader infinite 200 (excitation 400 nm, emission 460 nm).

Sphingosine kinase Activity Assay

Chang cells were seeded in 12-wells and grown to 70 - 80% confluence. The cells were infected as described. After the infection, the supernatant was removed accurately and the cells were quick-frozen in liquid nitrogen. 150 µl kinase buffer was added, and the cells were scrabbed of the plate. To further lyse the cells, they were sonicated for 10 min at 4° C. Sphingosine (10% octylglucopyranoside (w/v)) was also sonicated for 10 min. Next, 500 pmol of the sonicated sphingosine was added to 50 µl kinase buffer, as well as 1 µM ATP and 10 µCi [γ-P32] ATP. The mixture was added to the sonicated sample and incubated at 30° C and 350 rpm for 30 - 60 min. The sphingosinkinase activity was stopped by adding 20 µl 1 N HCl. Then 800 µl $CHCl_3$/CH_3OH/1N HCl (100:200:1), 240 µl $CHCl_3$ und 240 µl 2 M KCl were added. The samples were vortexed for 1 – 5 sec before a centrifugation step (14.000 rpm, 5 min at RT) was performed which leads to phase separation. The lower, organic phase was transferred to a fresh reaction tube and centrifuged in a SpeedVac for 1 – 2 h until the whole liquid was evaporated. The remaining hot sphingosine-1-phosphate was solved in 20 µl

CHCl$_3$/CH$_3$OH 1:1 and then transferred to a TLC plate in 4 µl steps. The plate was put in a TLC tank with running buffer and incubated for 1 – 2 h. After drying, a film was exposed to the TLC plate over night and finally the plates were analyzed using a phosphoimager (Typhoon 9200).

2.2.10 Lipidome analysis

Chang cells (2 x 10^5) were seeded in a 6-well plate one day before the experiment was performed and grown to a confluency of 70-90%. The cells were infected with N927 at a MOI of 50. To stop the infection, the cells were washed three times with warm Hepes-medium, before they were put on ice and washed two times with ice-cold PBS. The supernatant was removed carefully and 325 µl of methanol LC-MS Chromasolve® was added directly on the cells. Afterwards, the cells were scraped off with a cell scraper and the methanol-cell mixture was transferred in an eppendorf tube. The wells were washed with additional 325 µl methanol LC-MS Chromasolve® and the volume was added to the mixture in the eppendorf tubes. The tubes were kept on ice and closed with parafilm before frozen at -80° C. The cells in methanol were sent on dry ice to Burkhard Kleuser and Lukasz Japtok (Institut für Ernährungswissenschaften, Universität Potsdam, Arthur-Scheunert-Allee114-116, 14558 Nuthetal) for lipidome analysis via mass spectrometry.

2.2.11 *d*STORM

An imaging chamber was coated with poly-l-lysin. Therefore, the chamber was first incubated with 3 M KOH for 30 min, and washed with steril deionized water, followed by PBS and again steril deionized water. Afterwards the chamber was incubated with 0.05% poly-l-lysin for 10 min and washed once with water. Then, 1x10^8 *Neisseria* harvested from GC agar plates were pipetted into the chamber and incubated for 20 min at room temperature. The supernatant was removed and the chamber was washed once with PBS to remove unbound bacteria. Subsequently the bacteria were fixed with 3.7% PFA for 15 min at room temperature. The chambers were washed three times with PBS and finally the bacteria were kept in PBS. The next steps were performed by Anne Burgert in the department for biotechnology and biophysics (Lehrstuhl für Biotechnologie und Biosphysik, Biozentrum Am

Hubland, Julius-Maximilians Universität Würzburg). The bacteria were stained with an IgG anti-Ceramide C16/24 antibody (5 µg/ml in 5% BSA in PBS for 2 h) followed by an anti-rabbit Al647 secondary antibody (10 µg/ml in 5% BSA in PBS for 1 h). After a fixation step using 4% PFA with 0.3% glutaraldehyde for 30 min, the bacteria were analysed via dSTORM and a Ripley's H function as well as a morphological cluster analysis based on the results of Ripley's H analysis was carried out.

2.2.12 Statistical analysis

The software Microsoft Excel was used to calculate the statistical significance of the collected data with a two-tailed Student's t-test.

3 RESULTS

## 3.1 Investigation of NSM2 and its product ceramide in gonococcal infection

3.1.1 NSM2 is important for SREC-I/PorB$_{IA}$-mediated invasion

The SREC-I/PorB$_{IA}$-mediated invasion pathway of Ngo has been analysed in previous studies (Faulstich *et al.*, 2013). Subsequently, the involvement of NSM2 in this specific uptake mechanism was identified. NSM2 is crucial for the recruitment and activation of PI3K and therefore induces downstream signalling. The activation of NSM2 represents a very early step in the signalling cascade, which leads to the engulfment of the bacteria. Amongst other things, the role of NSM2 was investigated using inhibitor studies. GW4869 is a specific inhibitor directed against NSM2 and was first used to investigate the effect of NSM2 inhibition during invasion in epithelial cells (Chang cell line) (Faulstich *et al.*, 2015). To confirm the results, I repeated the infection experiments using HUVECs, a human umbilical vein endothelial cell line. The inhibitor treatment did not affect the adherence of the bacteria to the endothelial cells, but reduced the invasion to about the half (Figure 3.1 A). A western blot was performed using an antibody directed against SREC-I to verify the expression of the protein in the cells (Figure 3.1 B). The data suggest that NSM2 plays an important role in the phosphate-sensitive SREC-I/PorB$_{IA}$-mediated invasion not only in epithelial, but also in endothelial cell lines.
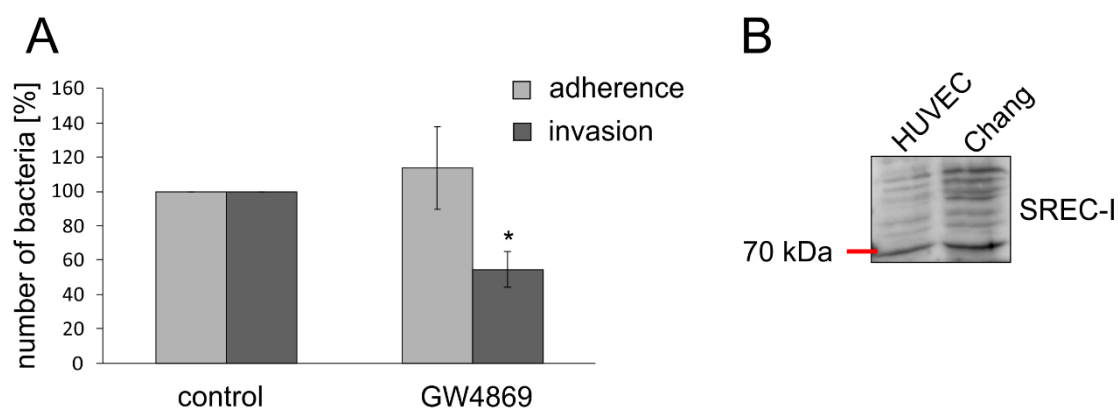
Figure 3.1: Neutral sphingomyelinase 2 plays a role in PorB$_{IA}$-dependent invasion in HUVECs. (A) HUVECs were pretreated with the NSM2-specific inhibitor GW4869 (1.6 µM) or DMSO as control for 2 h and infected with N927 (MOI 50 for 30 min) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments done in duplicate. Adherence and invasion of the control samples were set as 100%. $p < 0.05$ * (B) HUVECs express SREC-I. Cells were harvested and subjected to SDS-PAGE followed by western blot.

Besides the commercially available inhibitor GW4896, I also tested two different, yet unpublished NSM2 specific inhibitors, which were kindly provided by Prof. Dr. Christoph Arenz. Epithelial cells were infected after 2 h inhibitor treatment. In this approach GW4869 showed the strongest decrease in invasive bacteria compared with the inhibitors Eso48 and c11AG. Therefore, GW4869 was the chemical of choice for the inhibition of NSM2 in all future experiments (Figure 3.2).
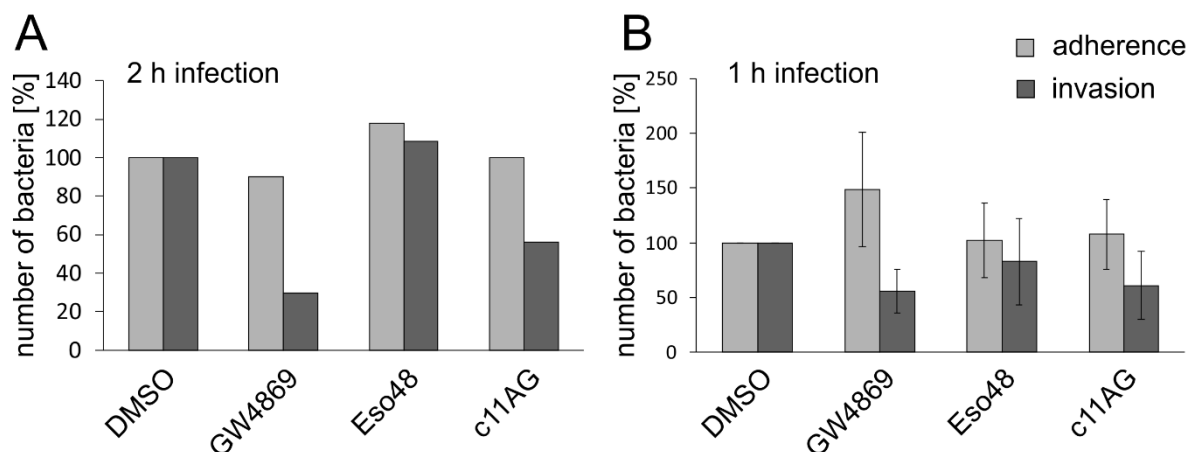

Figure 3.2: Different NSM2 inhibitors influence the PorB$_{IA}$-dependent invasion. Chang cells were pretreated with specific NSM2 inhibitors for 2 h prior to infection. The following concentrations were used: GW4869 1.6 µM, Eso48 3 µM, c11AG 4 µM. The cells were infected under phosphate-free conditions with N927 at MOI 50 either for 2 h (A) or 1 h (B). Adherence and invasion were quantified using gentamicin protection assay. The graph (A) shows one experiment, the graph (B) shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%.

3.1.2 Generation of an antibody against NSM2

The discovery of NSM2 as a key factor for PorB$_{IA}$-dependent invasion initiated further research projects to illuminate and narrow down the specific function and role of the enzyme in neisserial infection. Therefore, I aimed to develop tools for studying the protein. First, an antibody specific for NSM2 should be generated because the commercially available antibodies failed to recognize endogenous NSM2. Two different approaches were pursued: for one antibody, a synthetic peptide was used as an antigen, whereas another antibody was raised against the full-length enzyme. The peptide sequence of NSM2 used for immunization (KSSGQKGRKELLKGNGRR) was selected due to its good antigenicity, solubility and epitope prediction. Davids Biotechnologies, a company settled in Regensburg, Germany, performed

the synthesis and immunization of rabbits and the isolation of the antibodies. For the second antibody, it was planned to use the whole NSM2 protein as an antigen for the antibody production. However, it was not possible to express the whole protein in a soluble form in *E. coli*. Therefore, a practical student, Esther Rogalski, cloned the first third of the protein in an expression vector, transformed it in *E. coli*, expressed it and finally purified the protein. This part of the protein was also send to Davids Biotechnologies and the resulting antibody was named NSM2-1. Subsequently, the antibodies were tested using western blots. For this purpose, Chang cells were either transfected with a NSM2-GFP construct or with an empty vector as control transfection. The transfected cells as well as untreated Chang cells, either as a whole cell lysate or separated into cytosol and membrane fraction, were harvested and subjected to western blotting. Additionally, the purified protein NSM2-1 was subjected to western blotting. Both antibodies were used in a 1:100 dilution next to antibodies directed against GFP and His tag as controls (Figure 3.3 B). The peptide NSM2 antibody was concentrated and then directly compared to a 1:50 dilution of the unconcentrated antibody. The NSM2-1 antibody was purified in order to minimize the background and used for western blotting (Figure 3.3 A). The analysis of the blots revealed that the NSM2-1 antibody is able to recognize overexpressed NSM2. The size of the NSM2-GFP protein was confirmed by the blots incubated with a GFP antibody. The peptide NSM2 antibody also recognizes the overexpressed NSM2-GFP protein, but the background makes it impossible to be sure which band is the right one without comparing it directly with a control as GFP. Because of the high background, it is not possible to detect endogenous levels of NSM2 in Chang cells.
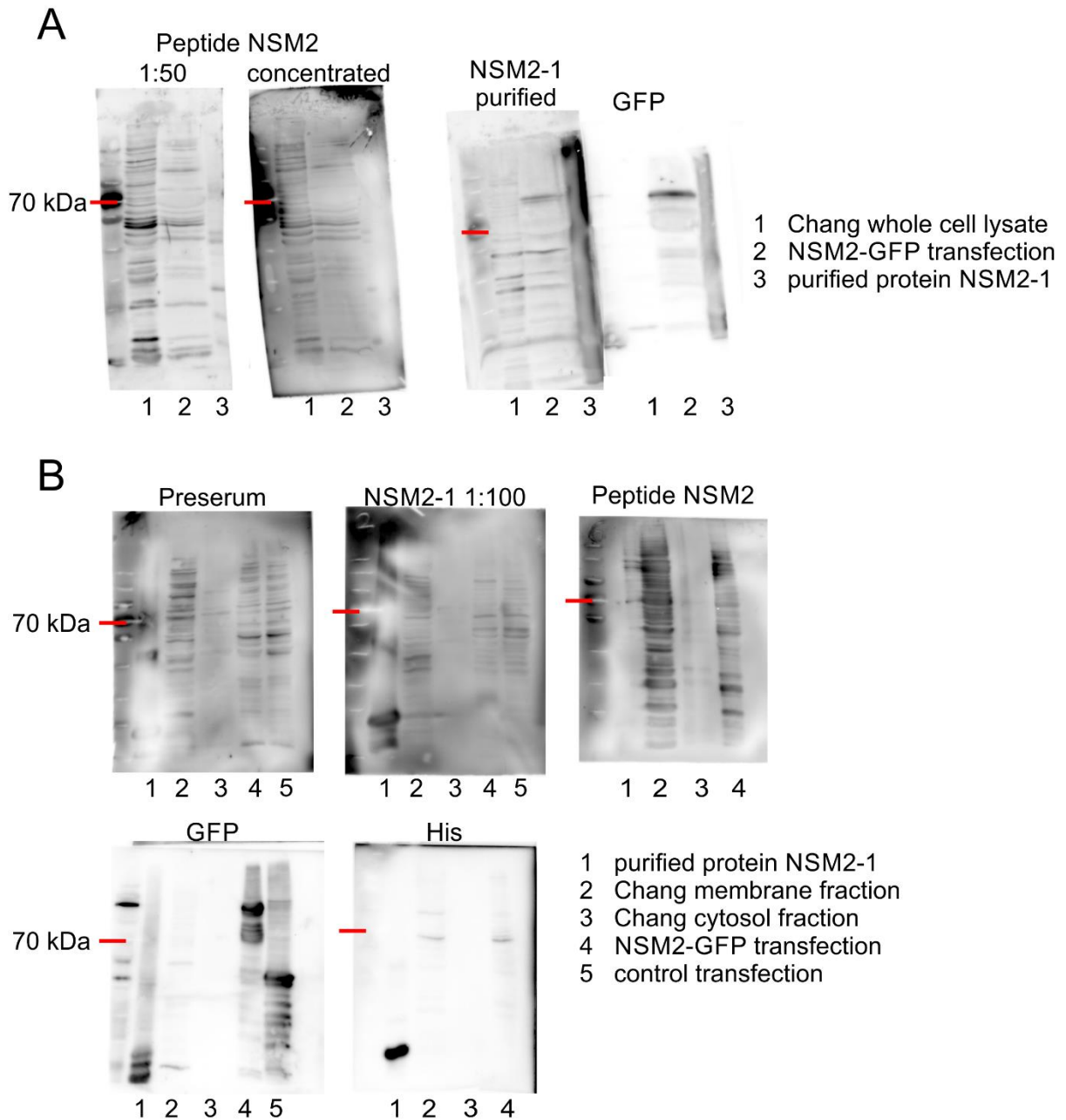
Figure 3.3: Testing of two antibodies directed against NSM2. (A) Chang cells were transfected with a NSM2-GFP construct. 36 h post-transfection, the cells were harvested. Chang cells, the transfected cells as well as the purified protein NSM2-1 were subjected to SDS-PAGE and western blot. The blots were analyzed using the peptide NSM2 antibody as well as the purified NSM2-1 antibody and an antibody directed against GFP as control. (B) Chang cells were transfected with a NSM2-GFP construct. 36 h after transfection, the cells were harvested. Chang cells were harvested and the membrane and cytosolic fraction was isolated. The purified protein NSM2-1, the transfected cells and a control transfection as well as the different Chang fractions were subjected to SDS-PAGE and western blotting. Antibodies directed against GFP and His-tag were used as controls. The preimmunization serum of the NSM2-1 antibody, as well as the NSM2-1 antibody and the peptide antibody of NSM2 were used to analyze the blots.

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3.1.3 RNA levels of neutral sphingomyelinases in different cell lines

The question arose if another cell line distinct from Chang cells would be more suitable for the designated approach. Therefore, I tested expression levels of neutral sphingomyelinase 1 to 3 in different cell lines that were used as neisserial infection models in publications or in our group (Figure 3.4). The results were normalized to the expression level of the respective enzymes in Chang cells. HUVECs showed a slightly decreased mRNA expression of all three neutral sphingomyelinases compared to Chang cells. In contrast, HEK 293T cells (human kidney epithelial cells) exhibit a log fold change of 0.5 in NSM1, 0.7 in NSM3 and a log fold change around 2.5 in NSM2 mRNA. The relative expression in this cell line is clearly increased, whereas the opposite is the case in HeLa2000. The results indicate that HEK 293T cells would be a good model when it comes to the investigation of NSM2.
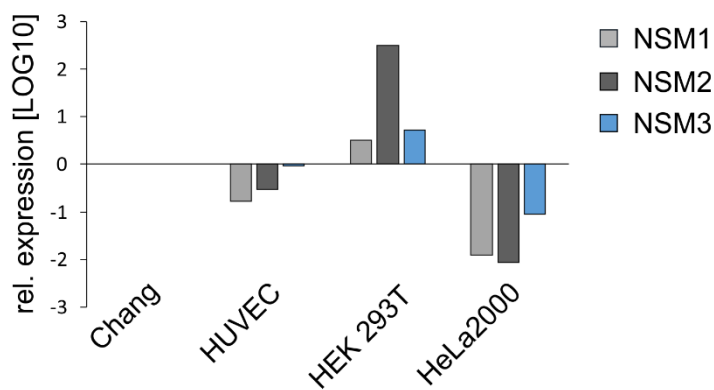

Figure 3.4: Comparison of the relative expression of NSM1, NSM2 and NSM3 mRNAs in different cell lines. Cells of different cell lines were harvested, the mRNA was isolated and converted into cDNA. A qRT-PCR was performed to evaluate the different expression levels of the mRNA of NSM1 to 3 in comparison to Chang cells.

3.1.4 Generation of a NSM2 knockout cell line using the CRISPR/Cas9 system

A karyogram was used to determine the karyotype of Chang cells. Chang cells are human conjunctiva epithelial cells, a tumor cell line which has been in culture for several decades. One characteristic of these cells is their potential for unlimited reproduction. This feature may lead to chromosomal aberrations. A karyogram of a healthy human male was compared with the karyogram of Chang cells (Figure 3.5). The result revealed severe chromosomal aberrations in Chang cells, including deletions, translocations and duplications. Some chromosomes were even multiplied as whole. Other chromosomes showed heavy

alterations that made it impossible to identify the chromosomes. Despite of the abberations, I decided to use Chang cells for the generation of NSM2 knockout clones, because it seemed feasible.
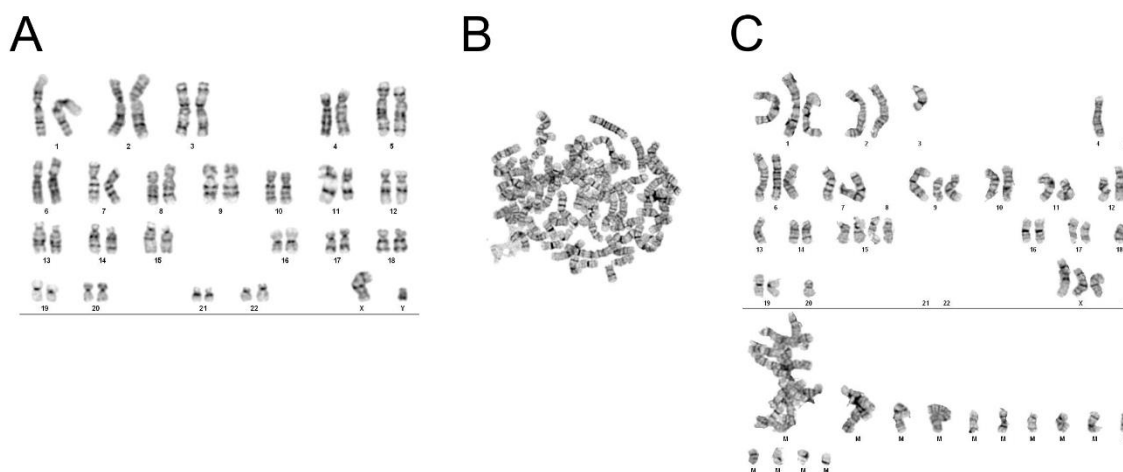

Figure 3.5: Karyogram. (A) Karyogram of a healthy human male. (B) Unordered chromosomes of cultured Chang cells. (C) Karyogram of cultured Chang cells.

The clustered regularly interspaced short palindromic repeats (CRISPR/Cas) system is the so called "immune system of bacteria and archaea" (Horvath & Barrangou, 2010). The adaptive immune system defends the microorganisms against viruses and plasmids. In the process, the invading nucleic acid is attacked in a sequence specific manner (Horvath & Barrangou, 2010). Shortly after its discovery the system became a promising, powerful and versatile tool for genome editing in diverse organisms (Mali *et al.*, 2013). The CRISPR/Cas9 system for genome editing is such a powerful tool, that the method initiated a new discussion about guidelines for gene editing in humans (Hirsch *et al.*, 2017). In this study, I aimed to produce a NSM2 knockout cell line using CRISPR/Cas9 following a published protocol (Ran *et al.*, 2013). Chang cells were transfected with two plasmids carrying the Cas9 protein coupled to GFP and one guide RNA each, directed against NSM2. The GFP positive cells were sorted and expanded as single clones for 2-3 weeks. The DNA of several clones was isolated and sent for sequencing. The clones were analyzed for deletions, which will result in the expression of a nonfunctional protein or no protein at all. Three promising clones were tested in infection experiments (Figure 3.6). The adherence of the gonococci to the NSM2 KO cell lines (clones) showed no significant differences compared to the untreated Chang cells. In contrast, there

was a clear and statistically significant effect on invasion in clone 7 and 11. The invasion was reduced to 20% in comparison to Chang cells. Interestingly, the inhibitor GW4869 revealed a strong effect on invasion not only in the Chang control cells, but also in the NSM2 knockout clones. Since the inhibitor is not cytotoxic for *Neisseria* in culture, this effect cannot be explained with GW4869 killing the bacteria (data not shown).
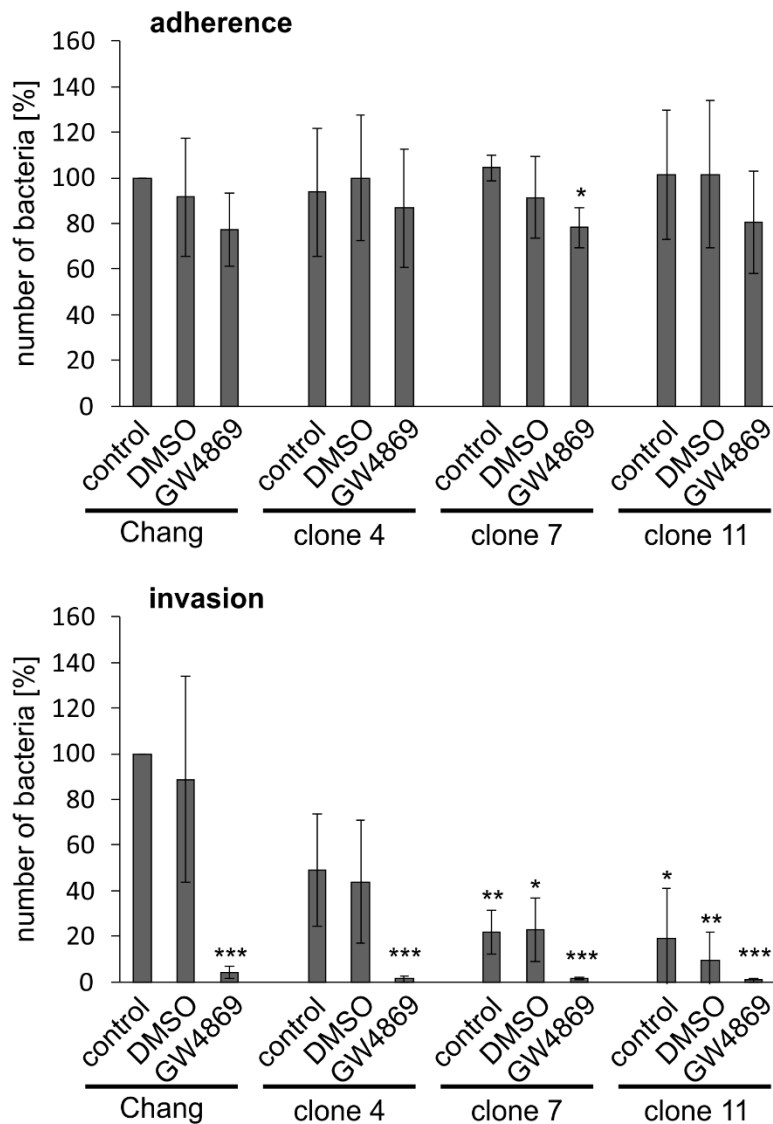
Figure 3.6: Testing of different NSM2 CRISPR/Cas9 knockout clones via Gentamicin protection assay. Chang cells were used to produce NSM2 knockout cell lines via the CRISPR/Cas9 system. 3 different single cell knockout clones as well as Chang cells as control were either left untreated or treated with DMSO or GW4869 (1.6 μM) for 1 h prior to infection. The cells were infected with N927 at MOI 50 for 1 h under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments done in duplicate. Adherence and invasion of the Chang control samples were set as 100%. p < 0.05 *, p < 0.01 **, p < 0.001 ***.

The output of the gentamicin protection assay is colony forming units. Therefore, the assay does not actually show the invasion of *Neisseria*, but the invasion and survival of the bacteria until the time point the host cells are lysed. In contrast, the infectivity assay (differential immunofluorescence staining) reveals the pure invasion. Clone number 7 and 11 were used to perform infectivity assays (Figure 3.7). The results obtained differed from the outcome of the gentamicin assay. Both clones didn't show a decrease in invasion in the untreated control samples and the samples treated with DMSO compared to Chang cells.
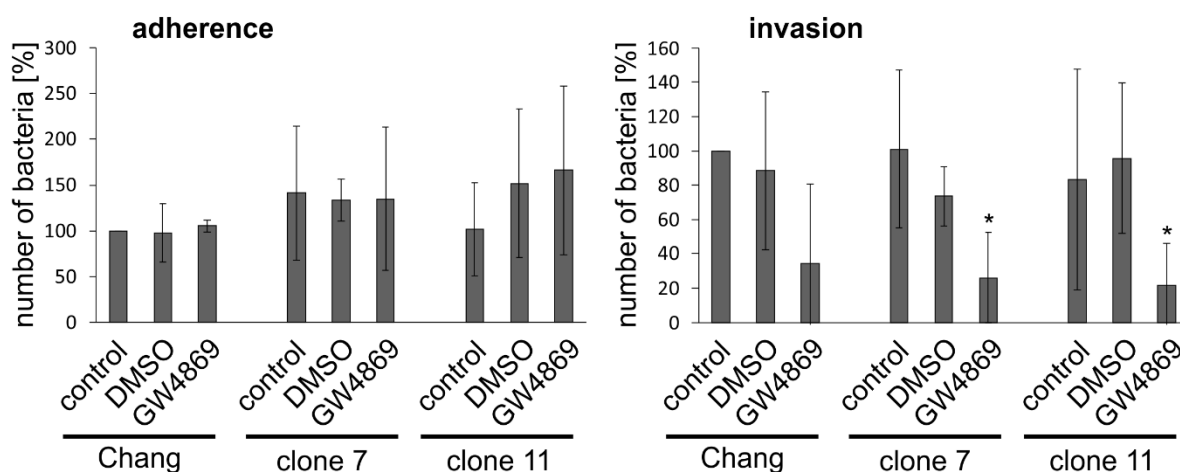

Figure 3.7: Testing of different NSM2 CRISPR/Cas9 knockout clones via differential immunofluorescence staining (infectivity assay). Chang cells were used to produce NSM2 knockout cell lines using the CRISPR/Cas9 system. 3 different single cell knockout clones as well as Chang cells as control were either left untreated or treated with DMSO or GW4869 (1.6 μM) for 1 h prior to infection. The cells were infected with N927 at MOI 10 for 1 h under phosphate-free conditions. Adherence and invasion were quantified using infectivity assay. The graph shows mean values ± SD of three independent experiments. Adherence and invasion of the Chang control samples were set as 100%. $p < 0.05$ *.

3.1.5 Complementation of the CRISPR/Cas9 NSM2 knockout cell line using shRNA

The next step was the complementation of the knockout cell lines with a NSM2-GFP construct. A lentiviral vector was used for the transduction of NSM2-GFP leading to a long-term expression of the protein. The transduced cells were sorted into single cell clones and expanded for several weeks before they were tested. The clones 7 and 11 as well as normal Chang cells were used for the complementation. Western blot was performed using an anti-GFP antibody to verify the transduction and expression of the NSM2-GFP construct (Figure 3.8 A). The untreated Chang cells as well as the knockout cell lines clone 7 and 11 did not

expressed GFP, whereas the different transduced cell lines express NSM2-GFP. Next, the clones were tested in infection, using the gentamicin assay (Figure 3.8 B). The adherence of the Ngo to different cell lines was quite consistent. In contrast, the complemented cell lines showed a higher invasion than the wildtype Chang cells. However, there was a discrepancy in invasion between the single experiments, resulting in a very high standard deviation. In addition, the earlier described effect of the decreased invasion of Ngo in the knockout cell lines (clone 7 and 11) could not be reproduced.
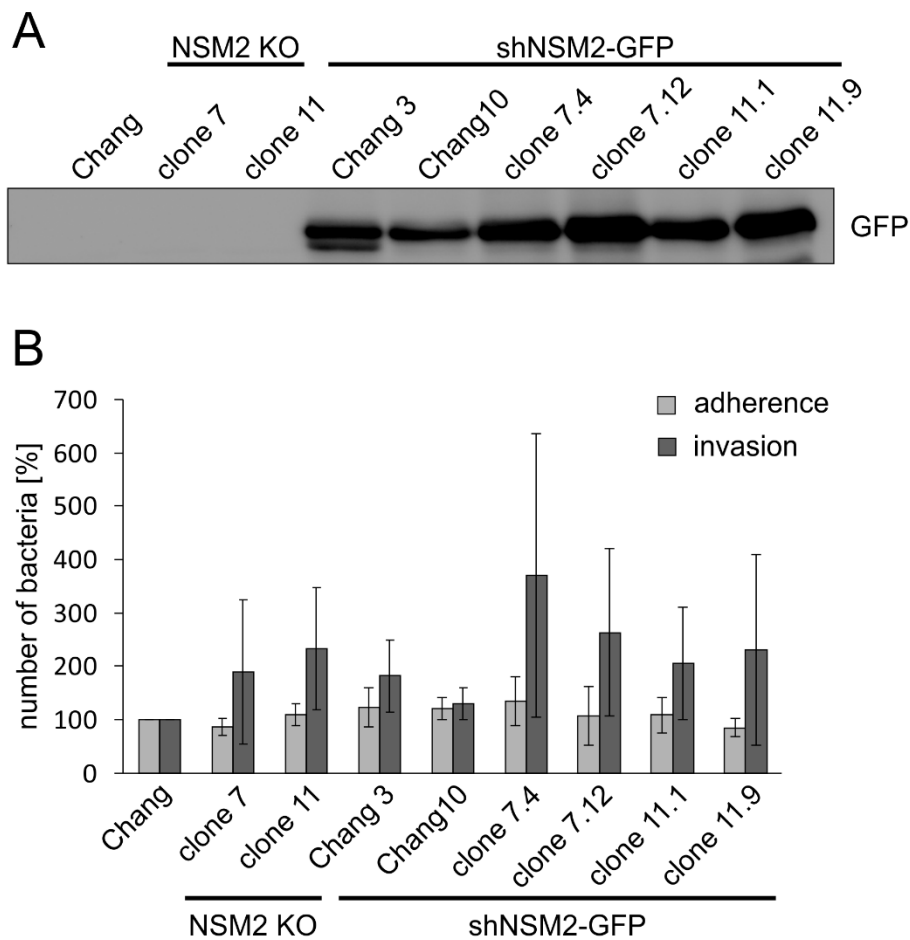
Figure 3.8: NSM2 CRISPR/Cas9 knockout cell lines were complemented with a NSM2-GFP construct. Chang cells as well as the NSM2 CRISPR/Cas9 knockout clones 7 and 11 were used to produce shRNA-based stable cell lines expressing NSM2 coupled to GFP. Different clones were tested. (A) Cells were harvested and analyzed by SDS-PAGE and western blot. (B) Different cell lines were infected with N927 at MOI 50 for 1 h under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments done in duplicate. Adherence and invasion of the untreated Chang samples were set as 100%.

3.1.6 Impact of ceramide on gonococcal infection

It has been shown, that NSM2 plays a crucial role in PorB$_{IA}$-dependent invasion. The enzyme converts sphingomyelin to ceramide and phosphorylcholine. The so build ceramide is important for signalling processes characteristic for the phosphate-dependent invasion (Faulstich, Dissertation 2013). Here, I aimed to address the question if more ceramide leads to an increased invasion of the gonococci. To this purpose, Chang cells were treated with bacterial sphingomyelinase (bSMase) derived from *Bacillus cereus*, a gram-positive bacterium which inhabits soil and can cause food poisoning. The bSMase hydrolyzes sphingomyelin to phosphocholine and ceramide and the catalytic reaction is dependent on divalent metal ions. Furthermore, the enzyme is a homologue of mammalian neutral SMase and mimics its actions (Ago *et al.*, 2006). The bSMase is supposed to produce ceramide and the influence of the activity of this enzyme was monitored using a gentamicin assay (Figure 3.9 A). The results indicated a significant impact on invasion when the cells were preincubated with the enzyme. In contrast to the expectations, the invasion in the preincubated cells decreased. This was also seen as a trend when the bSMase was added to cells simultaneously with the gonococci. Another strategy to find out if more ceramide leads to an increased invasion represented the addition of ceramide itself (Figure 3.9 B). In this case C16 ceramide was used. The addition of C16 ceramide led to an increased invasion, although the results were not statistically relevant. The effect of the NSM2 specific inhibitor GW4869 on the invasion could partly be restored by C16. There was still a decrease in invasion when the cells were treated with GW4869 and C16 ceramide, but the reduction in invasive bacteria was not significantly different from the one in untreated cells any more. Therefore, the two experiments described in figure 3.9 revealed contradictory results.
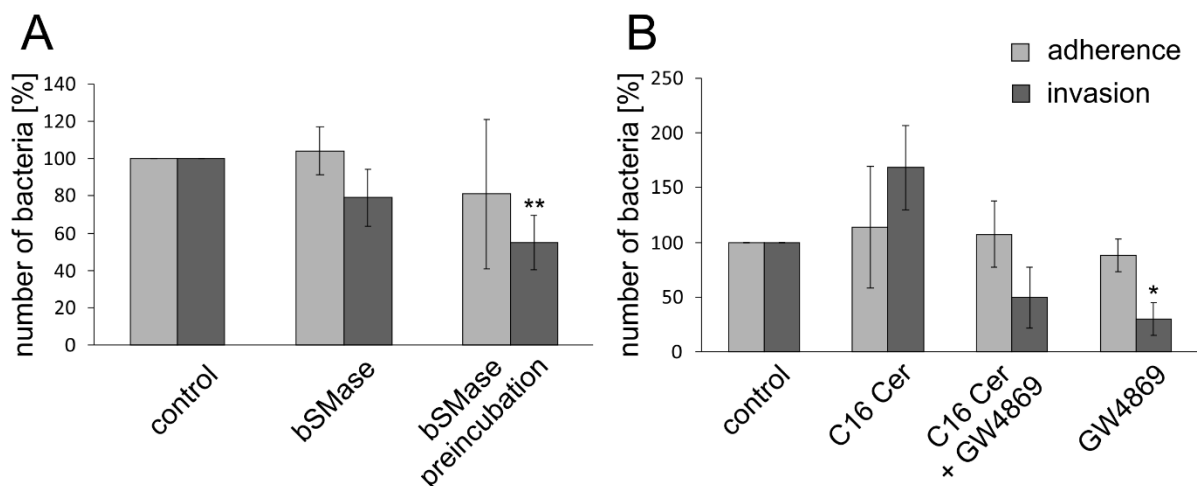
Figure 3.9: Change of ceramide levels influences PorB$_{IA}$-dependent invasion. (A) Chang cells were either pretreated with bacterial sphingomyelinase (bSMase), 50 μunits/ml for 30 min (preincubation) or the enzyme was added directly when the cells were infected. (B) Chang cells were pretreated as indicated with GW4869 (1.6 μM). 5 min before infection, C16 ceramide (50 nM) was added to the cells. (A+B) Cells were infected with N927 (MOI 50 for 30 min) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graphs show mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. $p < 0.05$ *, $p < 0.01$ **.

3.1.7 Staining of *N. gonorrhoeae* using a ceramide antibody

It has already been shown that ceramide is build due to the infection and can be visualized by fluorescence microscopy (Faulstich *et al.*, 2015). Those results were obtained using an IgM anti-ceramide antibody. At the revision of the experiment using an IgG antibody directed against ceramide 16 and 24, the results indicated a staining of the gonococci by the antibody. To prove this, *Neisseria* were stained using the C16/24 IgG antibody and subjected to super-resolution imaging by direct stochastic optical reconstruction microscopy (*d*STORM). The method enables the localization of single molecules by microscopy using photoswitchable fluorophores (van de Linde *et al.*, 2013). The pictures were taken and analysed by Dr. Anne Burgert. The results show a staining of the membrane in which the signal forms clusters (Figure 3.10 A). Those clusters were further analysed by conducting a standardized Ripley's L-function. The function clearly reveales a non-homogenous distribution of the molecules (figure 3.10 B). Hence, most of the ceramide molecules are existent in clusters. The function revealed a maximum at r ~ 100 nm. This peak shows the approximately average size of the clusters. The standardized Ripley's L-function is not made

for the identification of the exact size, instead it gives a value between the radius and the diameter. But the method definitely shows the distribution of the ceramide signals in clusters.
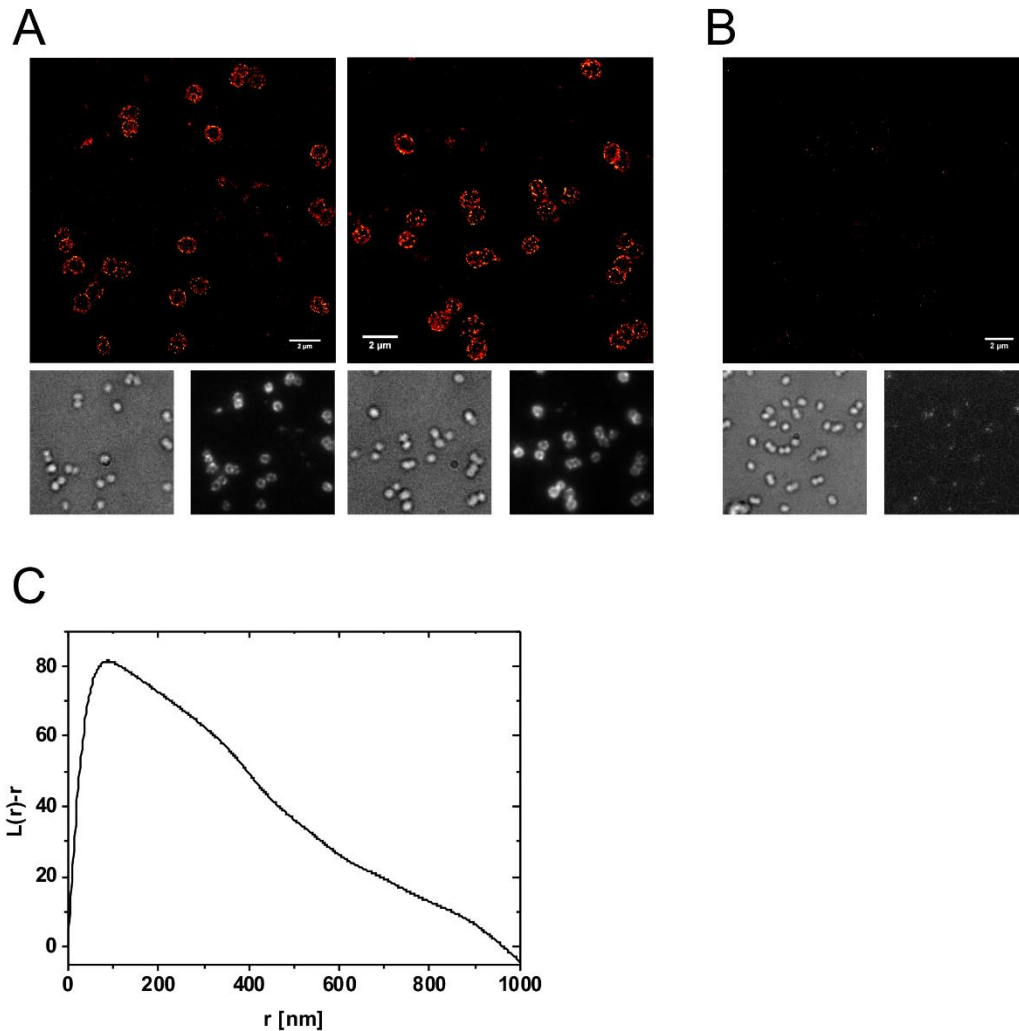
Figure 3.10: Staining of membranes of gonococci using an anti-C16/24 ceramide IgG antibody. N927 was fixed and stained using an anti-C16/24 ceramide IgG antibody as the primary antibody and a Al647 secondary antibody (A). N927 were fixed and stained only with the secondary antibody for control (B). The bigger pictures show *d*STORM pictures, whereas the small pictures show images by transmitted light (on the left side) and wide field fluorescence microscopy (on the right side). A standardized Ripley's L-function was performed based on the clusters seen in A (C).

3.2 Analysis of key players of the sphingolipid pathway in gonococcal infection

3.2.1 Gonococcal infection alters cellular sphingolipids

The visualization of the ceramide release due to infection proved to be more difficult than expected. Microscopy could not be the method of choice because the *Neisseria* were stained with the ceramide antibody. The FACS experiments, which were performed to measure the ceramide content in untreated versus infected cells (Faulstich *et al.*, 2015) are based on antibody binding, too. Therefore, the new method to be tried became mass spectrometry. The samples were prepared on site and then sent to our collaboration partners Dr. Lukasz Japtok and Prof. Dr. Burkhard Kleuser for lipidome analysis via mass spectrometry. One advantage of the method is its great sensitivity. Lipids in the scale of pmol can be detected. Another advantage is the detection of different ceramide species. It is possible that the total ceramide content does not change due to infection with *Neisseria*, but a specific species of ceramide changes. First, a testing of different time points was performed to evaluate the most suitable period for infection. 2 h infection time showed the biggest effect concerning changes in sphingolipid levels and was chosen for the following lipidome analysis. In this investigation, the levels of sphingomyelin, ceramide, sphingosine and sphingosine-1-phosphate were determined. The evaluation of the data obtained revealed that there is no significant change of sphingomyelin levels due to infection with *N. gonorrhoeae*, neither in total sphingomyelin nor in the individual sphingomyelin species. However, there was a trend visible: the levels increase slightly in the infected samples (Figure 3.11 A). The results for the single ceramide species showed the same trend as the sphingomyelin species. The infected cells contained more ceramide than the uninfected control samples. In the ceramide species 16, 18 and 20 the increase is even statistically significant (Figure 3.11 B). The relative amount of sphingosine is significantly bigger in infected than in uninfected Chang cells (Figure 3.11 C). The level of sphingosine-1-phosphate is mildly higher in infected than in uninfected cells, but this result is not statistically significant (Figure 3.11 D). In conlusion, the infection of Chang cells with gonococci leads to alterations in sphingolipid levels. In addition, gonococci harvested from GC agar plates were sent for mass spectrometry. However, no results could be obtained, indicating either sphingolipid levels under the limit of detection or the complete absence of sphingolipids in gonococci.
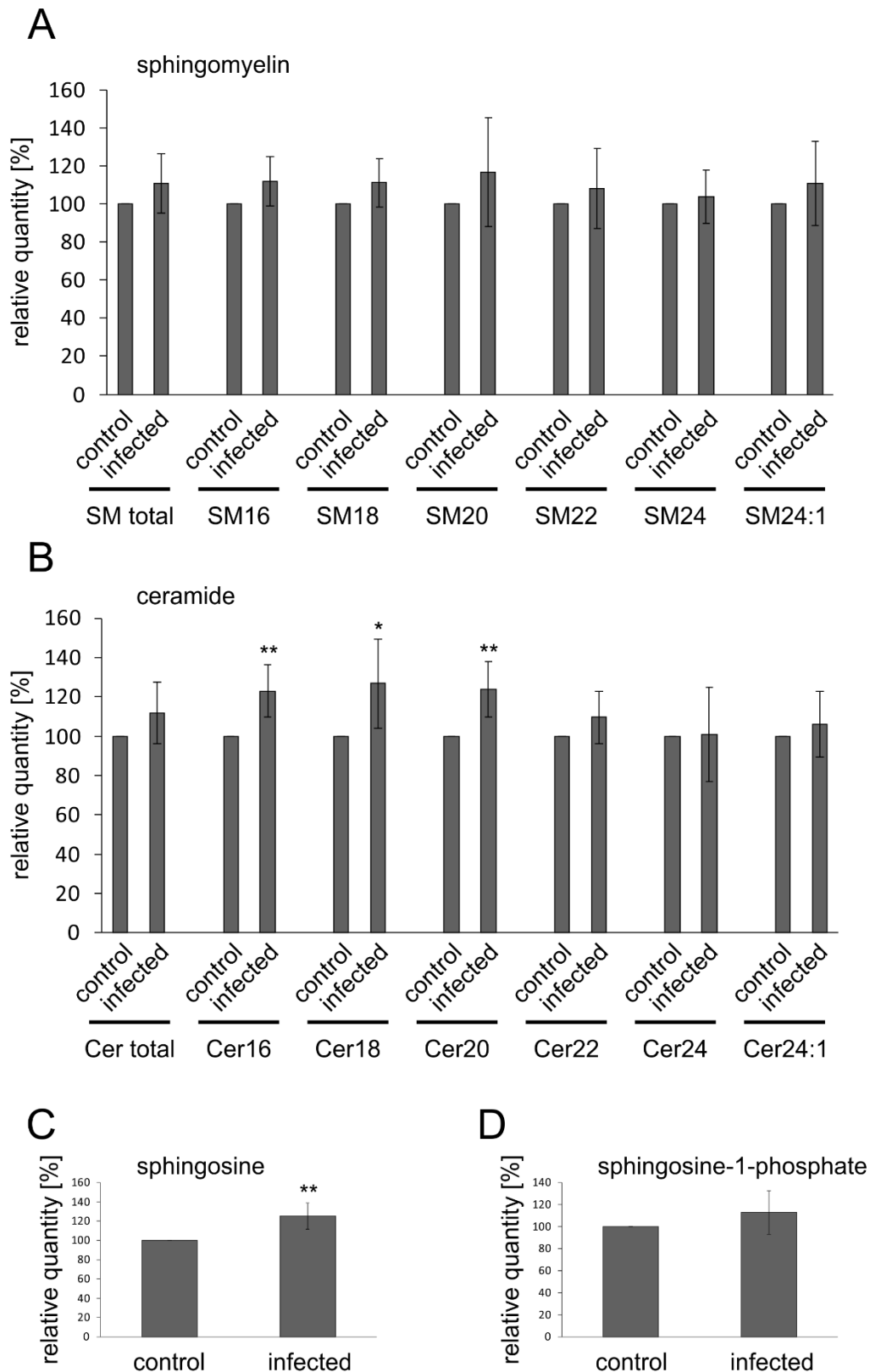
Figure 3.11: Infection with gonococci affects sphingolipids. Chang cells were infected with N927 (MOI 50 for 2 h) under phosphate-free conditions. The cells were harvested in methanol and analyzed via mass spectrometry (lipidome analysis). The graphs show mean values ± SD of six independent experiments. The uninfected control samples were set as 100%. p < 0.05 *, p < 0.01 **.

The graphs show the relative quantities of sphingomyelin (A), ceramide (B), sphingosine (C) and sphingosine-1-phosphate (D).

3.2.2 Sphingosine is toxic for gonococci

Ngo were tested for their susceptibility to several fatty acids and monoglycerides *in vitro* before. The experiments showed that a monoglyceride monocaprin inactivates the bacteria within one minute (Bergsson *et al.*, 1999). Sphingosine belongs to the group of sphingoid bases and possesses antibacterial activity against gram-positive as well as gram-negative bacteria (Fischer *et al.*, 2012). Here, I aimed to investigate the effect of pure sphingosine on gonococci since the levels of sphingosine were increased in cells during infection and could therefore affect the bacteria (Figure 3.11). First, the growth was measured using different concentrations of sphingosine (Figure 3.12 A). The lowest concentration showed no effect on bacterial growth, whereas the 10 µM concentration visibly slowed down the growth. The highest concentration showed a very strong effect on the bacteria, which was similar to the inhibitory effect of kanamycin. Next, the ability of the *Neisseri*a to survive the treatment with sphingosine and form new colonies on plate afterwards was tested (Figure 3.12 B). For this, a defined number of bacteria was exposed to 5 µM sphingosine, plated and counted the next day. The experiment was performed using two different media, HEPES and PPM+. The negatively affected the bacteria in both media compared to the untreated control and the bacteria treated with DMSO. The effect was statistically significant in both media, but it is stronger in HEPES than in PPM$^+$ medium. Taken together, the results indicate an antibacterial activity of sphingosine against *N. gonorrhoeae*.
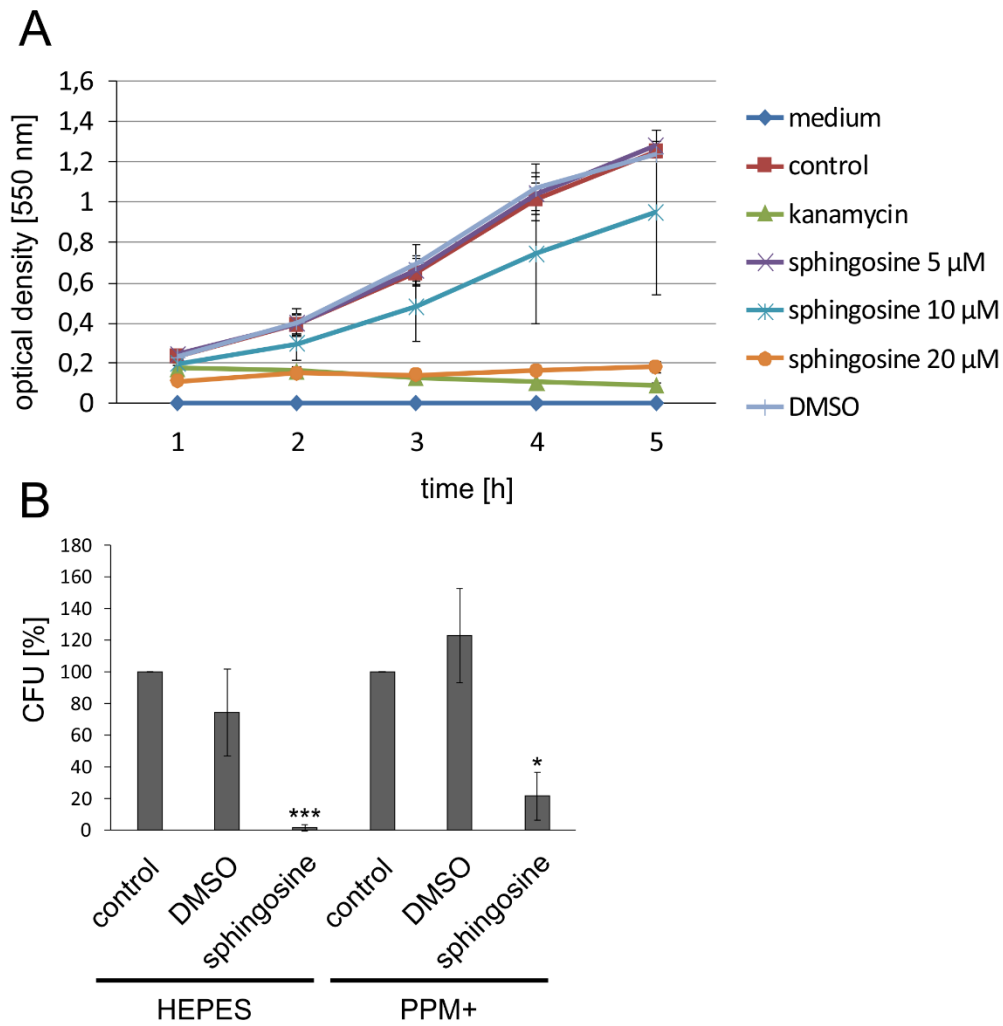
Figure 3.12: Sphingosine inhibits bacterial growth. (A) Bacterial growth was measured using neisserial strain N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows mean values ± SD of three independent experiments. (B) N927 were either cultured in HEPES or in PPM+ medium and treated with DMSO or sphingosine (5 µM) for 2 h. The bacteria were plated on GC agar plates and the colonies were counted the next day. The graph shows mean values ± SD of three independent experiments done in duplicate. The control samples were set as 100%. $p < 0.05$ *, $p < 0.001$ ***.

3.2.3 Role of sphingosine kinases in gonococcal infection

3.2.1.1 Inhibitor studies – SPHK2 plays a role in infection

The lipidome analysis revealed an increase of the sphingosine and sphingosine-1-phosphate levels in Chang cells due to infection (Figure 3.11). This increase argues for the involvement of sphingosine kinases, which convert sphingosine to sphingosine-1-phosphate. In the first instance, inhibitor studies were performed to address the question if the enzymes play a role in PorB$_{IA}$-mediated infection. The first inhibitor of choice was SKI-II. The commonly used substance inhibits both sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2) (Gao *et al.*, 2012, Neubauer & Pitson, 2013). The inhibitor has a drastic effect on the growth of gonococci (Figure 3.13 A). The smallest concentration used already results in a very strong growth defect of the bacteria. Higher concentrations show an effect comparable to kanamycin. In contrast, SKI-II has no cytotoxic effect on Chang cells up to a concentration of 20 µM (Figure 3.13 B). Next, the inhibitor was tested in phosphate-free infection experiments. The gentamicin assay revealed a statistically significant decrease in adherence as well as in invasion starting at a concentration of 10 µM (Figure 3.13 C). It is likely that this decline is caused by the direct effect of the inhibitor on the bacteria and not by the inhibition of the enzymes.
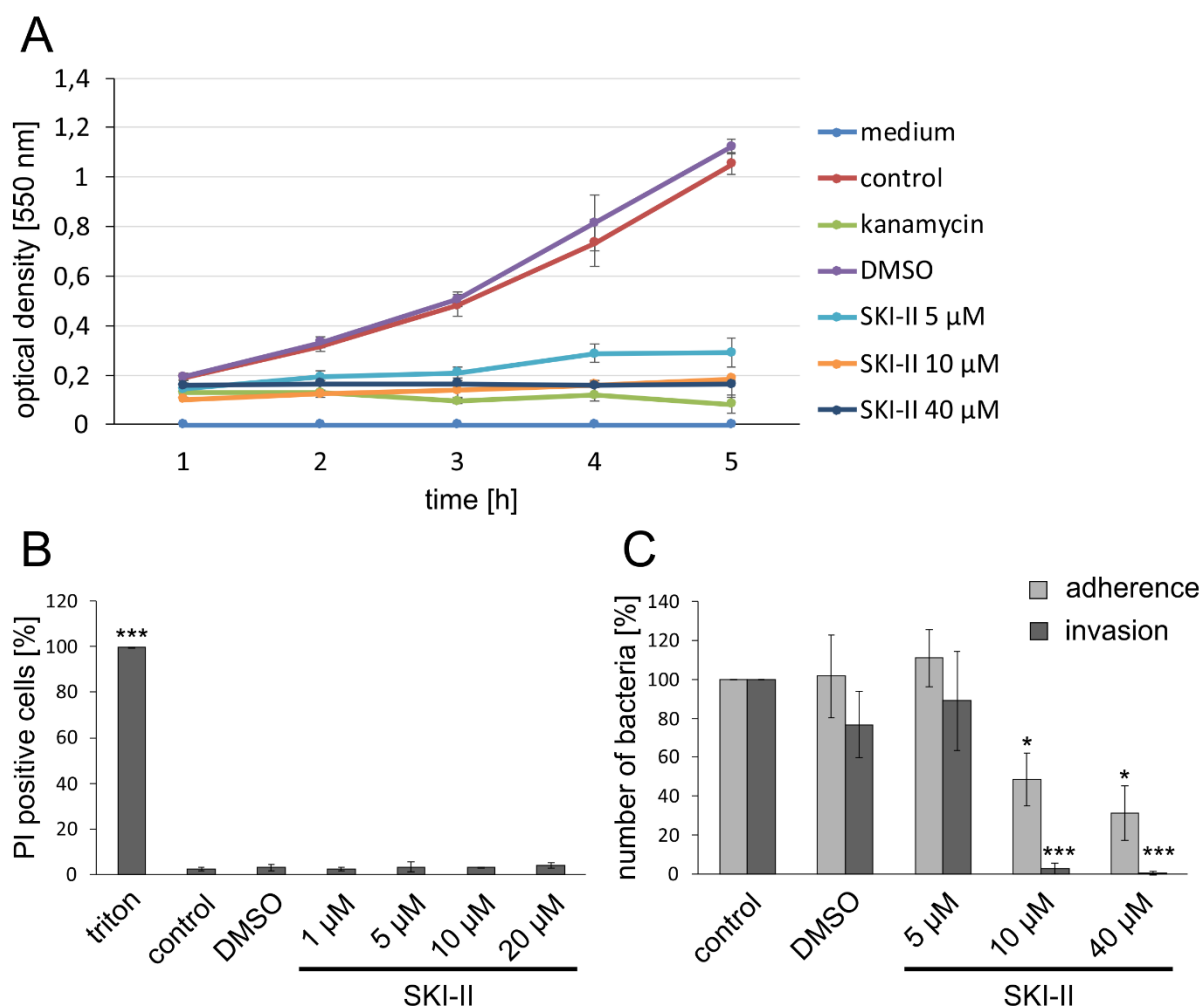
Figure 3.13: Sphingosine kinase inhibitor SKI-II influences Ngo and PorB$_{IA}$-dependent invasion. (A) Bacterial growth was measured using N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows mean values ± SD of three independent experiments. (B) Chang cells were used to conduct a cytotoxicity assay. The cells were treated with DMSO or the indicated concentrations of the inhibitor SKI-II for 2 h. 5 min before analysis Triton X-100 (0.1%) was added to one sample as positive control. The cells were stained with PI and analyzed via flow cytometry. The graph shows mean values ± SD of three independent experiments. $p < 0.001$ ***. (C) Chang cells were pretreated with the inhibitor SKI-II in the indicated concentrations or DMSO as control for 2 h and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. $p < 0.05$ *, $p < 0.001$ ***.

Since SKI-II had a direct effect on the bacterial growth, I decided to use other inhibitors, which target only SPHK1 or SPHK2. The use of these substances will result in detailed information about which of the two enzymes is involved in PorB$_{IA}$-mediated invasion. 5C which is also referred to as compound 5 in the literature, specifically inhibits the SPHK1 and

was synthesised in 2009 (Wong *et al.*, 2009). Besides *in vitro* studies, the inhibitor has already been proven suitable for *in vivo* experiments with mice. Treatment with 5C significantly decreases tumor growth in a mammary adenocarcinoma tumor model in BALB/c mice (French *et al.*, 2003). In this study, the inhibitor showed no effect on the growth of the bacteria (Figure 3.14 A). It exhibited also no cytotoxicity on Chang cells (Figure 3.14 B). The performed infection assays revealed neither a change in adherence nor in invasion due to inhibitor treatment (Figure 3.14 C).
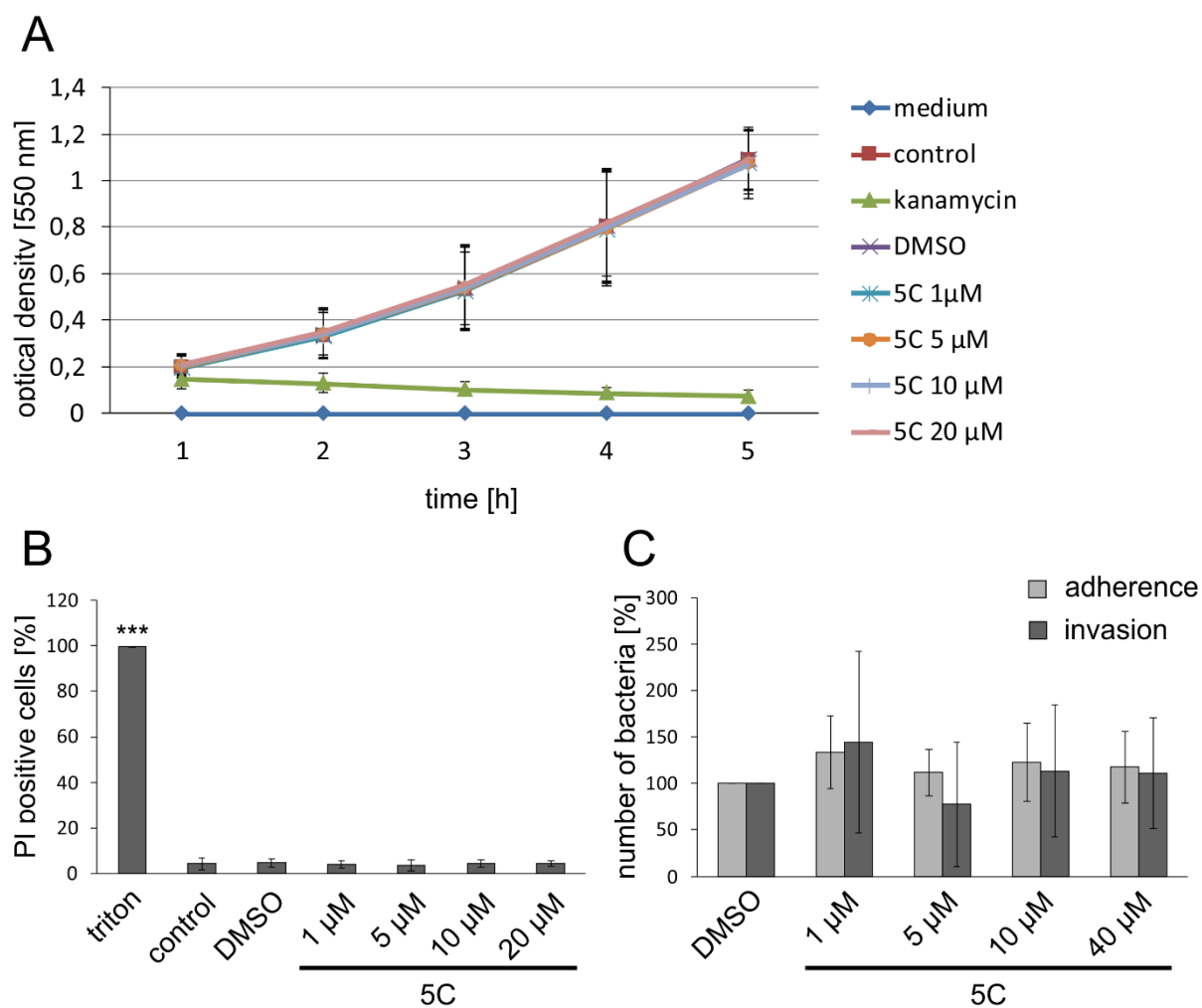
Figure 3.14: Sphingosine kinase 1 inhibitor does not affect PorB$_{IA}$-dependent invasion. (A) Bacterial growth was measured using N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows mean values ± SD of three independent experiments. (B) Chang cells were used to conduct a cytotoxicity assay. The cells were treated with DMSO or the indicated concentrations of the SPHK1 specific inhibitor 5C for 2 h. 5 min before analysis Triton X-100 (0.1%) was added to one sample as a positive control. The cells were stained with PI and analysed via flow cytometry. The graph shows mean values ± SD of three independent experiments. $p < 0.001$ ***. (C) Chang cells were pretreated with the inhibitor 5C in the indicated concentrations or DMSO as control for 2 h and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions.

Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%.

The SPHK2 was inhibited by K145. The substance is a selective SPHK2 inhibitor and was synthesised and characterized in 2013 (Liu *et al.*, 2013). It is competitive to sphingosine and suppresses sphingosine-1-phosphate levels *in vitro*. K145 has an apoptotic effect on U937 cells and was positively tested as potential anticancer agent in two different mouse models (Hu *et al.*, 2013, Neubauer & Pitson, 2013). Here, the inhibitor did not affect the bacterial growth (Figure 3.15 A). In contrast, K145 had a cytotoxic effect on Chang cells starting from the concentration of 10 µM and increasing with rising concentration (Figure 3.15 B). The infection was tested by gentamicin assay and revealed a minor decrease in the adherence due to rising concentrations of the inhibitor (Figure 3.15 C). This resulted in a significantly reduced adherence at 20 µM concentration of K145. This might be caused by the cytotoxic effect of the inhibitor. The treatment with K145 drastically affects the phosphate-sensitive invasion of the gonococci. Even at a low concentration of the inhibitor of 1 µM the invasion is statistically significantly reduced to about 30% compared to the DMSO control. At 5 µM concentration, the reduction was even stronger and only 3% of the bacteria invaded the cells.

Taken together, the results of the sphingosine kinase inhibitor studies indicate an important role of SPHK2 in the PorB$_{IA}$-mediated invasion, whereas SPHK1 seems not to be involved.
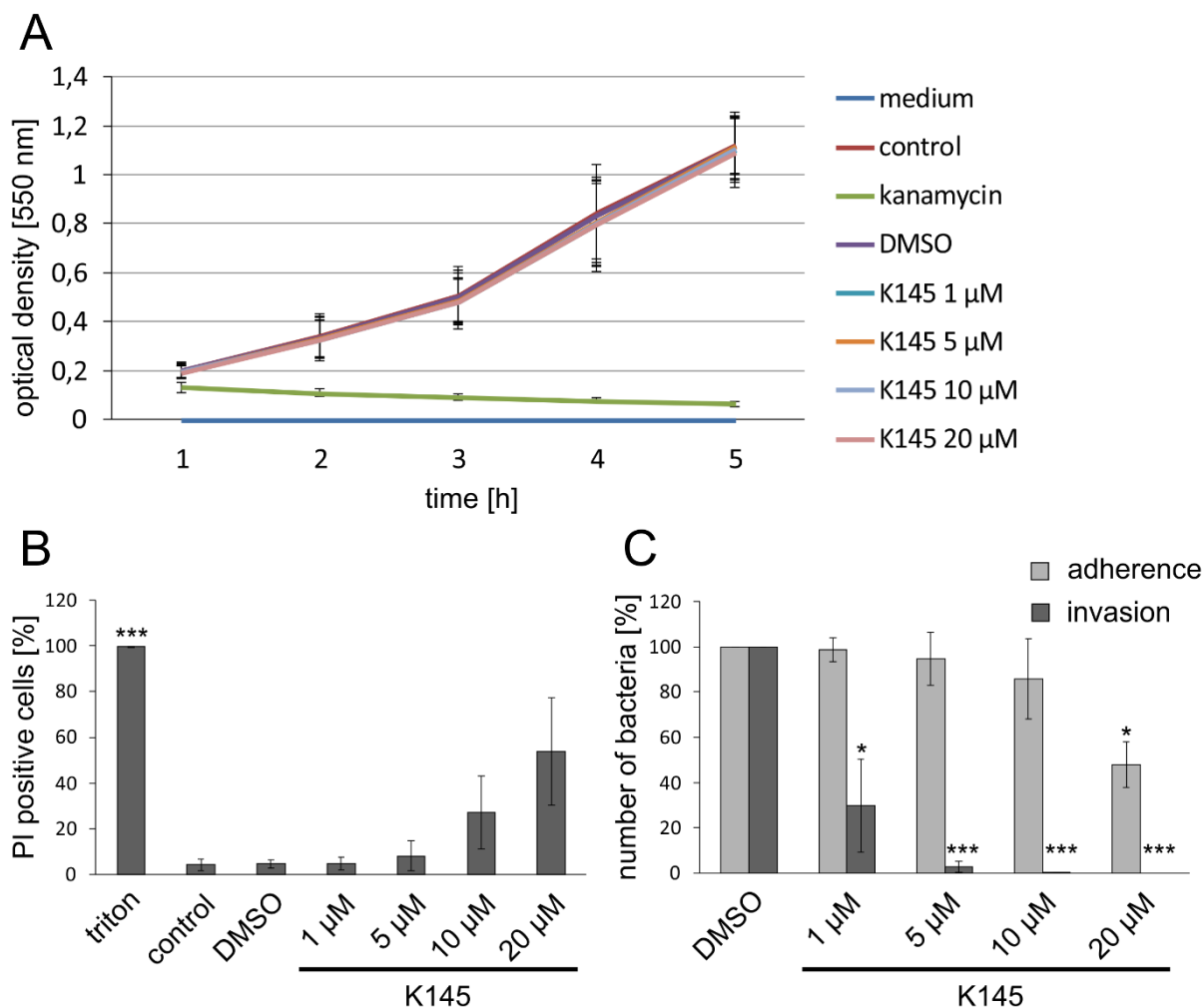
Figure 3.15: Sphingosine kinase 2 inhibition affects PorB$_{IA}$-dependent invasion. (A) Bacterial growth was measured using N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows mean values ± SD of three independent experiments. (B) Chang cells were used to conduct a cytotoxicity assay. The cells were treated with DMSO or the indicated concentrations of the SPHK2 specific inhibitor K145 for 2 h. 5 min before analysis Triton X-100 (0.1%) was added to one sample as a positive control. The cells were stained with PI and analysed using flow cytometry. The graph shows mean values ± SD of three independent experiments. $p < 0.001$ ***. (C) Chang cells were pretreated with the inhibitor K145 in the indicated concentrations or DMSO as a control for 2 h and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. $p < 0.05$ *, $p < 0.001$ ***.

3.2.3.2 Effect of SPHK2 inhibition on signalling and sphingolipid levels

Earlier studies performed in our group pointed out the significance of the PI3 kinase for phosphate-sensitive invasion of gonococci. The activation of the enzyme is required for the internalisation of the bacteria. In the signalling cascade leading to invasion, the PI3K acts

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downstream of NSM2 (Faulstich *et al.*, 2013, Faulstich *et al.*, 2015). The activated PI3K catalyses the conversion of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$], a plasma membrane lipid, to phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$]. The proteins AKT, a serine-threonine kinase, and PDK1, the phosphoinositide-dependent kinase 1, can bind to PI(3,4,5)P$_3$. Due to this binding, the two proteins come into proximity and PKD1 is able to phosphorylate AKT (Cantley, 2002). Therefore, the phosphorylation of AKT is used as a readout for the activation of PI3K. To detect the phosphorylated enzyme western blot analysis and an antibody directed against the phosphorylated form of AKT, pAKT, was used. I aimed to investigate the impact of the inhibition of SPHK2 not on the invasion itself, but on the signalling cascade leading to the invasion of the bacteria. The infected Chang cells treated with DMSO as control showed a significant increase of pAKT due to infection with N927 compared to the uninfected control (Figure 3.16). In contrast, the cells treated with the SPHK2-specific inhibitor K145 did not show an increase in pAKT levels due to infection. On the other hand, the blot revealed a higher basal level of pAKT in the uninfected control sample treated with K145 than the control sample treated with DMSO.
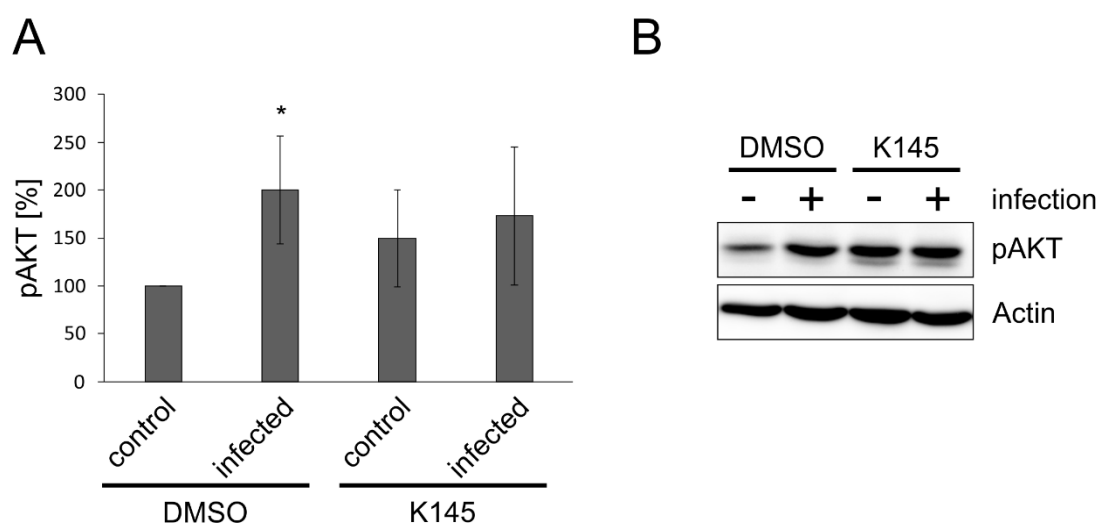
Figure 3.16: Sphingosine kinase 2 specific inhibitor K145 prevents infection-induced activation of PI3K. Chang cells were pretreated with DMSO as control or the inhibitor K145 (5 µM) 2 h before infection with N927 (MOI 50 for 1 h) under phosphate-free conditions. The cells were harvested and subjected to SDS-PAGE and western blot. (B) The activation of PI3K was analysed using an anti-pAKT antibody. (A) Quantification of the relative amount of pAKT based on experiment (B). The graph shows mean values ± SD of four independent experiments. The uninfected DMSO control samples was set as 100%. $p < 0.05$ *.

Furthermore, cells treated with the SPHK2 inhibitor K145 were sent for mass spectrometry to determine changes in sphingolipid levels due to the inhibitor treatment. The measurements revealed no changes in the levels of cellular sphingosine neither due to inhibitor treatment nor due to infection with gonococci (Figure 3.17 C). In contrast, K145 treatment resulted in a six-fold increase of S1P (Figure 3.17 D). Nevertheless, infection did not induce changes in S1P levels irrespective of the treatment with DMSO or K145. The ceramide levels of the cells treated with DMSO as a control show an increase when the cells were infected (Figure 3.17 B). Even though this increase is not statistically significant, it represents a trend, which was seen in the lipidome analysis performed earlier (Figure 3.11). In contrast, the ceramide levels of the cells treated with the inhibitor did not rise due to infection. In general, the relative quantities of ceramide are higher in the cells treated with K145 compared to the uninfected control cells. The effect is visible in every ceramide species as well as in the total ceramide levels. The same trend, but diminished, is present in the sphingomyelin levels (Figure 3.17 A). The cells treated with K145 show higher sphingomyelin levels compared to the cells treated with the DMSO control. While the cells treated with DMSO show an increase in the relative quantities due to infection, this effect cannot be observed in the cells treated with the SPHK2 inhibitor. There, the levels of sphingomyelin even seem to decrease due to infection.
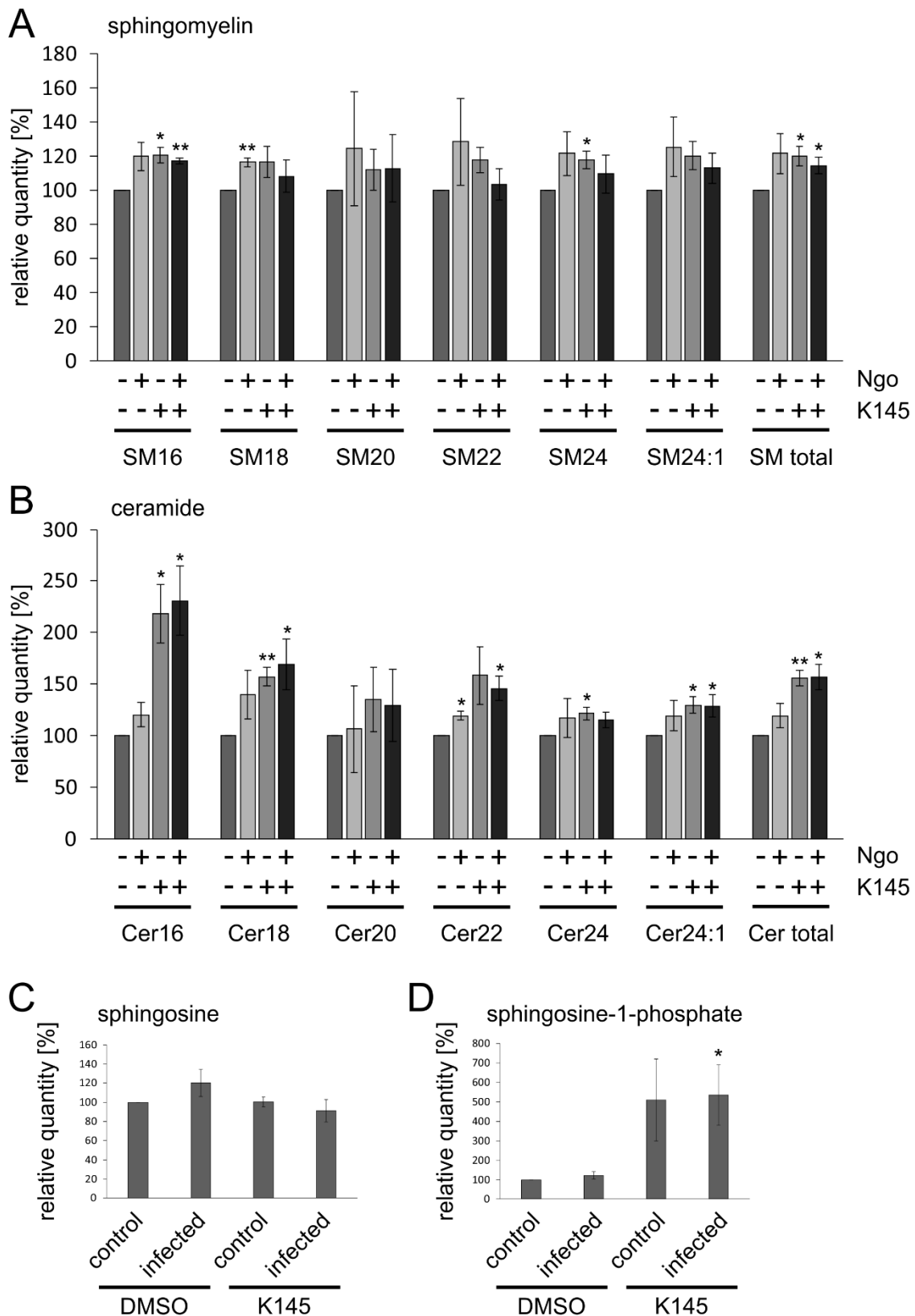
Figure 3.17: Changes in sphingolipid levels due to K145 SPHK2 inhibitor treatment. Chang cells were pretreated with DMSO as control or the inhibitor K145 (5 µM) 2 h before infection. Then, the cells were infected with N927 (MOI 50 for 2 h) under phosphate-free conditions. The cells were harvested in methanol and analysed via mass spectrometry (lipidome analysis). The graphs show mean values ± SD of three independent experiments. The uninfected control samples were set as 100%. p < 0.05 *,

p < 0.01 **. The graphs show the relative quantities of sphingomyelin (A), ceramide (B), sphingosine (C) and sphingosine-1-phosphate (D).

3.2.3.3 Effect of SPHK siRNA knockdown on infection and sphingolipid levels

The inhibitor studies were a first step to clarify the involvement of sphingosine kinases in PorB$_{IA}$-dependent invasion. As a second step, knockdown experiments were performed. The knockdown was introduced via siRNA pools directed against the RNA of SPHK1 (SKI) and SPHK2 (SKII). The infection experiments were conducted after an unusual long transfection time of 6 days. qRT-PCR revealed that after three days of transfection, the knockdown of one sphingosine kinase lead to a higher expression of the other sphingosine kinase. This effect could be avoided by a longer transfection time and the knockdown of both enzymes at the same time. The gentamicin assay showed that the knockdown of SPHK1 had no influence on invasion (Figure 3.18 A). The knockdown of SPHK2 showed a slight, but not significant, decrease of adherence and invasion. When SPHK1 and SPHK2 were down-regulated at the same time, there was a visible, but not significant decrease in adherence. The invasion of the bacteria was significantly decreased compared to samples transfected with a control siRNA pool. In contrast to the gentamicin assay, the differential immunofluorescence staining (infectivity assay) did not show any significant changes either in adherence nor in invasion of the bacteria (Figure 3.18 B). The knockdown was confirmed using qRT-PCR for quantification of the mRNA levels (Figure 3.18 C).
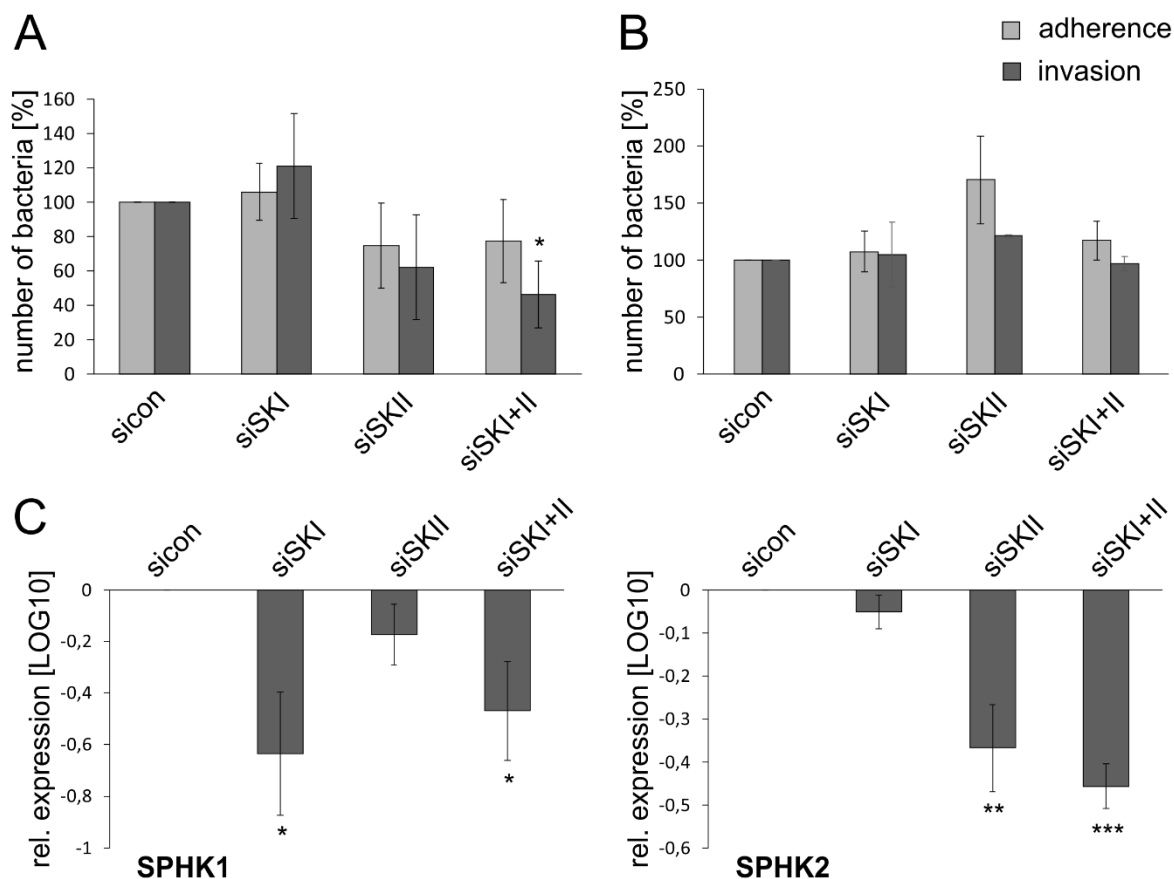
Figure 3.18: siRNA-mediated knockdown of sphingosine kinase 1 (SKI) and sphingosine kinase 2 (SKII) affects PorB$_{IA}$-dependent invasion. (A) Chang cells were transfected with siRNA against SKI, SKII or SKI+SKII two times on day 1 and day 3. 6 days post transfection 1, the cells were infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of four independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. (B) Chang cells were transfected with siRNA against SKI, SKII or SKI+SKII two times on day 1 and day 3. 6 days post transfection 1, the cells were infected with N927 (MOI 10 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using differential immunofluorescence staining. The graph shows mean values ± SD of two independent experiments. Adherence and invasion of the Chang control samples were set as 100%. (C) Chang cells were transfected with siRNA against SKI, SKII or SKI+SKII two times on day 1 and day 3. 6 days post transfection the cells were harvested, the mRNA was isolated and converted into cDNA. A qRT-PCR was performed to confirm the siRNA-mediated knockdown of SKI and SKII. The experiment was performed simultaneously to (A). The graph shows mean values ± SD of four independent experiments. The samples transfected with control siRNA (sicon) were set as basal level of expression. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

Lipidome analysis was used to observe the influence of the SPHK1 and SPHK2 knockdown on the sphingolipids. Due to the knockdown of the enzymes, the levels of S1P, the product of the two kinases, were statistically significantly decreased (Figure 3.19 D). At this point, the infection with gonococci did not affect the sphingosine levels. The relative quantities of sphingosine were not changed in a significant manner at all, independently of the siRNA used and the infection status (Figure 3.19 C). The knockdown of the sphingosine kinases showed a clear, partly statistically significant, effect on the sphingomyelin levels. The quantities of all sphingomyelin species were reduced (Figure 3.19 A). The measured ceramide levels showed the diminished effect observed in the sphingomyelin levels (Figure 3.19 B). The levels of ceramide were reduced in the cells transfected with SPHK1 and SPHK2 siRNA compared to the cells transfected with control siRNA. Furthermore, the infection with *N. gonorrhoeae* did not have any significant effect on the ceramide levels. In general, the original effect of increased ceramide, sphingosine and sphingosine-1-phosphate levels due to infection (Figure 3.11) was not existing in the cells treated with siRNA, neither in the cells treated with a control siRNA, nor in the cells transfected with siRNA directed against SPHK1 and SPHK2.
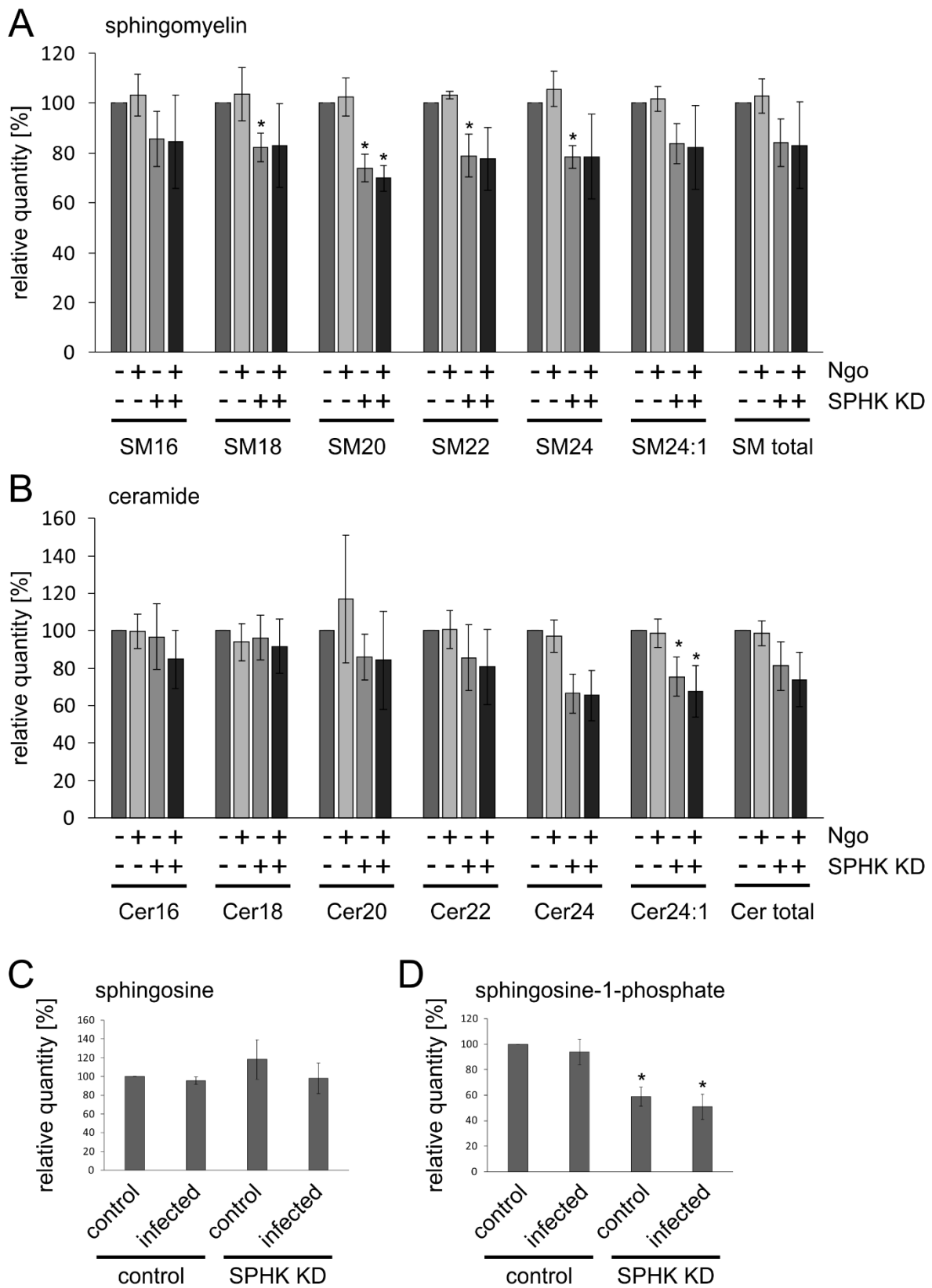
Figure 3.19: Changes in sphingolipid levels due to siRNA knockdown of SPHK1 and SPHK2. Chang cells were transfected with siRNA against SPHK1 and SPHK2 or a siRNA control two times on day 1 and day 3. 6 days post transfection 1, the cells were infected with N927 (MOI 50 for 2 h) under

phosphate-free conditions. The cells were harvested in methanol and analysed using mass spectrometry (lipidome analysis). The graphs show mean values ± SD of three independent experiments. The uninfected control samples were set as 100%. $p < 0.05$ *. The graphs show the relative quantities of sphingomyelin (A), ceramide (B), sphingosine (C) and sphingosine-1-phosphate (D).

3.2.3.4 Analysis of SPHK knockout MEFs

The findings gained with the knockdown experiments were to be confirmed by studies using knockout cell lines. Sphingosine kinase knockout mouse embryonic fibroblasts (MEFs) were kindly provided by Prof. Dr. Andrea Huwiler (Schwalm *et al.*, 2015). The infection was tested using gentamicin assay (Figure 3.20 A). The results revealed minor differences in adherence. The sphingosine kinase 1 knockout cells (SK1-/-) showed a small increase, while the sphingosine kinase 2 knockout cells (SK2-/-) showed a small decrease in adherence compared to the control (SK1+/+). In contrast, the invasion in the knockout cell lines is strongly increased compared to the control cell line. Western blotting and the following quantification of the blot indicated a rise of the pAKT levels in the infected compared to the uninfected cells in the SK1+/+ and in the SK1-/- cell line, whereas this rise did not occur in the SK2-/- MEFs. Of course, it must be taken into consideration, that the experiments were only conducted once (Figure 3.20 B, C).
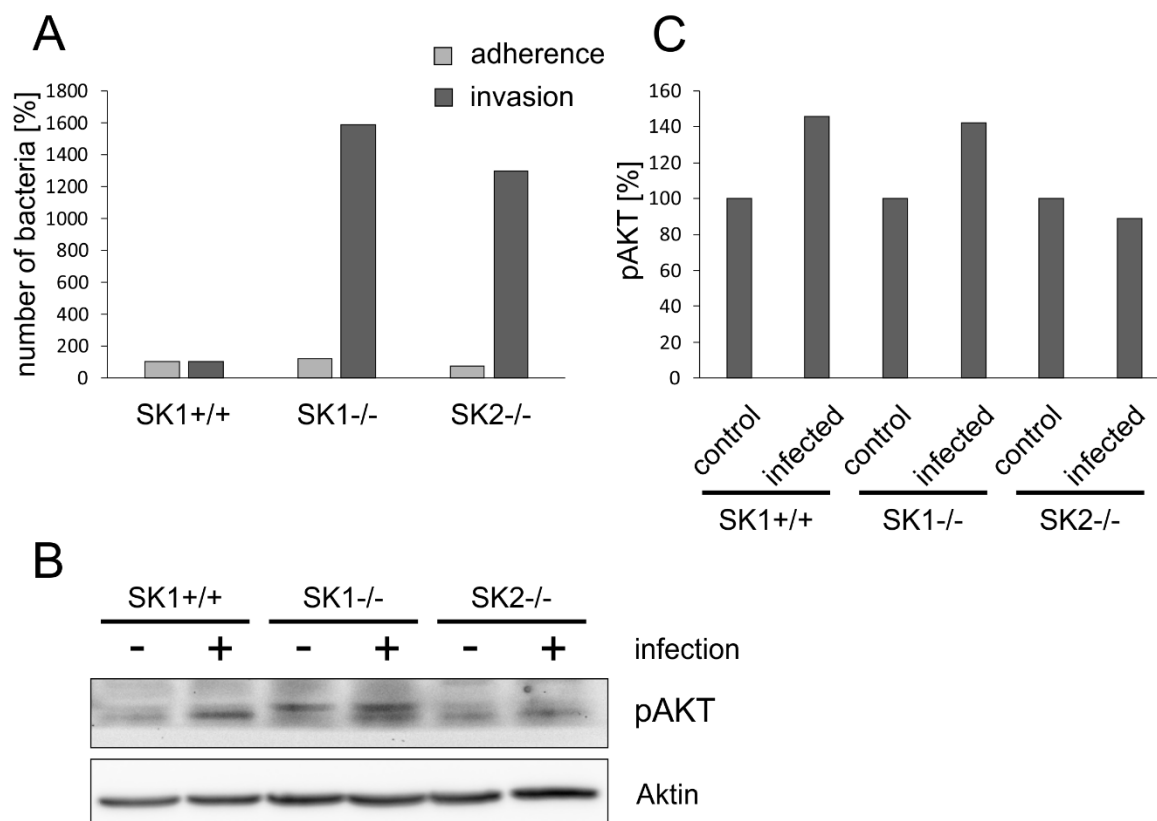
Figure 3.20: Testing of mouse embryonic fibroblasts (MEFs) compromising sphingosine kinase 1 (SK1-/-) and sphingosine kinase 2 (SK2-/-) knockouts. (A) MEFs were infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values of one experiment done in duplicate. Adherence and invasion of the control samples were set as 100%. (B) MEFs were infected with N927 (MOI 50 for 1 h). The cells were harvested and subjected to SDS-PAGE and western blot. The activation of PI3K was analysed using an anti-pAKT antibody. (C) Quantification of the relative amount of pAKT based on experiment (B). The uninfected control samples were set as 100%.

3.2.3.5 Testing of antibodies against SPHKs

To further investigate the role of sphingosine kinases, in particular SPHK1, antibodies from different companies were tested in western blotting (Figure 3.21). To this purpose, Chang cells were transfected with sphingosine kinase constructs, which were kindly provided by Prof. Dr. Dagmar Meyer zu Heringdorf (Pitson *et al.*, 2003, Alemany *et al.*, 2007). The expected size of the SPHK1-GFP protein was about 70 kDa, the one of the SPHK1-YFP protein was about 110 kDa. Endogenous SPHK1 should be 40 kDa, SPHK2 about 80 kDa big. The antibodies directed against SPHK1 were derived from the companies Abnova and Abcam. The antibodies against SPHK2 were derived from Abcam and Santa Cruz. Additionally, an anti-GFP antibody was used as a control. Both anti-SPHK1 antibodies recognized the

transfected SPHK1-GFP construct. The one from Abcam showed more background than the antibody from Abnova. Hence, the antibody from Abcam was not suitable for the detection of endogenous SPHK1. At the same time, the blot incubated with the Abnova antibody showed a protein band at 40 kDa in the Chang whole cell lysate, which was possibly native SPHK1. The antibodies directed against SPHK2 both showed much background, which made it impossible to detect endogenous SPHK2. While the antibody from Abcam did not even recognize the overexpressed SPHK2-YFP protein, the antibody from Santa Cruz could recognize the construct.
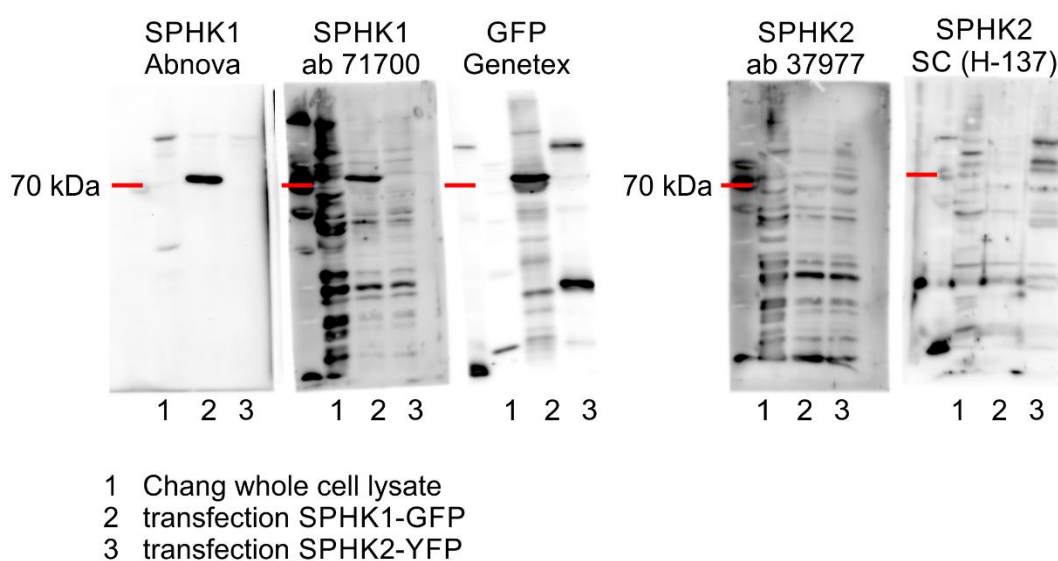
Figure 3.21: Testing of different antibodies directed against sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2). Chang cells were transfected with different constructs containing SPHK1 coupled to GFP or SPHK2 coupled to YFP. 36 h post transfection, the cells were harvested and subjected to SDS-PAGE and western blot. Antibodies from different companies directed against SPHK1 or SPHK2 as well as an antibody directed against GFP as control were used for analysis.

3.2.3.6 Establishment of a sphingosine kinase activity assay

At least one of the sphingosine kinases seems to play a role in PorB$_{IA}$-mediated invasion of gonococci. Given that the level of sphingosine-1-phosphate is increased due to infection (Figure 3.11), the activation of the SPHK is presumable. Here, I aimed to address the question if sphingosine kinases are activated by the phosphate sensitive invasion of gonococci. Therefore, I performed a SPHK activity assay. This very sensitive and accurate assay is based on the enzymatic activity of SPHK. Radioactive [γ-^{32}P]ATP is used for the

phosphorylation of sphingosine, which results in radioactively labelled sphingosine-1-phosphate (Kharel *et al.*, 2011). The molecule is reisolated by organic extraction and subjected to thin-layer chromatography. First, the assay was performed using a recombinant mouse sphingosine kinase 1 (Figure 3.22 A). The result revealed that the method was generally functioning. Then, Chang cells seeded in different wells were used for the assay (Figure 3.22 B). The control samples with the recombinant mouse SPHK again showed a signal at the appropriate height, in which sonification of the enzyme prior to the enzymatic reaction boosted the signal. In contrast, a clear signal was not visible either in the uninfected or in the infected cells. To increase the amount of the enzyme, the membranes and mitochondria from cells seeded in 15 cm dishes were isolated and subjected to the activity assay (Figure 3.22 C). Like in the approach tested before (Figure 3.22 B), no signals could be detected.
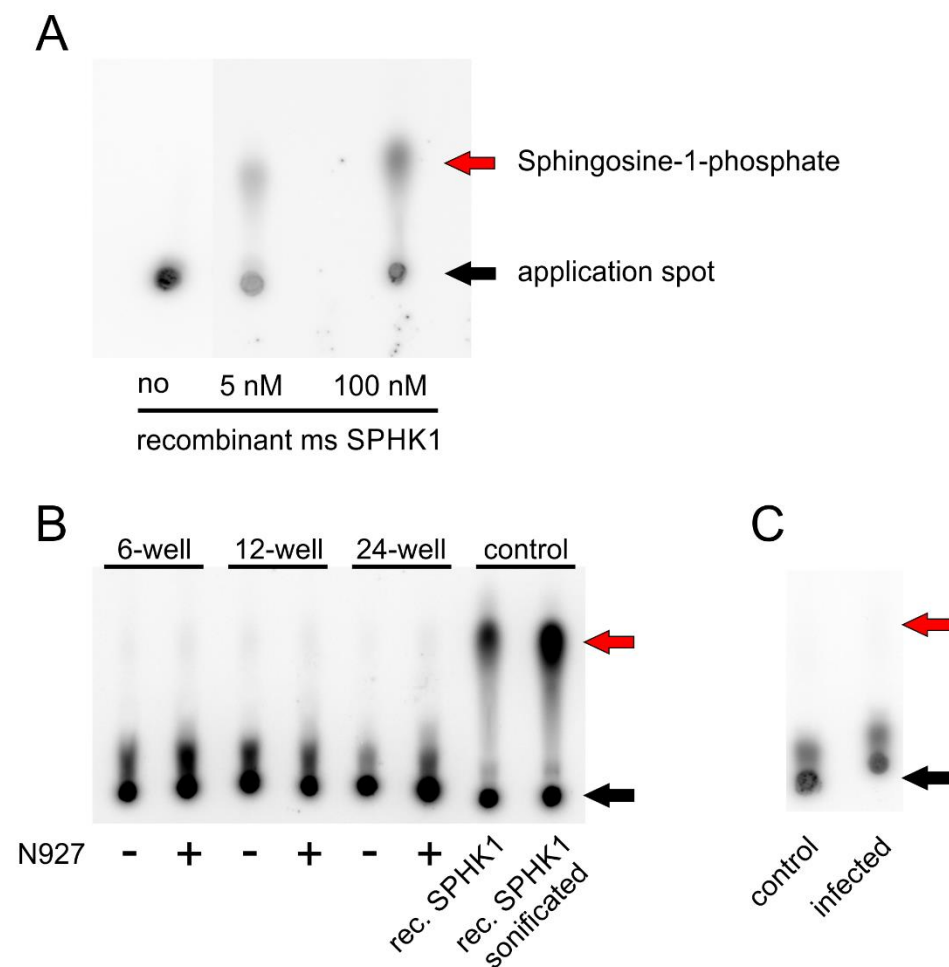
Figure 3.22: Sphingosine kinase (SPHK) activity assay. (A) Assay was performed using different concentrations of the recombinant mouse sphingosine kinase 1 enzym to establish a positive control.

(B) Chang cells were seeded in different wells and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. The cells were harvested and the SPHK activity assay was performed. The recombinant mouse SPHK1 (ms SPHK1) was used as a positive control with and without sonication. (C) Chang cells were seeded in 15 cm dishes. The cells were infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Mitochondria and membranes were isolated and used to perform the SPHK activity assay. Red arrows indicate the position of sphingosine-1-phosphate, black arrows indicate the position of the spot of application.

3.2.4 Role of Sphingosine-1-phosphate lyase in PorB$_{IA}$-mediated infection

The results shown previously indicated the involvement not only of the NSM2, but of the whole sphingolipid pathway in PorB$_{IA}$-mediated invasion. Therefore, I aimed to investigate a potential role of the sphingosine-1-phosphate lyase (S1P lyase, S1PL) in the signalling cascade leading to the uptake of gonococci. I started with inhibitor studies using an S1P lyase inhibitor named Compound A which was kindly provided by Prof. Dr. Burkhard Kleuser. Measurement of bacterial growth revealed an effect of Compound A on gonococci (Figure 3.23 A). At a 1 µM concentration of Compound A, the bacteria grew a little bit faster after 5 h than the untreated bacteria. In contrast, at a concentration of 5 µM and higher, the *Neisseria* grew slower. The compound was not cytotoxic to Chang cells (Figure 3.23 B). Next, the S1P lyase inhibitor was tested in infection experiments. While the adherence did not change, the phosphate sensitive invasion of N927 was increased due to inhibitor treatment (Figure 3.23 C). This effect started at the lowest concentration used (1 µM), and was significant at a concentration of 5 µM. The increase was still significant in higher concentrations, but it dropped compared to the value at a 5 µM concentration. In contrast, Compound A showed a different effect on invasion with N931 (PorB$_{IB}$, Pili⁻, Opa$_{50}$) (Figure 3.23 D). N931 uses an invasion pathway independent of PorB$_{IA}$ and phosphate. While the adherence was not affected, the invasion decreased at a 5 µM concentration of Compound A (p < 0,052).
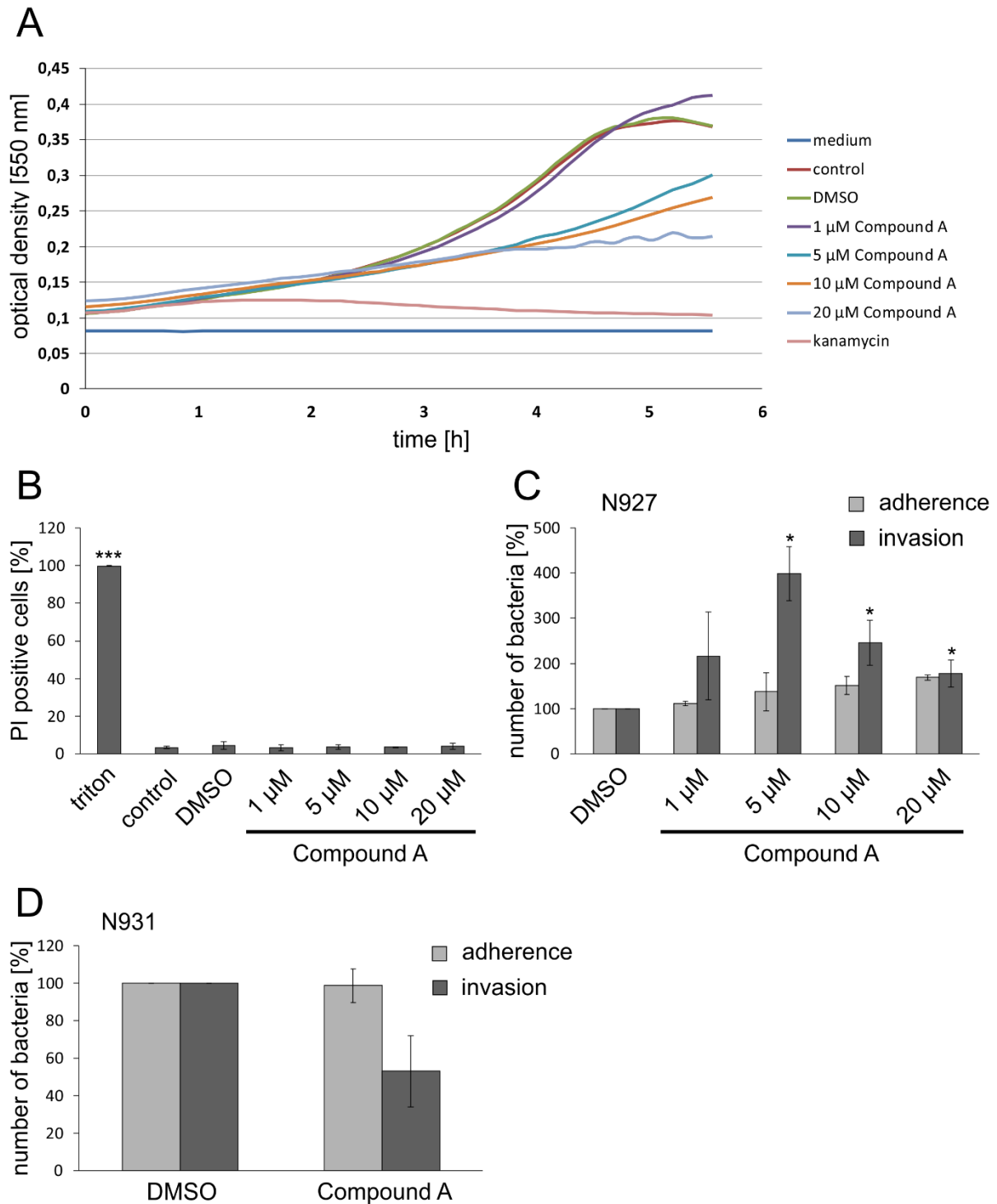
Figure 3.23: Effect of sphingosine-1-phosphate lyase inhibitor Compound A on neisserial infection.
(A) Bacterial growth was measured using N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows mean values ± SD of three independent experiments. (B) Chang cells were used to conduct a cytotoxicity assay. The cells were treated with DMSO or the indicated concentrations of the sphingosine-1-phosphate lyase specific inhibitor compound A for 2 h. 5 min before analysis Triton X-100 (0.1%) was added to one sample as positive control. The cells were stained with PI and analysed using flow cytometry. The graph shows mean values ± SD of three independent experiments. p < 0.001 ***. (C) Chang cells were pretreated with the inhibitor Compound A in the indicated concentrations or DMSO as control for 2 h and infected

with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments done in duplicate. Adherence and invasion of the control samples were set as 100%. $p < 0.05$ *. (D) Chang cells were pretreated with either DMSO or the inhibitor compound A (5 µM) for 2 h. The cells were infected with N931 (MOI 50 for 2 h). Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%.

The inhibitor studies revealed a possible role for the S1P lyase in gonococcal invasion. To investigate this further, infection experiments were performed using a S1P lyase HeLa knockout cell line. The knockout cell line ΔS1PL as well as the corresponding wildtype control cell line WT were constructed using the CRISPR/Cas9 system and kindly provided by Prof. Dr. Britta Brügger (Gerl *et al.*, 2016). The infection experiments were performed using the strains N927 (PorB$_{IA}$) and N931 (PorB$_{IB}$) to address the question if the S1P lyase is involved only in the PorB$_{IA}$-mediated, phosphate-sensitive invasion, or if the enzyme plays a role in other invasion pathways, too. A gentamicin assay performed with N927 revealed that the knockout of S1P lyase did not influence the adherence of the gonococci to the cells, whereas there was a significant decrease in invasion compared to the wildtype cells (Figure 3.24 A). This result could not be confirmed by differential immunostaining, because the experiment did not show any significant differences between the wildtype and knockout cells (Figure 3.24 B). The decrease in invasion observed in the gentamicin assay with N927 was also seen when the cells were infected with N931 (Figure 3.24 C, D). The results gained here for N927 were in conflict to those gained with the S1P lyase inhibitor Compound A (Figure 3.23). For this reason, the inhibitor was tested together with the wildtype and knockout cell lines (Figure 3.24 E, F). The treatment with the inhibitor did not affect the adherence neither to the wildtype nor to the knockout cells in a significant manner. While the invasion in the wildtype cell line was not altered due to inhibitor treatment, there was an effect on invasion in the S1P lyase knockout cell line. The decrease in invasion in the knockout cells compared to the wildtype cells was not significant anymore, when the cells were treated with Compound A (Figure 3.24 E). Compound A seemed to abrogate the effect caused by the absence of S1P lyase. Although the results concerning the role of the sphingosine-1-phosphate lyase in invasion of gonococci are conflictive when the inhibitor studies are

compared to the knockout studies, there is clear evidence that the enzyme does play a role in the uptake of the bacteria into cells.
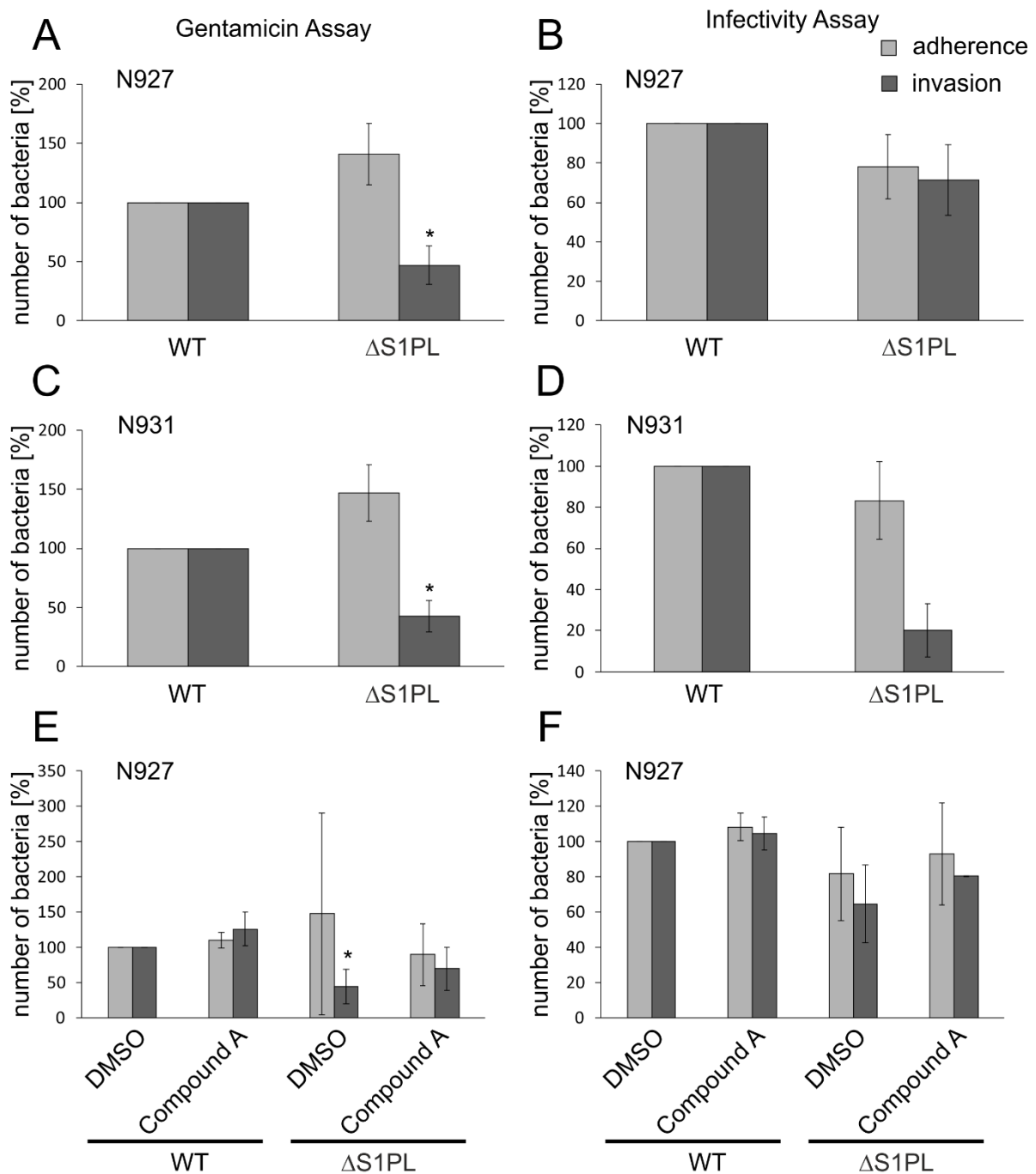
Figure 3.24: Sphingosine-1-phosphate lyase (S1PL) plays a role in neisserial infection. (A) Wildtype and ΔS1PL HeLa cells were infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in triplicate. Adherence and invasion of the control samples were set as 100%. (B) Wildtype and ΔS1PL HeLa cells were infected with N927 (MOI 10 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using differential immunofluorescence staining. The graph shows mean values ± SD of three independent

experiments. Adherence and invasion of the Chang control samples were set as 100%. (C) Wildtype and ΔS1PL HeLas were infected with N931 (MOI 50 for 3 h) under phosphate free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments done in duplicate. Adherence and invasion of the control samples were set as 100%. (D) Wildtype and ΔS1PL HeLa cells were infected with N931 (MOI 10 for 3 h) under phosphate-free conditions. Adherence and invasion were quantified using differential immunofluorescence staining. The graph shows mean values ± SD of two independent experiments. Adherence and invasion of the Chang control samples were set as 100%. (E) Wildtype and ΔS1PL HeLa cells were pretreated with DMSO or the S1PL specific inhibitor Compound A (5 µM) and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values ± SD of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. (F) Wildtype and ΔS1PL HeLa cells were pretreated with DMSO or the S1PL specific inhibitor Compound A (5 µM) and infected with N927 (MOI 10 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using differential immunofluorescence staining. The graph shows mean values ± SD of two independent experiments. Adherence and invasion of the Chang control samples were set as 100%. $p < 0.05$ *.

pAKT is used as readout for the activation of the PI3K which, is an important part of the signalling cascade leading to the uptake of the bacteria in the PorB$_{IA}$-mediated invasion. I aimed to investigate the influence of the S1P lyase knockout on this signalling cascade. Therefore, I infected wildtype and S1P lyase knockout cells with PorB$_{IA}$-expressing gonococci, harvested the cells, subjected them to western blotting and finally quantified the western blots (Figure 3.25 A, B). The results revealed a clear increase of the pAKT level in the infected wildtype cells compared to the uninfected control. The increased phosphorylation of AKT due to the activation of the PI3K in infected cells compared to uninfected cells is also visible in the S1PL knockout cell line. Although the difference is not significant because of the high standard deviation, the pAKT level in the infected wildtype cells is clearly higher than in the infected knockout cells. Lipidome analysis of the two cell lines was performed to confirm the knockdown and to investigate the consequences of the knockout on the sphingolipid balance. The data demonstrated a very strong and statistically significant increase of the sphingosine-1-phosphate (S1P) level in the S1PL knockout cell line compared to the wildtype cell line (Figure 3.25 C). Interestingly, the amount of S1P was not increased due to infection. The levels of sphingosine were similar in the wildtype and the knockout cells (data not shown). The knockout of the S1P lyase in HeLa cells leads to an accumulation of S1P and seems to influence the signalling cascade of the PorB$_{IA}$-mediated invasion.

A

B

C
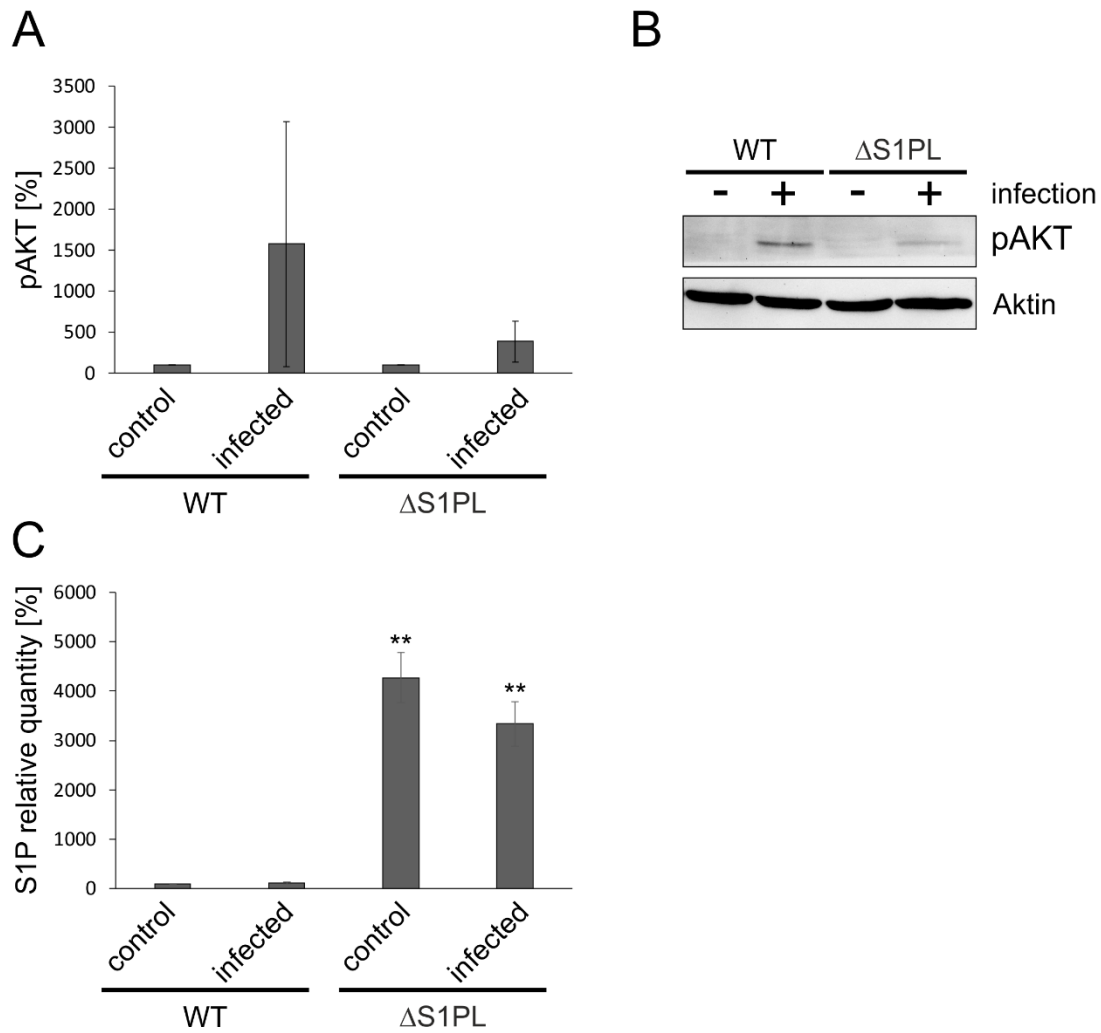
Figure 3.25: Activation of PI3K due to infection in sphingosine-1-phosphate lyase knockouts. Cells were infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. The cells were harvested and subjected to SDS-PAGE and western blot. (B) The activation of PI3K was analysed using an anti-pAKT antibody. (A) Quantification of the relative amount of pAKT based on experiment (B). The graph shows mean values ± SD of five independent experiments. The uninfected control samples were set as 100%. (C) Relative quantity of sphingosine-1-phosphate. Cells were infected with N927 (MOI 50 for 2 h) under phosphate-free conditions. The cells were harvested in methanol and analysed using mass spectrometry (lipidome analysis). The graphs show mean values ± SD of three independent experiments. The uninfected wild type control samples were set as 100%. $p < 0.01$ **.

3.2.5 Effect of external sphingosine-1-phosphate addition on gonococcal infection

Experiments performed earlier in this study revealed an influence of different enzymes of the sphingolipid pathway on gonococcal invasion. Two of those enzymes either produce sphingosine-1-phosphate (SPHK2) or degrade it (S1PL). S1P is one of the key sphingolipids in the "sphingolipid rheostat". The bioactive molecule can act as an intracellular messenger, but also as an autocrine and paracrine signalling molecule. It binds to G-protein-coupled receptors on the cell surface and can activate several downstream molecules. Therefore, I wanted to investigate the influence of extracellular S1P addition on invasion. The solubilized S1P was kindly provided by PD Dr. Andreas Weigert. First, the influence of S1P on bacterial growth was determined. The sphingolipid did not affect bacterial growth (Figure 3.26 A). The molecule showed no cytotoxic effect on Chang cells, either (Figure 3.26 B). Next, infection experiments were performed to investigate the impact of external S1P addition on the SREC-I/PorB$_{IA}$-mediated invasion under phosphate-free conditions. Gentamicin assays (Figure 3.26 C) revealed an increase in adherence as well as in invasion due to S1P addition compared to the control (1% BSA). The increase of the CFU in adherence and invasion is not statistically significant because of the high standard deviation (not shown), but the results showed the same trend in the three independent biological replicates. The inhibitor K145 inhibits the SPHK2 and has a strong effect on invasion. The treatment of cells with this substance should prevent the generation of S1P through SPHK2. I aimed to investigate if the external addition of S1P could rescue the effect of K145 on gonococcal invasion. The experiment revealed that the decrease of invasion due to K145 inhibitor treatment still occurs despite S1P addition (Figure 3.26 C). The performed infectivity assay (differential immunofluorescence staining), which shows the status quo after 1 h of infection, confirmed the data obtained by the gentamicin assay concerning the decreased invasion due to K145. The effect on invasion cannot be rescued by external S1P (Figure 3.26 D). The adherence to and invasion into the cells not treated with K145 show only a slight increase due to S1P addition compared to the control cells.
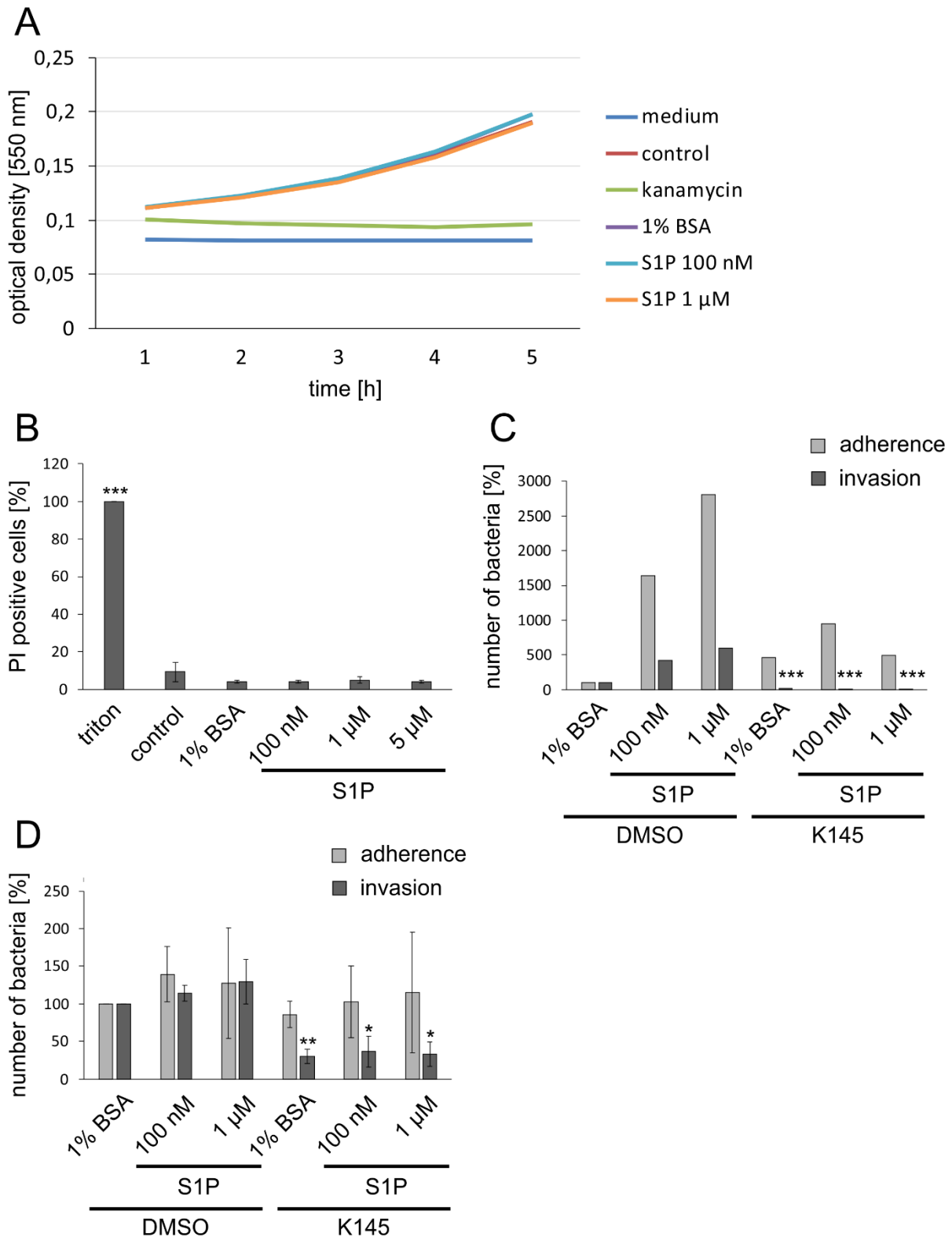
Figure 3.26: Effect of external sphingosine-1-phosphate (S1P) addition on neisserial infection.
(A) Bacterial growth was measured using N927. The bacteria were treated with different substances in the indicated concentrations. The graph shows one experiment. (B) Chang cells were used to conduct a cytotoxicity assay. The cells were treated with 1% BSA or the indicated concentrations of

S1P for 2 h. 5 min before analysis Triton A-100 (0.1%) was added to one sample as a positive control. The cells were stained with PI and analysed using flow cytometry. The graph shows mean values ± SD of three independent experiments. p < 0.001 ***. (C) Chang cells were pretreated with the inhibitor K145 (5 μM) or DMSO as control for 2 h, pretreated with S1P in the indicated concentrations for 30 min and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using gentamicin protection assay. The graph shows mean values of three independent experiments performed in duplicate. Adherence and invasion of the control samples were set as 100%. p < 0.001 ***. (D) Chang cells were pretreated with either DMSO or the inhibitor K145 (5 μM) for 2 h, pretreated with S1P in the indicated concentrations for 30 min and infected with N927 (MOI 50 for 1 h) under phosphate-free conditions. Adherence and invasion were quantified using differential immunofluorescence staining. The graph shows mean values of three independent experiments. Adherence and invasion of the control samples were set as 100%. p < 0.05 *, p < 0.01 **.

3.3 *N. gonorrhoeae* inhibits apoptosis in neutrophils

One characteristic of neutrophils is their short lifespan. After less than 24 hours in the bloodstream, they will go into apoptosis (McCracken & Allen, 2014). In the early stages of apoptosis, membrane changes occur, which include the exposure of phosphatidylserine (PS) to the cell surface. PS is bound by Annexin V, a phospholipid binding protein, with high affinity. Therefore, the Ca^{2+} dependent protein is used as marker for the detection of apoptotic neutrophils in this study (Vermes *et al.*, 1995). Previous studies already that *N. gonorrhoeae* are able to delay the onset of apoptosis (Chen & Seifert, 2011, Simons *et al.*, 2006). These results could be confirmed in this study (Figure 3.27 A). Different neisserial strains expressing either PorB$_{IA}$ (N927) or PorB$_{IB}$ (N138) can inhibit the apoptosis of neutrophils after 24 h in a statistically significant manner.

The specific neisserial factors responsible for this effect are unknown until now. Preliminary data gained in our lab suggested the involvement of secreted factors, because the supernatants of gonococci are sufficient to delay apoptosis (data not shown). Peptidoglycan, which is released in fragments by the bacteria during growth, was one possible candidate for the factor responsible. Therefore, I tested different peptidoglycan mutants which were kindly provided by Prof. Dr. Joseph Dillard. The mutants were all produced in a MS11 background. In the first mutant tested *amiC*, a peptidoglycan-degrading N-acetylmuramyl-L-alanine amidase was deleted. As the enzyme is involved in cell separation, the deletion mutant showed a derogated cell separation as well as impaired peptidoglycan fragment release. The mutant was not able to produce free disaccharides. Besides, it showed different

growth behaviour compared to the wildtype. The complementation of the mutant with wildtype *amiC* could rebuild the wildtype growth behaviour (Garcia & Dillard, 2006). It has been shown that Ngo can recycle peptidoglycan and additionally are able to regulate the peptidoglycan fragment metabolism. An important protein involved in this process is the AmpG permease. The enzyme is responsible for the transport of the fragments from the periplasm into the cytoplasm. The deletion of *ampG* resulted in an accumulation of peptidoglycan monomers in the supernatant (Garcia & Dillard, 2008). The *ampG* deletion mutant as well as the complemented mutant were tested in infection. A *ltgA ltgD* double mutant and the corresponding complemented mutant were also tested. The lytic transglycosylase A (LtgA) is responsible for the production and the lytic transglycosylase D (LtgD) for the release of peptidoglycan monomers in *N. gonorrhoeae*. As a result, the double mutant is not able to release peptidoglycan monomers. Instead, large soluble peptidoglycan fragments were found in the supernatant of the bacteria. In contrast to the *amiC* deletion mutant, the *ltgA ltgD* double mutant grows normally and the cell separation is not affected either (Cloud-Hansen *et al.*, 2008). Here, freshly isolated neutrophils were infected with the mutants discussed above (Figure 3.27 B). The wildtype strain MS11 shows a significant delay in the onset of apoptosis. The cells infected with the mutants showed less apoptosis than the uninfected cells, but because of the high standard deviation, a conclusion is not possible.

Numerous publications outline the role of NSM2 for neutrophils. Neutrophils, like all eukaryotic cells, possess sphingolipids as well as the associated enzymes. The neutral sphingomyelinase (NSM2) converts sphingomyelin to ceramide and is a crucial enzyme in the sphingolipid balance. It has also been shown that the inhibition of NSM2 in neutrophils via the sphingolactone-24 results in the neutralisation of the inhibitory effect of LPS on apoptosis. That means, the onset of the apoptosis is no longer delayed due to LPS treatment, when the NSM2 is inhibited in neutrophils (Lin *et al.*, 2011). Here, I aimed to investigate the effect of two different NSM2 inhibitors GW4869 and Eso48 on the inhibition of apoptosis in neutrophils due to infection with Ngo (Figure 3.27 C). The inhibition of the apoptosis is still visible in the PMNs treated with different concentrations of GW4869. In contrast, the effect vanished when the cells were treated with Eso48. Both inhibitors seem to influence the viability of the neutrophils, but the cytotoxic effect of Eso48 is stronger than the effect of GW4869.

In summary, the inhibitory effect of *N. gonorrhoeae* on apoptosis in neutrophils was confirmed in this study. It was not possible to clarify the factors leading to this effect on bacterial side, nor the role of NSM2 in this context.
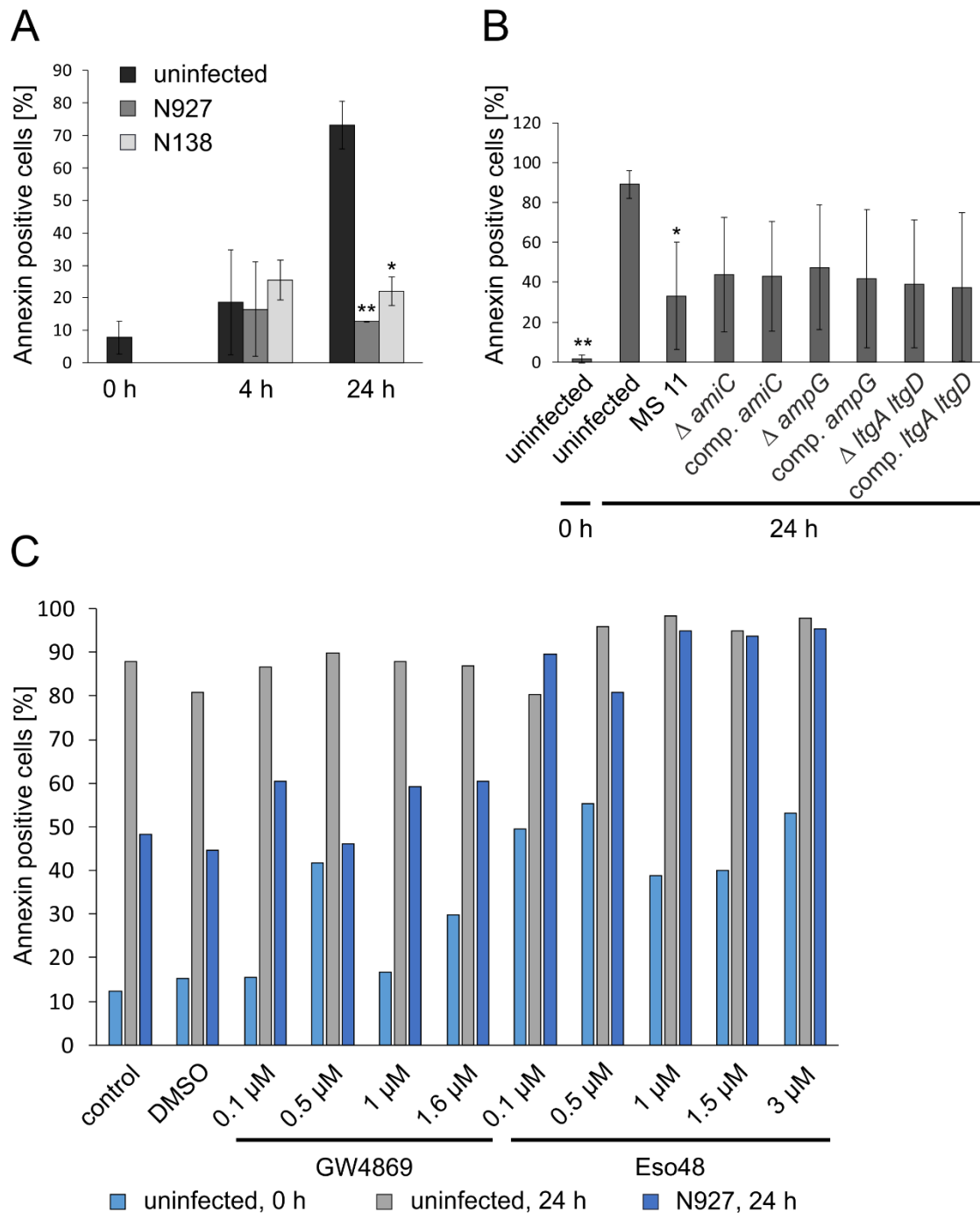
Figure 3.27: *N. gonorrhoeae* inhibits apoptosis in neutrophils. Neutrophils were isolated from fresh human blood. (A) The cells were infected with N927 or N138 (MOI 100) and either directly analysed or incubated for 4 h or 24 h. The neutrophils were stained and subjected to FACS analysis. The graph shows mean values ± SD of three independent experiments performed in duplicates. (B) The cells were infected with the wildtype strain MS11 or different mutant strains at MOI 100 and either directly analysed or incubated for 24 h. The neutrophils were stained and subjected to FACS analysis.

The graph shows mean values ± SD of three independent experiments. (C) The cells were pretreated with DMSO as control or the NSM2 specific inhibitors GW4869 or Eso48 in the indicated concentrations. The infection was performed with the Ngo strain N927 at MOI 50. The neutrophils were either analysed directly after the inhibitor treatment or incubated for 24 h. The cells were stained and subjected to FACS analysis. The graph shows one experiment. $p < 0.05$ *, $p < 0.01$ **.

4 DISCUSSION

During the last years, sphingolipids gained more and more attention as bioactive molecules, which regulate different cellular processes such as proliferation, apoptosis and intracellular trafficking. The balance of the sphingolipids ceramide, sphingosine and sphingosine-1-phosphate can determine cell fate. Hence, changes in the sphingolipid profile can lead to the development of severe pathologies. For this reason, the key players in the sphingolipid pathway may serve as potential drug targets (Hannun & Obeid, 2008, Neubauer & Pitson, 2013). Furthermore, various pathogens use the sphingolipid pathway and the involved enzymes for their own purposes. Causative agents of viral, bacterial, protozoan and fungal infections are able to manipulate the cellular sphingolipid metabolism to guarantee their survival and virulence (Arish *et al.*, 2015). Recent studies in our lab revealed the importance of the neutral sphingomyelinase 2 for SREC-I/PorB$_{IA}$-mediated gonococcal invasion (Faulstich *et al.*, 2015). NSM2 represents a part of the sphingolipid pathway and its role in infection suggests the possibility of *N. gonorrhoeae* needing or using additional members of the sphingolipid metabolism for the successful establishment of an infection.

4.1 The role of NSM2 and its product ceramide in infection

The NSM2 has been determined as the key factor for PorB-dependent invasion. The enzyme is required for the recruitment of the PI3K to caveolin, which leads to the activation of the PI3K-dependent downstream signalling and finally to the engulfment of the bacteria. The activation of the downstream signalling triggered by the binding of PorB$_{IA}$ to SREC-I requires the translocation of the cellular receptor in membrane rafts and the establishment of larger signalling platforms. This process is supported by the activity of NSM2 and the generation of ceramide (Faulstich *et al.*, 2015). This study aimed to investigate the role of NSM2 and its product ceramide in gonococcal infection in more detail. To this purpose, the involvement of the NSM2 in phosphate sensitive gonococcal invasion was confirmed by inhibitor studies in HUVECs. The invasion in HUVECs was clearly dependent on a functional NSM2, because the inhibition of the enzyme resulted in a strong decrease in invasion (Figure 3.1). The cell line can be used, since it expresses the required receptor SREC-I. Additionally this cell line represents a widely-used model system for endothelial cell function (Park *et al.*, 2006). Gonococci expressing PorB$_{IA}$ can enter the bloodstream and are associated with DGI (Cannon

et al., 1983). Hence, those bacteria come in close contact with the blood vessels-covering endothelial cells, they may attach and probably invade them. Therefore, HUVECs represent a suitable model for the simulation of a natural environment during DGI.

The importance of NSM2 for PorB$_{IA}$-mediated invasion was detected using the NSM2 inhibitor GW4869. This inhibitor was compared with two new NSM2-specific inhibitors Eso48 and c11AG (Figure 3.2). However, the biggest effect on gonococcal invasion was generated by the well-established GW4869, which is a potent, specific and non-competitive inhibitor of NSM2. The substance was and is widely used and acknowledged in different fields of research for the inhibition of NSM2 (Camare *et al.*, 2016, Gassert *et al.*, 2009, Chipuk *et al.*, 2012).

For further investigation of the role of NSM2 in invasion, antibodies directed against the enzyme were produced in this study and tested in western blot (Figure 3.3). The decision to generate the antibodies was made because the tested commercially available antibodies did not recognize endogenous NSM2 in Chang cells (data not shown). Unfortunately, the produced antibodies also were not able to detect the endogenous protein and showed a high background despite of purification. The NSM2-1 antibody was only able to detect its antigen when NSM2 was overexpressed.

Both antibodies, but especially, the peptide NSM2 antibody, show numerous bands instead of one defined band. The reasons for this could include posttranslational modifications of the protein. The NSM2 has several modified forms due to phosphorylation and palmitoylation, which could change the molecular weight and result in multiple bands on western blots (Filosto *et al.*, 2010, Tani & Hannun, 2007b). Additionally, alternative splicing could also lead to the indicated effect. According to the NCBI database, there are five different mRNA transcript variants existing. Another possibility includes the fragmentation of the target protein due to natural processes or the experimental procedure (Rauch, 2005). The occurrence of multiple bands in the western blot could also be a result of more typical problems with antibodies concerning specificity. Those problems include cross-reactivity and non-specific reactivity (Rauch, 2005). The produced antibodies are directed against human NSM2 and were generated using rabbits, that also express NSM2. The cDNA of the rabbit NSM2 shares 89% identity with the human NSM2. The protein shows even 90% similarity with the human enzyme according to the NCBI database. The high similarity between the

proteins could reduce the antigenic potential of the peptide and the first part of the human NSM2 used for the immunisation of the rabbits resulting in antibodies with poor specificity.

Many researchers offer hands-on advice in different forums like research gate to enhance the quality of western blots. For example, the blocking solution could be changed in order to lower the background, the first as well as the secondary antibody concentration could be titrated to achieve the best possible result and the membrane could be switched from PVDF to nitrocellulose, which gives less background. All experimental adjustments should be tested in the future, because a functional antibody would be a powerful tool to clarify the role of the NSM2 in gonococcal invasion.

Besides, the reason for the antibodies inability to recognize endogenous NSM2 could be another one: the amount of endogenous NSM2 in the used cells could be too low. The testing of several cell lines concerning their NSM RNA levels revealed differences in the NSM2 RNA levels (Figure 3.4). While Chang and HUVEC cells show a similar amount of RNA transcripts, the expression of NSM2 in HEK 293T is highly increased and in HeLa2000 highly decreased. Therefore, HEK 293T, but not the HeLa cell line, could be suitable for experiments that tend to investigate the NSM2. HEK 293T cells are derived from human kidney cells. In kidney cells, the expression of NSM2 is comparatively high, but the estimated protein expression is very low according to GeneCards, a human gene database. This finding strengthens the argument that it is not possible to correlate the mRNA level to the level of protein. A high transcription rate does not directly indicate a high amount of protein, because of several regulatory processes (de Sousa Abreu *et al.*, 2009). Hence, it did not make sense to perform the infection experiments in HEK 293T cells, although the NSM2 mRNA levels were highest in this cell line, since the protein expression in the tried and tested Chang and HUVEC cells could still be higher.

Chang cells were also used to generate a NSM2 knockout cell line using the CRISPR/Cas9 system. The knockout cell line represents a powerful tool to study the impact of NSM2 on $PorB_{IA}$-mediated invasion. Three promising knockout clones were tested in infection. The gentamicin assay revealed no influence of the lacking NSM2 on bacterial adherence (Figure 3.6). Only clone 7 treated with GW4869 differs from this observation, but the result could not be confirmed by the infectivity assay, which shows no effect on adherence either (Figure 3.7). Hence, the NSM2 knockout does not influence gonococcal adherence. This result is in

line with the infection experiments performed in earlier studies using the NSM2 inhibitor GW4869 as well as with the knockdown experiments using siRNA directed against NSM2 (Faulstich *et al.*, 2015). In contrast, the invasion is affected by the NSM2 knockout (Figure 3.6). Clone 4 shows only a small decrease in invasion, indicating an unsuccessful knockout. Perhaps not all alleles of the NSM2 gene were destroyed by the CRISPR/Cas9 system resulting only in a kind of knockdown instead of the target full knockout. Another possibility is that the repair of the caused double strand break resulted in a small in frame deletion, which could lead to the expression of an incomplete NSM2 protein that possesses a sphingomyelinase rest activity. The other two tested clones show a significant decrease in invasion, which may stand for a successful and complete knockout of the NSM2. Although, the clones 7 and 11 show a bigger decrease in invasion, there are still bacteria left, which can enter the cells. When the NSM2 is inhibited in wild type Chang cells, there are also some invasive gonococci left, but the percentage is considerably lower. One possible explanation for the remaining intracellular bacteria is the usage of an alternative invasion pathway. In this case, however, the percentage of intracellular bacteria should be as high as it is the case after the inhibitor treatment. Another possibility is a failed generation of a full knockout. The single cell clones still show a rest activity of the NSM2, which mediates the entry of the bacteria into the cells. In case of a successful knockout, the cells could try to compensate the loss, for example through a higher activity of other sphingomyelinases. Those enzymes could produce the needed ceramide and therefore restore the invasion of the gonococci in parts. This hypothesis would be based on the assumption that not the protein NSM2, but its activity and therefore its product ceramide is crucial for the effective invasion of the bacteria into human cells.

The negative effect on invasion due to the knockout of NSM2 could not be confirmed using differential immunofluorescence staining (Figure 3.7). The invasion was only affected when the clones were treated with the NSM2 inhibitor GW4869. The differential immunofluorescence staining enables distinguishing of extra- and intracellular bacteria. The method shows the status quo of the infection after one hour. In contrast, the gentamicin assay is a survival assay. The bacteria have to survive for 2 to 3 hours in the cells after invasion and simultaneously keep their ability to replicate and grow on GC agar plates. Under the premise of a successful knockout, the differing results of the two methods implicate that the NSM2 and its activity is only important for the survival of the bacteria, but

not for the entry of the bacteria into the cells. Hence, rather ceramide, the product of the enzyme, than the protein itself may be important for gonococcal invasion and survival.

On the other hand, the decrease of invasion seen in the differential immunofluorescence staining in the Chang cells due to inhibitor treatment shows that the NSM2 seems to have an impact on invasion, not on intracellular survival. Furthermore, previous studies suggest a role of NSM2 in the early signalling events leading to bacterial uptake. The enzyme is important for the recruitment of the PI3K and the activation of the downstream signalling, which finally results in the engulfment of the gonococci (Faulstich et al., 2015). Additionally, the activity of NSM2 and the generation of ceramide leads to the establishment of membrane rafts, which are known to be hijacked by many intracellular microorganisms as a general port of entry into cells (Faulstich et al., 2015, Manes et al., 2003)

A phenomenon, which should not be neglected, is the so called off-target effect. The off-target effect describes the non-specific recognition and digestion at non-targeted regions by the CRISPR/Cas9 system (Horii & Hatada, 2015). In general, the specificity of the CRISPR/Cas9 nucleases is achieved by the specific binding of the 20-base-pair guide RNA to the DNA target. Meanwhile, there are several research groups reporting highly active nucleases with imperfectly matched RNA-DNA interfaces. Single, double and even up to five mismatches are tolerated, which leads to a high frequency of off-target effects in human tumor cell lines. According to the data, the toleration of mismatches seems to be sequence-dependent, sensitive to the position, the number and distribution of mismatches (Horii & Hatada, 2015, Fu et al., 2013, Hsu et al., 2013, Pattanayak et al., 2013). Therefore, in this study, the occurrence of off-target effects, which can produce undesirable phenotypes, has to be taken into consideration. Such a phenotype could have a negative impact on the survival of gonococci inside the cells resulting in the decrease in gonococcal invasion observed in the gentamicin assay (Figure 3.6).

The generated knockout cell lines were complemented with a NSM2-GFP construct using shRNA. The complementation should rescue the bacterial invasion and therefore prove that the original outcome is caused by the NSM2 knockout and not by an off-target effect. The successful complementation and hence, expression of the NSM2-GFP protein was confirmed by western blot (Figure 3.8). Next, gentamicin assays were performed to examine the effect on gonococcal invasion. While the complementation did not affect the adherence of the

gonococci to the cells at all, the invasion was increased. This result stands for a successful complementation of the NSM2 knockout cell lines with a NSM2-GFP construct. However, the original decrease in invasion due to the NSM2 knockout by the CRISPR/Cas9 system could not be reproduced. The clones 7 and 11, which were used as controls in the infection experiment, showed an increase in invasion compared to the untreated control Chang cells instead of the originally observed decrease.

There are different possible explanations for the disappearance of the decrease in invasion due to the NSM2 knockout. One explanation could be the flexibility of tumor cells to adapt to new circumstances. The cells could have compensated for the loss of the NSM2 by upscaling the expression of other sphingomyelinases, which are able to take over the tasks of the NSM2 and can generate ceramide. That would be a hint that not the actual NSM2 protein, but its product ceramide is important for gonococcal invasion. The easiest way to achieve an adaptation or compensation may be the up- or downregulation of a protein with similar function or a paralog. For example, the inhibition of the kinesin motor protein Eg5, which is essential for spindle assembly, is compensated by the upregulation of kinesin Kif15 in HeLa cells. In this case, Kif15 can replace all essential functions of Eg5. Furthermore, there are also known more complex feedback mechanisms, which can compensate for gene loss. Those mechanisms involve regulatory networks such as the *egfl7* knockout in zebrafish, where a whole set of proteins is upregulated due to the loss of the gene (Cerikan *et al.*, 2016, Tanenbaum *et al.*, 2009, Rossi *et al.*, 2015). Cell-intrinsic adaptations in order to compensate a loss of function have also been described for *DOCK6*, a gene coding for a RAC1/CDC42 guanine nucleotide exchange factor and associated with the Adams-Oliver syndrome. The cells compensate the gene disruption by reducing other factors, which results in increased levels of active RAC1 and CDC42. Interestingly, this adaptation does not appear in siRNA knockdown experiments (Cerikan *et al.*, 2016). Therefore, the results obtained by siRNA knockdowns and whole knockouts by gene disruption through CRISPR/Cas9 differ. This effect has not only been observed in human cells, but also in mouse and *Arabidopsis*. It is assumed that the different timescale of the approaches may be responsible for the effect and that adaptation occurs over time (Cerikan *et al.*, 2016, Morgens *et al.*, 2016, Rossi *et al.*, 2015). In this study, the compensation of the gene disruption of NSM2 over time could be an explanation for the disappearance of the first observed decrease in gonococcal invasion.

However, further experiments are required to identify potential proteins that could replace NSM2.

Another possible explanation for the observed effect could be the abnormal nature of immortalized cell lines. The karyogram of Chang cells used for the generation of the NSM2 knockout revealed severe chromosomal aberrations (Figure 3.5). These differences compared to a normal set of chromosomes make it impossible to determine the number of *Smpd3* alleles present in Chang cells without further tests. The higher the number of alleles, the less efficient is the disruption of the target gene using the CRISPR/Cas9 system. Hence, the method is most efficient in haploid cell lines (Horii & Hatada, 2015). On the one hand, the abnormal nature of the Chang cells could contribute to the compensation and, therefore, explain the effect observed in gonococcal infection. On the other hand, the chromosomal aberrations could decrease the chances of a complete knockout of NSM2. This could mean that the knockout has never been complete, which would fit to results obtained using the differential immunofluorescence staining that showed no effect on gonococcal invasion due to the NSM2 knockout (Figure 3.7). The hypothesis of an incomplete knockout could also be in line with the gentamicin assay results (Figure 3.6). The remaining 20% invasion could be explained by a single remaining *Smpd3* locus, whose expression was upregulated over time and enabled to restore the gonococcal invasion.

The knockout of the NSM2 can affect the cellular signalling, but also the biophysical properties of the plasma membrane (Milhas *et al.*, 2010a). In general, gentamicin cannot cross the plasma membrane of human cells. However, the knockout of the NSM2 could alter the biophysical properties of the plasma membrane and result in a different permeability for gentamicin. Therefore, the antibiotic could reach intracellular bacteria and kill them. Due to the different experimental set up of the gentamicin assay compared to the differential immunofluorescence staining, the changes in membrane permeability could explain the unequal results obtained in the original infection experiments (Figure 3.6 and 3.7). In the future, the membrane permeability could be checked by PI staining.

4.2 The role of ceramide in infection

Ceramide, the product of NSM2, plays an important role in the SREC-I/PorB$_{IA}$-mediated invasion due to the formation of membrane microdomains and the initiation of downstream

signalling (Faulstich *et al.*, 2015). In this study, I aimed to investigate the influence of ceramide on gonococcal invasion independent of the activity of NSM2. The working hypothesis included the idea that more ceramide correlates with a higher invasion. Therefore, bSMase was added to the human cells. This method is a useful and common tool for the investigation of the effects of endogenous ceramide generation (Milhas *et al.*, 2010a). The hypothesis could not be confirmed, because the addition of bSMase to the cells resulted in decreased invasion of the gonococci, while there was no significant effect on adherence observed (Figure 3.9 A). The generation of ceramide due to bSMase treatment has recently been confirmed using *d*STORM for the visualization of ceramides with high spatial resolution. The bSMase treatment increases the overall ceramide concentration in the plasma membrane of different tested cell lines as well as the quantity and the size of ceramide-rich-platforms (Burgert *et al.*, 2017). Therefore, it can be assumed, that the bSMase treatment also increases the ceramide content in the plasma membrane of the Chang cells used in this study. The function of ceramide not only as a structural component of the plasma membrane but also as a second messenger may explain the decrease in gonococcal invasion. The bSMase mimics the mammalian nSMase producing ceramide and causing differentiation, development, aging and apoptosis (Ago *et al.*, 2006). Therefore, it changes the fine-tuned balance of sphingolipids in human cells disturbing the signalling events necessary for gonococcal invasion. Other than that, the bSMase can not only catalyse sphingomyelin, but is also capable of hydrolysing other membrane lipids like phosphatidylcholine, extending its effects on the plasma membrane. It can even be cytotoxic for some mammalian cell types including erythrocytes (Milhas *et al.*, 2010a). Hence, the adverse effects on human cells can negatively influence gonococcal invasion. In further experiments, a cytotoxic effect of the bSMase on Chang cells should be excluded as well as a direct negative effect on *N. gonorrhoeae*.

Instead of adding an enzyme that generates ceramide, the ceramide can also be added directly to the cells to investigate its effect on gonococcal infection. For this reason, infection experiments were performed using cells pretreated with C16 ceramide (Figure 3.9 B). The addition of C16 ceramide showed no effect on bacterial adherence, hence, ceramide does not play a role for the initial attachment of the bacteria to the cells. In contrast, C16 ceramide caused an increase in invasion by trend and can even counteract the effect of the NSM2 inhibitor GW4869 to some extent. In further experiments, a direct effect of the C16

ceramide on *N. gonorrhoeae* should be excluded. The advantage of the direct addition of C16 ceramide are the diminished side effects compared to the treatment with bSMase, which were discussed above. The C16 ceramide is directly incorporated without further effects on the plasma membrane. However, it is not known in which dimension the incorporation of the molecule happens. Recent studies reveal an inefficient incorporation of C16 ceramide in the membrane of primary T cells (Collenburg *et al.*, 2016). A more efficient incorporation might result in an enhanced positive effect on gonococcal invasion, supporting the theory that the ceramide production is the crucial step for the PorB$_{IA}$-mediated invasion neither than the NSM2 as scaffold protein. The minor effect of C16 ceramide on invasion could also show that C16 is not important for the bacterial engulfment. Instead, another ceramide species could be essential. However, C16 seems to play a role in invasion, since the lipidome analysis pointed out an involvement of the ceramide (Figure 3.11). Taken together, the results obtained in these experiments using either bSMase or C16 ceramide are contradictory, allowing no final assessment.

4.3 Gonococci possess ceramide

Ceramide is a crucial structural component of mammalian membranes. In this study, the question arose if ceramide is not only essential for human membranes, but also represents a component of the gonococcal membrane. *d*STORM pictures revealed the staining of gonococci by a C16/24 ceramide IgG antibody (Figure 3.10). The staining is unlikely the result of an unspecific binding of the antibody to gonococcal surface structures, since the antibody was extensively tested and declared to specifically recognize ceramide (Krishnamurthy *et al.*, 2007). Therefore, *N. gonorrhoeae* seems to possess ceramide. The search for homologous genes in gonococci encoding for enzymes necessary for the biosynthesis of ceramide in the NCBI database revealed no hits. Hence, the gonococci seem to rather take up the ceramide than synthesise its own. The uptake of ceramide could be verified by feeding the bacteria with synthetic azido-functionalized ceramides, which allow bio-orthogonal click-reactions to label incorporated ceramides using fluorescent dyes (Collenburg *et al.*, 2016). The source of the ceramide should be determined. In this study, the bacteria were grown on GC agar plates, which contain meat enzymatic digests. Hence, the plates can provide the ceramide. It would be interesting to investigate if human cells could also serve as ceramide source for

gonococci. The exchange of lipids between a pathogen and its host has already been described for *Borrelia burgdorferi*. The pathogen can extract lipids from the plasma membrane of host cells and incorporate them (Crowley *et al.*, 2013). In the case of *B. burgdorferi*, the lipid exchange could contribute to the pathogenesis of the caused disease. In contrast, the question why the gonococci include ceramide from the medium remains unclear until now. The incorporated ceramide is not equally distributed, but forms about 100 nm big clusters. The formation of those clusters could be random due to physical properties of the gonococcal membrane, but it is more likely that the bacteria take up ceramide for a reason, which makes the localisation in clusters necessary. The localization of ceramides in platforms with a size of about 75 nm has recently been shown in different human cell lines using super-resolution imaging (Burgert *et al.*, 2017).

4.4 Gonococcal infection increases ceramide and sphingosine

The ceramide increase due to SREC-I/PorB$_{IA}$-mediated invasion has already been shown using antibody-based methods (Faulstich *et al.*, 2015). In this study, the actual amount of ceramide in human cells before and after gonococcal infection has been measured. Simultaneously, the amount of other sphingolipids connected to ceramide through the sphingolipid pathway was detected, giving a precise picture of the changes in the sphingolipid balance due to infection (Figure 3.11).

According to the results, the amount of sphingomyelin was increased in infected cells by trend. This is counterintuitive, because the activation of the NSM2 and the following catalysis of SM to ceramide were expected to decrease the SM level. SM is by far the most abundant sphingolipid in animal cell membranes (Christie, 2014) and the total amount of SM in the tested Chang cells is very high compared to that of the other sphingolipids (raw data not shown). It is assumed, that the activation of the NSM2 occurs mainly locally at the attachment and internalisation site of the gonococci. For this reason, the conversion of a comparably small amount of the molecule may be of no consequence for the total SM levels. Furthermore, it has to be taken into account, that the measurement in this study was conducted two hours after infection, whereas the SREC-I/PorB$_{IA}$-mediated invasion is significantly faster. The gonococci invade human cells already 15 to 30 min after the addition to the cells. Thus, the SM levels have enough time to recover. This effect has been described

in baby hamster kidney cells, where the initially decrease of SM levels due to sphingomyelinase treatment was rescued within a few hours due to the rapid turn-over of plasma membrane SM (Slotte *et al.*, 1990).

The increasing levels of ceramide in infected cells is recognizable in all ceramide species and in Cer16, 18 and 20, the increase is statistically significant. Earlier in this study, I could show the ability of gonococci to incorporate ceramide from their growth medium (Figure 3.10). This incorporated ceramide could explain the mild increase due to infection seen in all examined ceramide species. The significantly higher ceramide levels cannot be explained by this effect. They are the result of the NSM2 activation due to gonococcal infection. Mammals possess over 200 structurally distinct ceramides, which can be differentiated by structural modifications. Meanwhile, the awareness rises that different ceramide species may execute different functions (Hannun & Obeid, 2011). Therefore, the question why only the levels of those three species are increased due to gonococcal infection cannot be answered in this study.

The sphingosine level also increases due to infection with *N. gonorrhoeae*. The higher amount of sphingosine could be explained with the increased ceramide levels. To control and restore the ceramide levels to a normal standard, the ceramide has to be depleted amongst others by ceramidases to sphingosine. Therefore, the increase of sphingosine could be a side effect of the normalization of the ceramide levels after infection. Although the increase of sphingosine and therefore the activation of other enzymes of the sphingolipid pathway besides the NSM2 could only represent the natural degradation pathway of ceramide, this activation could also be important for the gonococcal invasion. Hence, the increase of sphingosine and the increase of sphingosine-1-phosphate could indicate the involvement of the whole sphingolipid pathway in SREC-I/PorB$_{IA}$-mediated invasion.

4.5 Sphingosine kills *Neisseria gonorrhoeae*

Sphingosine belongs to the group of sphingoid bases and possesses antibacterial activity against gram-positive as well as gram-negative bacteria. Therefore, it plays an important role in innate antimicrobial activity of both, skin and mucosal surfaces (Fischer *et al.*, 2012). However, the susceptibility of *N. gonorrhoeae* to sphingosine has not been tested before. In

this study, the toxic effect of sphingosine on PorB$_{IA}$-expressing gonococci was shown (Figure 3.12). The bacterial growth was prevented in a dose-dependent manner starting at a concentration of 10 µM. In contrast, in the plating experiment already a 5 µM concentration showed a significant effect on bacterial survival. The explanation could offer the different experimental set up. In the growth curve experiment the large number of bacteria can compensate the low concentration of sphingosine, whereas in the plating experiment, there was a defined, much smaller number of bacteria used. Therefore, there were more sphingosine molecules present per gonococcus at the same concentration, resulting in this dramatic effect. The susceptibility of gonococci to sphingosine is even clearer in HEPES-medium compared to PPM$^+$ medium. The media differ in their composition and purpose. While HEPES is a phosphate-free infection medium, which contains ingredients important for cells, PPM$^+$ is a medium specific for the needs of gonococci. Therefore, the nutrient supply for gonococci is only guaranteed in PPM$^+$ medium, making them a slightly more robust against sphingosine. To this date, the specific mechanism, which leads to the killing of gonococci by sphingosine is unknown. Microscopic investigation of *S. aureus* and *E. coli* treated with sphingosine revealed both extra- and intracellular damage. Furthermore, intracellular inclusions were observed, which probably reflect lipid uptake (Fischer *et al.*, 2013). The antimicrobial effect of sphingosine on *S. aureus* takes place on a physiological level mainly acting at the plasma membrane (Bibel *et al.*, 1993). Additionally, sphingosine has the ability to act on bacteria on the transcriptional level. Sphingosine was also shown to be involved in *P. aeruginosa* infection. The opportunistic pathogen infects mainly the human lung and is able to detect host-derived sphingosine by a sphingosine-responsive transcription factor. The direct binding of sphingosine to the transcription factor resulted in the enhanced transcription of a protein necessary for the survival of the bacterium in the murine lung (LaBauve & Wargo, 2014). Nevertheless, sphingosine prevents lung infections by *P. aeruginosa* (Pewzner-Jung *et al.*, 2014).

4.6 SPHKs in PorB$_{IA}$-mediated invasion

4.6.1 SPHK2 plays a role in PorB$_{IA}$-mediated invasion

The lipidome analysis of infected versus non infected cells (Figure 3.11) revealed a potential important role in gonococcal invasion of further enzymes involved in the sphingolipid

pathway besides NSM2. To this purpose, different enzymes of the pathway were objects of research in this study, starting with the sphingosine kinases. Both sphingosine kinases were inhibited using the dual inhibitor SKI-II (Figure 3.13). The substance inhibits SPHK2 and targets SPHK1 by enhancing the degradation of the enzyme leading to an increased probability of other direct targets (Gao *et al.*, 2012, Neubauer & Pitson, 2013, Ren *et al.*, 2010). Since SKI-II had a strong negative effect on the survival of the bacteria, the decrease in adherence and invasion is not a consequence of the inhibition of the two enzymes SPHK1 and SPHK2, but is a toxic effect of SKI-II on the gonococci. Interestingly, the lowest concentration of 5 µM shows a drastic effect on bacterial growth, but does not influence bacterial adherence and invasion in infection experiments in a significant way. SKI-II has the ability to increase the total ceramide levels in A498 kidney cells by 30-60% (Gao *et al.*, 2012). This ceramide increase could also appear in Chang cells and counterbalance the negative effect on gonococci at a low concentration of the inhibitor. However, the concentration of 5 µM could simply be too low to cause an effect.

Thereupon, specific sphingosine kinase inhibitors were used, which target only one of the two enzymes. The SPHK1 specific inhibitor 5C, which inhibits the levels of generated S1P (Wong *et al.*, 2009), showed no effect on bacterial growth, on the viability of the used Chang cells and on gonococcal adherence and invasion (Figure 3.14). Therefore, the SPHK1 does not seem to play a role in phosphate-dependent invasion. In contrast, SPHK2 seems to have a great impact on gonococcal invasion (Figure 3.15). The experiments performed with K145, a selective inhibitor of SPHK2 revealed a drastic effect on gonococcal invasion in Chang cells without significantly affecting the cell viability.

4.6.2 Effect of SPHK2 inhibition on sphingolipids

Besides the reduction of neisserial invasion, the SPHK2 inhibitor K145 also led to changes in the cellular sphingolipid levels (Figure 3.17). The results revealed that the inhibitor does not only affect the levels of the sphingolipids directly involved in the enzymatic reaction of the SPHK2. Instead, it prevents the small increase in the sphingolipid levels due to gonococcal infection seen in the controls of all analysed sphingolipid species. Furthermore, the levels of sphingomyelin and ceramide are increased due to inhibitor treatment. Hence, a disturbance at one point in the sphingolipid pathway seems to influence the whole sphingolipid balance

of the cells. As the balance is fine-tuned, those changes could determine the cell fate (Neubauer & Pitson, 2013). In addition, the lipidome analysis revealed a dramatic increase of the SPHK2 product S1P due to SPHK2 inhibition by K145. This result is not only counterintuitive but also contradictory to the literature, since K145 suppresses the S1P level in U937 cells and SPHK2 inhibitors in general seem to lower the S1P levels in cultured cells (Liu *et al.*, 2013, Kharel *et al.*, 2012). A possible explanation for the high S1P level provides the secretion of S1P. The molecule can act as paracrine and autocrine signalling molecule, and in contrast to the cellular S1P, the circulating S1P levels are elevated due to SPHK2 inhibition (Liu *et al.*, 2013, Kharel *et al.*, 2012). The infection medium used in this study lacks the ingredients necessary for the secreted S1P to go in solution. Instead, the S1P adheres to the surface of the cells and is measured and analysed together with the cellular S1P. Therefore, the levels of S1P in this study are extremely high due to K145 treatment.

The experiments performed with K145, a selective inhibitor of SPHK2 showed a drastic effect on gonococcal invasion starting at the lowest concentration (Figure 3.15). In opposition to the invasion, the adherence is only affected at the highest concentration. This effect can be explained by the cytotoxicity of K145 at high concentrations. The strong effect on invasion can be an evidence for the importance of the SPHK2 for gonococcal engulfment. Even though, the inhibitor is described and widely used specifically against SPHK2, off-target effects might occur, which could interfere with the gonococcal invasion. Furthermore, it cannot be excluded that the effect on gonococcal invasion is triggered by the disturbance of the whole sphingolipid pathway. The inhibitor influences the whole sphingolipid balance, which could affect the signalling cascade necessary for the entry of the gonococci into human cells.

4.6.3 K145 activates the AKT Signalling

The treatment with the SPHK2 specific inhibitor K145 affects the cellular signalling cascade that is necessary for the engulfment of the *Neisseria* (Figure 3.16). The phosphorylation of AKT due to the activation of the PI3K during the gonococcal invasion does not take place anymore when the SPHK2 is inhibited. Therefore, the inhibition of the SPHK2 seems to result in an interruption of the signalling processes of bacterial invasion. The missing activation of the PI3K shows that the functional SPHK2 is involved in the early signalling events leading to

bacterial entry into human cells upstream of the PI3K. Interestingly, the western blots revealed a higher basal level of pAKT in the uninfected samples treated with K145. Hence, K145 could lead to a permanent phosphorylation and therefore, activation of AKT. This effect is contradictory to the results obtained in other studies. It has been shown, that SPHK inhibitors including K145 inhibit the ERK and AKT signalling pathways and respectively the phosphorylation of the enzymes as a downstream event of the SPHK inhibition (Liu *et al.*, 2013). However, S1P activates the PI3K/AKT pathway (Igarashi & Michel, 2001). Therefore, the high basal level of pAKT can be a result of the very high S1P level in this study due to K145 treatment.

4.6.4 SPHK siRNA knockdown affects gonococcal invasion

Knockdown experiments using siRNA were conducted to verify the involvement of SPHK2 in PorB$_{IA}$-mediated invasion (Figure 3.18). The long transfection time of 6 days was chosen, since the control qRT-PCR revealed a compensation of the knockdown through the upregulation of the other SPHK, respectively (data not shown). The qRT-PCRs were performed to confirm the SPHK knockdown at the RNA level. The verification on protein level was not possible, because the tested antibodies directed against SPHK1 or SPHK2 were not able to recognize the endogenous proteins (Figure 3.21). Possible reasons for the unspecific binding of the antibodies and the appearance of multiple bands have already been discussed in connection with the NSM2 antibodies (4.1).

The gentamicin assay showed no significant involvement of the SPHKs in adherence. While the knockdown of the SPHK1 did not influence the gonococcal invasion, the downregulation of the SPHK2 revealed a decreasing trend in invasion. In contrast to the decrease in invasion due to the K145 inhibitor treatment, the effect of the SPHK2 downregulation is very weak. The reason for this, might be a poor knockdown, which was confirmed by qRT-PCR. The knockdown of SPHK2 alters the sphingolipid recycling pathway and results in the reduced conversion of sphingosine to ceramide (Maceyka *et al.*, 2005). Since ceramide plays an important role in the PorB$_{IA}$-mediated invasion, the decrease in ceramide levels due to the downregulation of SPHK2 could explain the negative effect on invasion. Conversely, the knockdown of SPHK1 increases ceramide levels, possibly leading to the small increase in invasion seen in the gentamicin assay (Hait *et al.*, 2009, Faulstich *et al.*, 2015). The

downregulation of both SPHK1 and SPHK2 had a significant negative effect on invasion. The double knockdown was performed to exclude the possibility of compensation. Despite their opposing physiological roles, there are hints that the SPHK isoforms are able to compensate the deficit of the other enzyme to some extent (Takabe *et al.*, 2008).

The differential immunofluorescence staining revealed no significant effect neither on adherence, nor on invasion. However, it has to been taken into consideration that this assay is not very sensitive. In general, the effects on invasion seen in gentamicin assays are extenuated or not visible when the differential immunofluorescence assay is used. However, there is no effect on invasion due to the siRNA knockdown, which could argue for a role of the SPHKs only in the intracellular survival of the bacteria, but not in the actual entry into the human cells. On the other hand, the results regarding the signalling cascade necessary for the invasion of the gonococci obtained using the inhibitor K145 showed the involvement of the SPHK2 in the early signalling events leading to the engulfment of the bacteria. Therefore, it is unlikely, that the enzyme just plays a role in intracellular survival and not in invasion. A differential immunofluorescence staining using the SPHK2 specific inhibitor K145 has to be performed in order to address the question.

4.6.5 SPHK siRNA knockdown decreases sphingolipid levels

The lipidome analysis revealed that the knockdown of the two SPHKs at the same time has consequences for all sphingolipids in the sphingolipid pathway and not only for the product of the downregulated enzymes (Figure 3.19). That fact concurs with the result of the lipidome analysis after the treatment with the SPHK2 specific inhibitor K145. However, the application of K145 and the SPHK knockdown lead to different consequences for the sphingolipid pathway. While K145 in general increased the sphingolipids, the downregulation of SPHK1 and SPHK2 resulted in decreased levels of sphingomyelin, ceramide and sphingosine-1-phosphate, partly in a statistically significant manner. Only sphingosine shows no strong reaction to the siRNA knockdown. In contrast to the knockdown performed here, which leads to the downregulation of both SPHKs, the inhibitor K145 inhibits only the SPHK2. Therefore, the differences between the two lipidome profiles could be a result of the SPHK1 activity. In the case of the SPHK2 inhibition, SPHK1 could compensate the loss to some extent. This observation was made in knockout mice. The

single SPHK1 or SPHK2 knockout mice showed a normal development, whereas the double knockout was embryonically lethal, indicating a compensation of the lacking enzyme in the single knockout mutants (Takabe *et al.*, 2008, Mizugishi *et al.*, 2005). In the knockdown experiment, the decrease of S1P due to the downregulation of the two SPHKs was expected, whereas the decrease in SM and ceramide cannot be explained. The original effect on the sphingolipid levels due to infection (Figure 3.11), specifically the increase of SM, ceramide and sphingosine cannot be observed in the knockdown experiment. The *Neisseria* seem to have no impact on the sphingolipid levels. Perhaps the influence of the knockdown on sphingolipid levels is stronger than the effect of the gonococci and therefore, prevents or supresses the original reaction to the infection. However, the samples transfected with the control siRNA do not show the original increase either. Since an off-target effect leading to this result is unlikely, the effect seems simply to be not reproducible.

The involvement of only one SPHK in gonococcal invasion points to a role of the sphingolipid pathway exceeding the natural degradation of ceramide, which was built due to NSM2 activation. The involvement of SPHK2 but not of SPHK1 was surprising regarding the localization of the enzymes. While SPHK2 is mainly located in intracellular compartments, SPHK1 is located in the cytosol and is additionally translocated to the plasma membrane due to activation (Igarashi *et al.*, 2003, Johnson *et al.*, 2002). Therefore, this enzyme would be in close proximity to the site of action when gonococci try to invade human cells. On the other hand, SPHK2 can also be located in the cytosol and would therefore be able to fulfil a function in gonococcal invasion (Igarashi *et al.*, 2003). While a broad range of pathogens makes use of the SPHK1 in order to enhance their infection or even enable their survival, there are not many pathogens known that utilize the SPHK2 (Arish *et al.*, 2015). Up to date, there is only one virus known, which interacts with the SPHK2. The chikungunya virus replication complex co-localizes with the SPHK2 enzyme during infection (Reid *et al.*, 2015). In contrast, pathogens causing fungal, protozoan, viral and bacterial infections target the SPHK1 during infection (Arish *et al.*, 2015). *Mycobacterium tuberculosis* represents a bacterium that specifically inhibits the SPHK1 in order to block calcium signalling and phagosome maturation in human macrophages (Malik *et al.*, 2003), whereas *Mycobacterium smegmatis* increases the activity of the SPHK1, which results in the enhancement of pro-inflammatory and the suppression of antimicrobial factors (Arish *et al.*, 2015, Prakash *et al.*, 2010, Yadav *et al.*, 2006). In the described case of *Mycobacterium*, the pathogen changes the

activity of the enzyme SPHK1. In the case of *N. gonorrhoeae*, however, it is yet unknown if the bacteria alter the activity of the SPHK2 or if only a functional enzyme is needed for a successful invasion.

4.6.6 Consequences of SPHK knockout in MEFs for gonococcal invasion

The inhibitor and knockdown experiments should be complemented by experiments using knockout cell lines. For this reason, mouse embryonic fibroblasts lacking either SPHK1 or SPHK2 were used for infection experiments (Figure 3.20). While the knockouts did not affect the adherence of the gonococci to the cells, the invasion was drastically increased compared to the control. This result is contradictory to the results obtained with the inhibitor and knockdown experiments. However, it is not unusual that different cell lines behave differently. The activation of the PI3K due to the infection was observed in the control cells and in the SPHK1 knockout cells, but not in the SPHK2 knockout cells. The situation concerning the signalling is different in cells treated with the SPHK2-specific inhibitor K145, where the pAKT basal level was clearly increased. Hence, both experiments showed that a lack of SPHK2 or its function prevents the phosphorylation of AKT as a result of gonococcal infection. However, the MEFs have already been in a very high passage when they were delivered. Furthermore, the experiments were only conducted once, which is why it is not possible to come to a conclusion. In general, mouse cells may not be a good model for neisserial infection, since the pathogen is restricted to human hosts and the progress of the infection differs in mice compared to humans.

4.6.7 Establishment of a SPHK Activity Assay

In this study, the involvement of at least one of the SPHKs in gonococcal invasion could be proven. Subsequently, the activation of the enzyme due to the infection with *N. gonorrhoeae* should be investigated. Therefore, a sphingosine kinase activity assay was successfully established (Figure 3.22). The sample preparation for the activity assay includes sonication. The controls using recombinant SPHK1 (Figure 3.22 B) reveal the importance of this step for the successful execution of the assay. Unfortunately, the activation of the SPHK due to gonococcal invasion could not be investigated using this assay, since the Chang cells

did not show any signal, independent of the infection. Assuming, that the amount of SPHK in the probes is too low to detect a signal, I increased the quantity of the cells. However, the number of the cells used for the assay did not alter the results. Additionally, the number of the cells is limited, since the separation of the lipids is faulty when the TLC plate is overloaded. Therefore, mitochondria and membranes were isolated from 15 cm dishes to increase the amount of enzyme in the reaction. Since the SPHKs are amongst others mainly located in the cytosol, the enrichment of the enzymes with this method can only be effective when the enzymes are translocated to the plasma membrane due to activation by the gonococci. The purification of the plasma membrane and mitochondria did not alter the results. There was still no signal detectable. In the future, the assay should be repeated using a different cell line, which shows a higher expression of the SPHKs or the enzymes could be overexpressed.

4.7 S1PL plays a role in PorB$_{IA}$-mediated invasion

The sphingosine-1-phosphate lyase is known as the gatekeeper of the lipid metabolic flow and represents the only exit point for sphingolipid intermediates (Aguilar & Saba, 2012). Therefore, the inhibition of the enzyme leads to a strong increase of the S1P levels in cells. In this study, the S1P lyase-specific inhibitor Compound A was used to investigate the potential role of the enzyme in PorB$_{IA}$-mediated invasion (Figure 3.23). Since the S1P lyase is located in the ER, it is more likely, that the enzyme is not directly involved in gonococcal invasion, but its product S1P could play a pivotal role (Ikeda *et al.*, 2004, Van Veldhoven & Mannaerts, 1991).

The gentamicin assays performed with N927 revealed a strong increase in invasion due to the inhibitor treatment. The increase in invasion peaks at a concentration of 5 µM and then starts to drop as the concentration rises, although the invasion is still significantly higher compared to the DMSO control. The drop can be explained by the negative effect of Compound A on *Neisseria*. The inhibitor prevents the S1PL from working and leads to an increased S1P level in the cells. In general, S1P is important for the regulation of cell growth and for the suppression of apoptosis (Cuvillier *et al.*, 1996). The suppression of apoptosis could facilitate the survival of the bacteria within the cells after the invasion. Because of the experimental setup, which includes only two hours of infection before the bacteria are

plated, this scenario is unlikely to cause the observed effect. Another hypothesis involves the linkage of S1P to cytoskeletal rearrangements (Takabe *et al.*, 2008). S1P can be secreted and, therefore, the sphingolipid can bind to EDG-1, a G protein coupled receptor and together with the transactivation through AKT, this binding results in Rac activation, actin assembly and endothelial cell migration (Lee *et al.*, 2001). The activation of Rac1 and actin rearrangements are requirements for the invasion of gonococci (Faulstich *et al.*, 2013). Hence, the increased S1P level due to the inhibition of the S1PL could support the intracellular processes leading to the engulfment of the bacteria. Furthermore, secreted S1P can bind to S1P receptors in an autocrine or paracrine fashion and activates the PI3K and AKT, which results in cell survival. The PI3K and its activation is a pivotal part of the signalling cascade necessary for gonococcal invasion (Arish *et al.*, 2015, Faulstich *et al.*, 2013). Its activation could enhance the bacterial uptake.

While the inhibition of the S1PL increases the invasion of N927, the invasion of N931 is decreased. Those conflictive results are based on the different invasion pathways used by the different strains. N927 (PorB$_{IA}$, Opa$^-$, Pili$^-$) uses the SREC-I/PorB$_{IA}$-mediated invasion pathway. This phosphate-sensitive invasion is triggered by a signalling cascade involving the NSM2 and its product ceramide, as well as caveolin, the PLCγ1, the PI3K, PKCμ and Rac1, resulting in cytoskeletal rearrangement and the uptake of the bacteria (Faulstich *et al.*, 2013, Rechner *et al.*, 2007). In contrast, the Opa$_{50}$-expressing strain N931 (PorB$_{IB}$, Opa$_{50}$, Pili$^-$) enters human cells through the binding to HSPG receptors. This invasion pathway involves the PKC, PC-PLC and the ASM (van Putten & Paul, 1995, Grassmé *et al.*, 1997). Due to the different signalling molecules and cascades involved in the two invasion pathways described, the consequences of the inhibition of the S1PL by Compound A on invasion are distinct. While the PorB$_{IA}$-mediated invasion is enhanced, the Opa$_{50}$/HSPG-triggered invasion is inhibited. Nevertheless, both pathways are dependent on the S1PL and therefore have to share common features. Since both invasion pathways include sphingomyelinases, which produce ceramide and react to the S1PL inhibition, this common ground could be the involvement of the sphingolipid pathway.

The inhibition of the S1PL resulted in an increase in invasion in PorB$_{IA}$-mediated infection. In contrast, the experiments conducted with the S1PL deficient HeLa cell line ΔS1PL revealed a decrease in the phosphate-sensitive invasion (Figure 3.24). The gentamicin assay showed no

significant effect on adherence, but a clear decrease in invasion. Whereat, the differential immunofluorescence staining revealed a decrease in adherence as well as invasion due to the S1PL knockout. The reasons for the partly considerably fluctuations between the results obtained with the gentamicin assay versus the differential immunofluorescence staining have already been discussed earlier (4.6.4). There are different explanations for the opposing impact on invasion in the inhibitor and knockout experiments. On the one hand, the effect of the inhibitor on invasion can be caused by a side effect. However, Compound A was tested and the impact on lipids verified the functionality of the substance (personal communication with Prof. Burkhard Kleuser). On the other hand, the knockout of an enzyme is mostly connected to major consequences for the cells. Therefore, the lipidome and cellular processes of ΔS1PL could be changed due to the knockout and result in different consequences for the PorB$_{IA}$-mediated invasion compared to the inhibitor treatment. However, the cell line was tested and only small adaptations in the lipid and protein compositions were found (Gerl *et al.*, 2016). The discrepancies could also be explained by general differences between cell lines. The inhibitor studies were performed in Chang cells, whereas the ΔS1PL cell line is derived from HeLa cells. Additionally, the expression pattern of S1P receptors on the surface of the two cell lines could differ and result in the activation of different intracellular signalling pathways, which may affect the gonococcal invasion in opposing ways (Strub *et al.*, 2010). The negative effect on invasion due to S1PL knockout may be the result of a disturbed balance of the sphingolipid rheostate. Besides the drastic increase in S1P levels (Figure 3.25), lipidome analysis revealed decreased levels of sphingosine, ceramide and sphingomyelin due in the S1PL knockout cell line (data not shown). Those changes could interfere with the signals necessary for a successful invasion of the gonococci. Again, the levels of sphingolipids do not change due to infection. The possible reasons therefore have been discussed earlier.

The disturbance of the signalling cascade leading to the engulfment of the bacteria could be confirmed by analysing the activation of the PI3K (Figure 3.25). The experiment showed a clear decrease of phosphorylated AKT in the S1PL deficient cells compared to the wildtype due to infection. Hence, the activation of the PI3K in the knockout cells is weak and results in a small number of invasive gonococci compared to the wildtype cells.

In contrast to the PorB$_{IA}$-mediated invasion, the lack of a functional S1PL affected the HSPG-triggered invasion in the same way whatever method was used (Figure 3.24). The decrease in invasion of N931 was even clearer using the knockout cell line compared to the inhibitor treatment. The differential immunofluorescence staining confirmed the results obtained by gentamicin assay. Therefore, the S1PL is involved in the Opa$_{50}$/HSPG-triggered invasion and seems to be important for a successful invasion of human cells. Furthermore, the enzyme plays a role in PorB$_{IA}$-mediated invasion.

To elucidate the discrepancy between the results obtained with inhibitor treatment versus the S1PL knockout cell line in the PorB$_{IA}$-mediated invasion, infection experiments were performed using ΔS1PL and Compound A both at the same time. The wildtype ΔS1PL cells showed a small increase in invasion due to the inhibitor treatment. However, compared to the increase in invasion due to the inhibitor seen in Chang cells, this effect is negligible. The observation points out the possibility of an off-target effect in Chang, but not in HeLa cells. The knockout cells treated with the inhibitor did not show a significant decrease in invasion anymore. Nevertheless, the invasion is still decreased compared to the wildtype control. The small increase in invasion compared to the ΔS1PL DMSO-treated samples could either be a result of the inhibitor treatment, showing its functionality or the numbers are a result of the natural variability of the assay. The differential immunofluorescence staining shows by trend similar results compared to the gentamicin assay related to invasion, whereat the numbers are less significant.

4.8 Impact of external S1P addition on gonococcal invasion

This study revealed an effect of higher S1P levels in cells on invasion. Until now, it is not completely clear how this effect is accomplished, because more S1P leads to an increase in invasion in Chang cells, whereas the invasion is decreased in the HeLa ΔS1PL cell line. This might be in connection to different expression patterns of S1P receptors since S1P can act in an autocrine as well as a paracrine manner. Therefore, I analysed the effect of an external addition of S1P on invasion (Figure 3.26). The differential immunofluorescence staining showed no significant influence either on gonococcal adherence or on invasion, but S1P addition led to a trend in increase of both parameters. Additionally, the gentamicin assay showed the same trend, but a stronger increase in adherence and invasion. However, also in

this case, the effect was not significant due to the high standard deviation. The addition of S1P seems to somehow enhance the adherence of the bacteria to the cells. It is not clear, if the effect arose from changes in the cells or in the bacteria due to S1P. In this study, I could show that *Neisseria* are able to take up ceramide, while sphingosine is toxic for them (Figure 3.10 and 3.12). This shows that members of the sphingolipid pathway can interact with the bacteria. Therefore, it is also possible, that S1P has a direct effect on gonococci, which has consequences for adherence and invasion but does not influence the bacteria growth in liquid culture (Figure 3.26).

On the one hand, the increased invasion seen in the infection experiments can be a logical consequence of the increased adherence. When more bacteria bind to the cells, more bacteria have the chance to invade the cells. On the other hand, the S1P can be incorporated by the membrane of the human cells and change its composition resulting in a facilitated invasion of the bacteria. For example, external S1P triggers the recruitment of several membrane raft proteins, including the PI3K, and enhances the levels of tyrosine phosphorylation. Furthermore, other membrane raft components like caveolin-1 are recruited to the plasma membrane (Zhao *et al.*, 2009). Caveolin-1 and the PI3K both play an important role for the PorB$_{IA}$-mediated invasion of gonococci. Therefore, the enhanced recruitment could facilitate the engulfment of the bacteria.

The external S1P might increase the accessibility of or the affinity to receptors on the human cells leading to a higher adhesion and invasion. Furthermore, the sphingolipid could interact with a yet unknown phosphate-sensitive co-receptor important for the PorB$_{IA}$-mediated invasion, as has been proposed in a recently published dissertation (Reimer, 2017).

Another possibility is that the sphingolipid could bind to its surface-exposed cellular receptors and trigger intracellular signalling pathways, which result in the enhanced engulfment of the bacteria. Different connections between S1P receptors and signalling molecules important for gonococcal engulfment have been detected. The S1PR$_1$ affects AKT and Rac in the context of vascular barrier regulation in endothelial cells. In this example, the transactivation of the receptor leads to the activation of the described signalling (Singleton *et al.*, 2006). In contrast, the S1PR$_2$ mediates endothelial cell dysfunction by inhibiting the PI3K/AKT signalling pathway under high glucose conditions in a diabetes model (Liu *et al.*, 2016). Furthermore, the invasion of another pathogen is connected to extracellular S1P. The

phagocytosis of the pathogenic fungus *Cryptococcus neoformans* by alveolar macrophages is enhanced by extracellular S1P. According to this report, the sphingolipid binds to $S1PR_2$ resulting in an increase of the antibody-mediated phagocytosis through the regulation of the expression of the phagocytic FCγ receptors (McQuiston *et al.*, 2011).

The SPHK2 specific inhibitor K145 has a drastic effect on gonococcal invasion (Figure 3.15). I aimed to investigate whether the external addition of S1P can rescue the negative effect of K145 on invasion. Both infection experiments showed that this was not possible. Contradictory to the literature (Kharel *et al.*, 2012, Liu *et al.*, 2013), this study revealed an increase of S1P due to K145 treatment. Therefore, the negative effect of K145 is not due to a lack of S1P in general. It is already known, that the subcellular location of the SPHK2 and hence, the location of its product S1P, has a great impact on its physiological role (Neubauer & Pitson, 2013). Therefore, the inhibition of the SPHK2 might lead to less S1P being present at the right place of action. The importance of the location of S1P for the right mode of action might explain why external S1P is not able to rescue the negative effect of the SPHK2 inhibition on gonococcal invasion.

Since S1P showed no significant effect on invasion and the addition could not rescue the negative effect of the SPHK2 inhibitor K145 on invasion, S1P seems not to act as a para- or autocrine signal in $PorB_{IA}$-mediated invasion, but as an intracellular messenger.

4.9 *N. gonorrhoeae* inhibits apoptosis in neutrophils

Neisseria are able to delay the onset of apoptosis in PMNs. The bacteria inhibit spontaneous as well as intrinsically- or extrinsically-induced apoptosis and therefore extend the life span of the neutrophils (Chen & Seifert, 2011, Simons *et al.*, 2006). Those studies were performed using strains differing in their expression of Opa proteins and their capability to form pili. The results indicate that both parameters do not change the apoptosis inhibition. However, no attention has been paid to porin. Therefore, it was not known if the expression of $PorB_{IA}$ in contrast to $PorB_{IB}$ influences the effect. Porin has already been connected to the modulation of apoptosis before. For example, PorB from meningococci was shown to interact with mitochondria and protect immune cells from apoptosis (Massari *et al.*, 2000). In gonococci, the translocation of porin to the mitochondria has been linked to the modulation of

apoptosis, although, the mechanism remains unknown (Criss & Seifert, 2012, Muller *et al.*, 2000, Kozjak-Pavlovic *et al.*, 2011). In this study, I aimed to elucidate that question if the serotype of porin has an impact on the delay of apoptosis in PMNs by performing infection experiments with primary PMNs using neisserial strains expressing either PorB$_{IA}$ (N927) or PorB$_{IB}$ (N138) (Figure 3.27 A). The inhibition of the apoptosis was clearly shown to be independent of the serotype of porin.

The apoptosis of PMNs can be inhibited by lipopolysaccharide (LPS) of *E. coli* via phosphorylation of p38 MAPK. The challenging of the neutrophils with LPS leads to the induction of the NSM2 and the production of ceramide, which is metabolized to S1P resulting in the delayed onset of PMN apoptosis via p38 MAPK activation (Nick *et al.*, 2002, Lin *et al.*, 2011). However, the concrete mechanism, how the life span of PMNs is prolonged by gonococci is unknown until now. Preliminary data collected in our lab excluded neisserial LOS, but pointed to an involvement of secreted factors, including peptidoglycan. Hence, different peptidoglycan mutants were tested in this study, however, revealed no differences in apoptosis inhibition compared to the wildtype due to the high standard deviation (Figure 3.27 B). The reasons for the high standard deviation are based on the special phenotypes that the mutants showed in the experiments. The phenotypes were mostly characterized by differences in growth behaviour (Garcia & Dillard, 2008, Cloud-Hansen *et al.*, 2008). Furthermore, the mutants showed drastic clumping in the liquid culture due to a disturbed cell separation (Garcia & Dillard, 2006), which complicated the determination of the optical density in order to adjust the volume needed for the required MOI. Therefore, it would be helpful to use methods for the normalization of the results. In the future, western blotting could be used to quantify the actual amount of *Neisseria* used for infection by their PorB content. The number could then be used to normalize the results obtained in the FACS-based apoptosis assay.

The experiment conducted showed the typical significant inhibition of apoptosis due to the infection with the MS11 wildtype strain (PorB$_{IB}$, Pili$^+$, Opa$^-$). One would expect all three complementation strains to reveal the same result as the MS11 wildtype. Actually, the numbers are quite similar compared to the wildtype, but the differences to the uninfected control are not significant due to the high standard deviation. If the natural secretion of peptidoglycan fragments, which might play a crucial role in gonococci-host interactions

(Sinha & Rosenthal, 1980), would play a role in the delayed onset of apoptosis in PMNs due to gonococcal infection, at least one of the mutants should influence the apoptosis inhibition. The deletion of amiC results in a lack of free disaccharides. Would these disaccharides be of importance, the rate of PMNs undergoing the programmed cell death should be higher compared to MS11. The lack of the two lytic transglycosylases A and D leads to the release of large soluble fragments instead of peptidoglycan monomers and should therefore also result in an increase in neutrophil death. In contrast, the deletion of ampG should enhance the inhibitory effect, since none of these effects could be seen in the results obtained in this study, peptidoglycan seems to play no major role in the inhibition of apoptosis in neutrophils. However, peptidoglycan fragments are important for the modulation of the innate and adaptive immune response. The molecules bind to NOD1 and NOD2, which are cytosolic pattern recognition receptors and intracellular regulators of the immune response during gonococcal infection (Mavrogiorgos *et al.*, 2014, Kaparakis *et al.*, 2010).

In PMNs, the NSM2 is responsible for 99% of the total sphingomyelinase activity and is largely associated with the plasma membrane. The enzyme plays an important role in many neutrophil functions, such as oxidant generation and release, Fcγ receptor-mediated phagocytosis, and apoptosis. Furthermore, NSM2 activity is crucial for orienting chemotactic migration in neutrophils (Sitrin *et al.*, 2011, Hinkovska-Galcheva *et al.*, 1998, Mansfield *et al.*, 2002). In addition, the inhibition of the NSM2 seems to abolish the effect of gonococci on the onset of apoptosis in neutrophils. Those experiments were performed using sphingolactone-24 as NSM2 inhibitor (Lin *et al.*, 2011). In this study, I used two different NSM2 inhibitors to investigate the effect (Figure 3.27 C). The uninfected samples treated with either DMSO, GW4869 or Eso48 were analysed to investigate the cytotoxic effect of the inhibitors on the neutrophils. The results revealed a toxic effect of the inhibitors on the primary cells, whereas the toxicity was not linked directly to the concentration of the substances. One would expect an increasing number of dead cells associated with the increase in concentration, however, this was not the case. An explanation for the effect could be the so called NETosis. This process describes the cell death of PMNs due to the generation of neutrophil extracellular traps (NETs) (Zawrotniak & Rapala-Kozik, 2013). The NETs are formatted to degrade virulence factors and kill invading bacteria. They consist of granule proteins and chromatin, which together form extracellular fibres (Brinkmann *et al.*,

2004). However, the NET formation can not only be triggered by microorganisms, but also by various other stimuli, including cytokines, autoantibodies, nitric oxide, platelets and monosodium urate crystals (Kaplan & Radic, 2012). Therefore, the NSM2 inhibitors used in this study could trigger NETosis. As the NET formation represents a chain reaction, which is not necessarily dependent on the concentration, it could explain the differing cytotoxic effect. This hypothesis can be proven by microscopy or the measurement of the extracellular DNA release.

The uninfected samples analysed after 24 h revealed a death rate of 80 to 95%, demonstrating that the short life span of PMNs is not dependent on the NSM2. The analysis of the infected samples after 24 h clearly showed the inhibition of the apoptosis due to gonococcal infection. However, the decrease in Annexin positive cells is smaller compared to results seen in (Figure 3.27 A). The reason for this, might be the decreased MOI. While the first experiments were conducted using a MOI of 100, this experiment was performed using only a MOI of 50. The sample treated with the GW4869 showed the inhibition of the apoptosis, indicating that a functional NSM2 might not be necessary for this process. In contrast, the infected neutrophils treated with Eso48 showed high mortality after 24 h. There are two possible explanations for this effect. On the one hand, the inhibition of the apoptosis could be prevented by the NSM2 inhibition. This hypothesis is contradictory to the results obtained with the other NSM2-specific inhibitor GW4869. On the other hand, the neutrophils could be dying, because of the cytotoxic effect of Eso48. Therefore, it is not possible to come to a conclusion concerning the involvement of NSM2 in PMN apoptosis inhibition due to gonococcal infection.

4.10 Conclusion and outlook

In the present study, I aimed to define the impact of NSM2 and investigate the role of other members of the sphingolipid pathway on PorB$_{IA}$-mediated invasion. The involvement of the NSM2 could be confirmed by inhibitor experiments. Unfortunately, the generated antibodies directed against NSM2 do not represent a suitable tool for the detailed investigation of the enzyme. In contrast, a NSM2 knockout cell line produced by the CRISPR/Cas9 system is of great value. The knockout cell lines generated and tested in this study revealed a great impact of the NSM2 on phosphate sensitive invasion. However, the results were

inconsistent. Therefore, I would propose the generation of a new knockout cell line by the CRISPR/Cas9 system, applying different guide RNAs. The knockout cells could then be complemented using both the functional enzyme and enzymatic inactive variants of the NSM2 to further elucidate the role of the enzyme in gonococcal invasion. Furthermore, the changes in the lipidome composition of the knockout as well as in the complemented cells should be analyzed. Even though, the involvement of the enzyme could be confirmed, it is still unknown how the enzyme is activated in the context of infection. This question should also be addressed in the future.

The lipidome analysis conducted in this study revealed an increased trend in the levels of ceramide, sphingosine and sphingosine-1-phopshate, indicating the involvement of the whole sphingolipid pathway in infection. The increase in ceramide could be the result of the activation of the NSM2. The visualization of the ceramide release due to gonococcal infection pointed to the incorporation of exogenous ceramide by the gonococci. This incorporation was investigated using dSTORM and a ceramide-specific anti-C16/24 IgG antibody. In the future, these results should be confirmed using click-chemistry (Collenburg $et~al.$, 2016). Clickable ceramide analogues ω-azido-C_6-ceramide and ω-azido-C_{16}-ceramide could be used to verify the incorporation and even trace the association during the infection of human cells. In this study, I could show the toxic effect of sphingosine on gonococci. Since the lipidome analysis revealed an increase of sphingosine due to infection in human cells, it would be interesting to investigate the consequences of high intracellular sphingosine levels for the engulfed bacteria.

In the next part of my work, I could show the involvement of another key enzyme of the sphingolipid pathway, the sphingosine kinase 2, in $PorB_{IA}$-mediated invasion, using inhibitor as well as siRNA knockdown studies. Since the subcellular localisation of the SPHK2 is not restricted to the cytosol, and the activation of the SPHK1 is dependent on the translocation to the plasma membrane, it would be interesting to investigate if the activation of the SPHK2 due to infection is also connected to translocation. Since antibodies directed against SPHKs and tested in this study proved to be insufficient to recognize endogenous SPHK, overexpression constructs should be used to perform experiments. The SPHK activity assay was established, but revealed no results for Chang cells. Therefore, the assay should be performed using the mentioned overexpression constructs or different cell lines, which

possess a higher amount of SPHK. Additionally, a SPHK2 knockout cell line using the CRISPR/Cas9 system should be generated to further investigate the role of the enzyme in infection. The production of knockout cell lines can be recommended for all enzymes involved in the sphingolipid pathway including ceramidases, which have not been addressed so far.

Moreover, the S1PL plays a role in the uptake of gonococci in both the phosphate sensitive as well as the Opa-triggered invasion. Although, the inhibitor studies performed using Chang cells revealed contradictory results, since the PorB$_{IA}$-mediated invasion was increased whereas the Opa-HSPG triggered invasion was decreased, the knockout HeLa cell line showed the same results for both pathways. Therefore, it would be useful to generate a knockout cell line in Chang cells to test the inhibitor and the knockout in the same genetic background. The inhibition and knockout of the S1P lyase influenced the Opa$_{50}$-mediated invasion in a negative way. Furthermore, this pathway is dependent on the activation of the ASM and the release of ceramide (Grassme *et al.*, 1997). Hence, the involvement of additional members of the sphingolipid pathway like the SPHKs in this invasion mechanisms is possible and should be investigated.

The signalling experiments conducted in this work allow the placement of the NSM2 activation and the following signalling cascade of the sphingolipid pathway in the context of the gonococcal invasion. The NSM2 as well as the SPHK2 and the S1PL seem to play an important role in the early stages of the signalling events leading to the engulfment of the bacteria in the phosphate sensitive invasion, since those events take place before the activation of PI3K.

The external addition of S1P should give a first hint in order to answer the question whether the molecule acts as a para/autocrine or as an intracellular signalling molecule in PorB$_{IA}$-mediated invasion. This question could not be answered completely. The results pointed to S1P as an intracellular signaling molecule, since the addition did not change the invasion in a significant manner. Additionally, the effect of the SPHK2-specific inhibitor K145 could not be rescued by the S1P addition. More experiments should be conducted to exclude the para- and autocrine signalling mechanism. For example, the export of S1P could be prevented or the different receptors of the molecule could be blocked by antagonists preventing the binding of S1P and thereby locking off the downstream signalling.

Furthermore, the role of NSM2 was investigated in the inhibition of apoptosis in neutrophils due to gonococcal infection. In this work, I could confirm the inhibitory effect described in the literature and could show its independency of neisserial surface structures like porin and Opa proteins. The connection of the delayed onset of apoptosis and the functional NSM2 could not clearly be shown due to the cytotoxic effect of the NSM2 inhibitors. Unfortunately, the question which neisserial factor is responsible for the inhibition of the apoptosis in PMNs has to remain elusive, since the experiments conducted with peptidoglycan mutants revealed no changes.

Altogether, I could show that the sphingolipid pathway is involved in the PorB$_{IA}$-mediated infection of *Neisseria* in human cells. However, this is just the beginning of a very interesting and complex research topic, since many details concerning for example activation and the connection to the signalling cascade leading to the engulfment of the bacteria remain elusive.

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6 APPENDIX

6.1 Abbrevations

μ	micro
APL	anionic phospholipids
APS	ammonium persulfate
ASM	acid sphingomyelinase
ATP	adenosine triphosphate
ATP	adenosine triphosphate
B. burgdorferi	*Borrelia burgdorferi*
BSA	bovine serum albumin
bSMase	bacterial sphingomyelinase
Cav1	caveolin-1
cDNA	complementary DNA
CEACAM	human carcinoembryonic antigen-related cell adhesion molecules
cfu	Colony forming unit
cm	centimeter
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
ddH$_2$O	double-distilled water
DGI	disseminated gonococcal infection
dH$_2$O	distilled H$_2$O
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxynucleosid triphosphate
***d*STORM**	Direct Stochastic Optical Reconstruction Microscopy
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EED	Embryonic ectodermal development
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulared kinase
EtOH	ethanol
FACS	fluorescence-activated cell scanning
FCS	fetal calf serum
Fig.	figure

GC	gonococcus
GFP	green fluorescent protein
Gp96	glycoprotein 96
GPCR	G protein coupled receptors
gt	goat
GTP	guanosine triphosphate
h	hour(s)
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSPG	heparan sulphate proteoglycans
IB	immunoblotting
IF	immunofluorescence
Ig	immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	kilobase
kDa	kilodalton
KO	knockout
KO	knockout
l	liter
L. pneumophila	*Legionella pneumophila*
LAMP1	Lysosomal-associated membrane protein 1
LDL	low density lipoprotein
LOS	lipooligosaccharide
LPDI	low phosphate-dependent invasion
LPS	lipopolisaccharides
LtgA	lytic transglycosylase A
LtgD	lytic transglycosylase D
M	molar
MEFs	mouse embryonic fibroblasts
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
ms	mouse
n	nano
N. gonorrhoeae	*Neisseria gonorrhoeae*
NAATs	nucleic acid amplification tests
NCBI	National Center for Biotechnology Information
NETs	neutrophil extracellular traps
Ngo	*Neisseria gonorrhoeae*
NSM1	neutral sphingomyelinase 1
NSM2	neutral sphingomyelinase 2

NSM3	neutral sphingomyelinase 3
N-terminal	amino-terminal
OD$_{550}$	optical density measured at a wavelength of 550 nm
Opa	opacity-associated
p	pico
P$^-$	non-piliated phenotype
P. aeruginosa	*Pseudomonas aeruginosa*
P$^+$	piliated phenotype
PAGE	polyacrylamide gel electrophoresis
pAKT	phosphorylated AKT
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PilC, D, E, F	pilus-associated proteins C, D, E, F
PKC	protein kinase C
PKD1	PKCμ, protein kinase C μ
PLCγ1	phospholipase Cγ 1
PMN	polymorphonuclear leukocyte
PorB$_{IA}$	PorB subtype A
PorB$_{IB}$	PorB subtype B
PPM	proteose peptone medium
PPM$^+$	PPM medium supplemented with vitamin mix and NaHCO3
PS	phosphatidylserine
PVDF	polyvinylidene-fluoride
qRT-PCR	quantitative real-time PCR
Rac1	ras-related C3 botulinum toxin substrate
rb	rabbit
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
rpm	revolutions per minute
RT	room temperature
S. aureus	*Staphylococcus aureus*
S1P	sphingosine-1-phosphate
S1PL	sphingosine-1-phosphate lyase
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	secondes
shRNA	short hairpin RNA
siRNA	small interfering RNA

SM	sphingomyelin
SMases	sphingomyelinases
SPHK	sphingosine kinase
SPHK1	sphingosine kinase 1
SPHK2	sphingosine kinase 2
SREC-I	scavenger receptor expressed on endothelial cells 1
STI	Sexually transmitted infection
T4P	Type IV pili
TbpA	transferrin binding protein A
TBS	Tris buffered saline
TEMED	tetramethylethylenediamine
TLC	thin-layer chromatography
TLRs	toll-like receptors
TNFα	tumor necrosis factor-α
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
v/v	volume per volume
VRP	viral replicon particles
w/v	weight per volume
WHO	World Health Organization

6.2 Publications and presentations

Publication

Faulstich, M., **Hagen, F.**, Avota, E., Kozjak-Pavlovic, V., Winkler, A.C., Xian, Y., Schneider-Schaulies, S., and Rudel, T. (2015). Neutral sphingomyelinase 2 is a key factor for PorB-dependent invasion of Neisseria gonorrhoeae. Cellular microbiology *17*, 241-253.

Poster presentations

Hagen, F., Kozjak-Pavlovic, V., Rudel, T., (2015). Sphingolipids in gonococcal infection. SphingoFOR2123 workshop Sphingolipids in infection control and beyond, Würzburg.

Hagen, F., Weigand, M., Japtok, L., Kozjak-Pavlovic, V., Rudel, T., (2016). The role of neutral sphingomyelinases in gonococcal low phosphate-dependent invasion. International Pathogenic Neisseria Conference (IPNC) 2016, Manchester, UK.

Hagen, F., Weigand, M., Japtok, L., Kozjak-Pavlovic, V., Rudel, T., (2016). Sphingolipids in gonococcal infection. Eureka! 2016 International Symposium organized by the Students of the Graduate School of Life Sciences, Würzburg.

6.3 Danksagung

6.4 Curriculum Vitae

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01/2014 – 03/2017 **Graduation** in the Department of Microbiology, Biozentrum, Universität Würzburg

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10/2007 – 03/2011 **Bachelor of Science in biology** in the Department of Zoology and Evolutionary Biology, Universität Regensburg

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Supervisor: Prof. Dr. Jürgen Heinze

6.5 Declaration of independence

Affidavit

I hereby confirm that my thesis entitled "Sphingolipids in gonococcal infection" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Sphingolipide in der Gonokokkeninfektion" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, den