Interaction of *Streptococcus suis* with neutrophil extracellular traps (NETs)

**THESIS**
Submitted in partial fulfilment of the requirements for the degree

**DOCTOR OF PHILOSOPHY**
(PhD)

awarded by the University of Veterinary Medicine Hannover

by

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Hannover

Hannover, Germany 2015
“What we know is a drop, what we don’t know is an ocean.”

Isaac Newton

Meinen Eltern, Eugen und Oliver
Parts of the thesis have already been published previously at scientific meetings, conferences or journals:

**Oral presentations**


*de Buhr, N.*, von Köckritz-Blickwede, M. and Baums, C. G. “Interaction of *Streptococcus suis* with neutrophil extracellular traps”, Seminar on Infection Biology, Centre for Infection Medicine, University of Veterinary Medicine Hannover, Hannover 2015


**Poster presentations**


Publications [see Chapter 3]


Publications (in preparation)


Sponsorship:

This work was funded by a fellowship of the Ministry of Science and Culture of Lower Saxony (Georg-Christoph-Lichtenberg Scholarship) within the framework of the PhD program ‘EWI-Zoonosen’ of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology and Translational Medicine (HGNI). Further this project was financially supported by the Niedersachsen-Research Network on Neuroinfectiology (N-RENNT) of the Ministry of Science and Culture of Lower Saxony.
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List of Abbreviations

%  percentage
µg  microgram
²H₂O  deuterium oxide
aggNETs  aggregated NETs
AMP  antimicrobial peptide
BBB  blood-brain-barrier
BCSF  blood-cerebrospinal fluid-barrier
BLMB  brain-leptomeningeal-barrier
BMEC  brain microvascular endothelial cells
C.f.u.  colony forming units
C3b  complement component 3b
CD  cluster of differentiation
CNS  central nervous system
CPEC  choroid plexus epithelial cells
CR  complement receptor
CSF  Cerebrospinal fluid
CWS  cell wall sorting signal
DAPI  4’,6-diamidino-2-phenylindole
DltA  D-alanine-poly (phosphoribitol) ligase subunit 1
DNA  deoxyribonucleic acid
*E. coli*  *Escherichia coli*
e.g.  latein: exempli gratia (for example)
EF  extracellular factor of *S. suis*
EndA  endonuclease A of *S. pneumoniae*
EndAsuis  endonuclease A of *S. suis*
ERK  extracellular signal-regulated kinase
*et al.*  latein: *et alii*
FBPS  fibronectin and fibrinogen binding protein of *S. suis*
Fc  fragment crystallisable
GAS  Group A Streptococcus
h  Hour
*H. influenzae* B  *Haemophilus influenzae* B
H₂O₂  hydrogen peroxide
hBD  human β-defensin
HIBC  human choroid plexus papilloma
IdeS  immunoglobulin degrading enzyme
IdeSsuis  immunoglobulin M degrading enzyme of *S. suis*
IFN-γ  interferon gamma
Ig  immunoglobulin
IL  Interleukin
IUPAC  International Union of Pure and Applied Chemistry
L  Liter
*L. monocytogenes*  *Listeria monocytogenes*
LIF  leukemia inhibitory factor
LPG  Lipopolysaccharide
LPS  Lipopolysaccharide
MAPK  mitogen activated protein kinase
MCP-1  monocyte chemotactic protein-1
MEK  mitogen-activated protein kinase
MGP  marginal granulocyte pool
min  Minute
MI  Milliliter
MMP 9  metalloproteinase 9
MPO  Myeloperoxidase
MRP  muramidase-released protein
*N. meningitidis*  *Neisseria meningitidis*
n.s.  non significant
NADPH  nicotinamide adenine dinucleotide phosphate
NE  neutrophil elastase
NETs  neutrophil extracellular traps
NucB  nuclease from *Bacillus licheniformis*
OFS  opacity factor of *S. suis*
PAD4  protein arginine deiminase
Pg  Pictogram
PgdA  Peptidoglycan N-deacetylase of *S. suis*
ph  power of hydrogen
PKC  protein kinase C
PMA  phorbol-12-myristate-13-acetate
PMN  polymorphonuclear leukocytes
ROS  reactive oxygen species
RPMI  Roswell Park Memorial Institute
*S. pneumoniae*  *Streptococcus pneumoniae*
*S. pyogenes*  *Streptococcus pyogenes*
*S. suis*  *Streptococcus suis*
SAO  surface antigen one
SD  standard deviation
SEM  standard error of the mean
SLY  Suilysin of *S. suis*
SLE  systemic lupus erythematosus
SpyCEP  *S. pyogenes* cell-envelope protease
SsnA  secreted nuclease A of *S. suis*
*Staph. aureus*  *Staphylococcus aureus*
STs  sequence types
SVV  systemic vasculitis
T  Time
TEER  transepithelial electrical resistance
TJ  tight junction
TLR  toll like receptor
TNFα  tumor necrosis factor alpha
WT  wildtype
ZO  zonula occludens
Δ  Delta
1 General Introduction

*Streptococcus* (*S.* *suis*) is one of the most important pathogens in pigs and an emerging zoonotic agent, causing meningitis and other pathologies. The pathogenesis of *S. suis* meningitis and the reaction of the innate immune system is poorly understood. Nevertheless, infiltrations with high numbers of neutrophils are typical for lesions induced by *S. suis* infection [Fig. 1-1, (Beineke *et al.*, 2008)]. It was demonstrated by *in vitro* experiments with cell culture models of the blood-cerebrospinal fluid (CSF)-barrier that *S. suis* is able to cross this barrier and that neutrophil granulocytes follow *S. suis* in the “CSF compartment” (Steinmann *et al.*, 2013). A recently identified defense mechanism of the innate immune system against different pathogens is the formation of neutrophil extracellular traps (NETs). NETs are formed by the release of a decondensed chromatin from neutrophils. Histones, antimicrobial peptides and granule proteins are bound to these web-like structures leading to the killing pathogens entrapped in these NETs [Fig. 1-2, (Brinkmann, 2004)]. Interestingly, some pathogens are described to possess DNases as a defense mechanism against NET entrapment and numerous DNases have been identified in streptococci (Molloy, 2006). Until now, the role of NETs during *S. suis* infection and in the CSF compartment has not been studied so far.

This project aimed to investigate the interaction of *S. suis* and NETs in general and the function of NETs in the CSF compartment.

**Figure 1-1** Histological finding in the brain of a intranasally with *S. suis* serotype 2 strain 10 infected piglet: Severe diffuse suppurative meningitis. Bar = 10 µm. Reprinted from (Beineke *et al.*, 2008) with permission from Elsevier.

**Figure 1-2** Neutrophil extracellular traps (NETs) trapping *S. suis*. Visualization is done with immunofluorescence microscopy. DNA = Hoechst (blue), NETs = Alexa Fluor® 488 (green), *S. suis* = Alexa Fluor® 633 (red)
1.1 **Streptococcus suis**

*S. suis* is an important porcine pathogen that belongs to the family of Streptococcaceae. It is characterized as a Gram-positive coccus, growing on sheep blood agar with an alpha hemolysis. Depending on the differences of the capsule-antigens up to date 35 serotypes are described (Gottschalk, 2012; Goyette-Desjardins et al., 2014) and of these serotypes 2 and 9 are the most important in Europe (Wisselink, 2000). The natural host is the pig and an acute course of a *S. suis* infection is associated with different clinical signs like lameness, fever, central nervous system (CNS) dysfunctions or dyspnoea (Straw et al., 2006). Depending on the possible different localizations of *S. suis*, the pathological findings of diseased piglets can vary between meningitis, arthritis, endocarditis or pneumonia (Clifton-Hadley & Alexander, 1980; Staats et al., 1997). Furthermore, *S. suis* is a commensal on the mucosa in the upper respiratory tract, the genital tract and the intestine (Higgins et al., 1990; Robertson & Blackmore, 1989; Swildens et al., 2004). Often different *S. suis* genotypes are found on tonsils of healthy piglets (Arends et al., 1984; Baums et al., 2007; Clifton-Hadley & Alexander, 1980). Carrier pigs without clinical signs play an important role as infection source. The upper respiratory tract and in particular the tonsils are considered to be the main entry site for *S. suis* in pigs (Williams et al., 1973). Horizontal (oronasal) but also vertical transmission (perinatal infection) of *S. suis* is common (Amass et al., 1997; Berthelot-Hérault et al., 2001; Robertson & Blackmore, 1989; Staats et al., 1997). *S. suis* is also an important zoonotic agent. The risk for a *S. suis* infection in humans is increased for people with close contact to pigs, like farmers, butchers or veterinarians (Arends & Zanen, 1988; Lun et al., 2007). Notably, more than 96 % of cases in humans are meningitis, septicemia or septic shock (Lun et al., 2007). Streptococcal toxic shock-like syndrome caused by *S. suis* infection was observed for the first time in two outbreaks in humans in the Jiangsu Province, China (1998) and the Sichuan Province, China (2005) (Lun et al., 2007; Tang et al., 2006; Yu et al., 2006). The clinical course is reminiscent to the streptococcal toxic shock-syndrome caused by infection with group A streptococci (Todd et al., 1978). Both *S. suis* outbreaks in China followed local disease outbreaks in pigs (Yu et al., 2006), which underlines the zoonotic potential. In a review about pig-borne infections, *S. suis* is considered a zoonotic pathogen with a high risk for transmission from pigs to humans. This assessment was based on disease burden, the host specificity of the pathogen and the mortality rate in humans. In comparison to other bacteria the zoonotic significance of *S. suis* was estimated to be the highest (Pappas, 2013). With over 90 % of all worldwide reported clinical *S. suis* infections in humans, Asia is most affected. Different authors describe *S. suis* as an emerging zoonotic agent in Asia (Gottschalk et al., 2010; Goyette-Desjardins et al., 2014; Lun et al., 2007). Interestingly, in 2014 a new research project pointed out that an infection route of *S. suis* over the gastro-intestinal tract is possible. The authors concluded that *S. suis* should be considered as a food borne pathogen (Ferrando et al., 2015).
Numerous virulence factors and virulence associated factors of *S. suis* have been investigated in recent years (Baums & Valentin-Weigand, 2009; Fittipaldi et al., 2012). In the following part only a short overview about some important factors that mediate host-pathogen interaction is explained.

The polysaccharide capsule is an important virulence factor of *S. suis*, as demonstrated in animal experiments. The capsule protects *S. suis* against phagocytosis (Charland et al., 1998; Smith et al., 1999) and it is discussed that the capsule is involved in escaping out of neutrophil extracellular traps (NETs) (Zhao et al., 2015). Though unencapsulated mutants were avirulent in experimental infections, recent data indicate that unencapsulated *S. suis* strains lead more often to endocarditis (Lakkitjaroen et al., 2011). Indications are given that the capsule of *S. suis* is not enough for full virulence (Vecht et al., 1991b) and in addition is only slightly immunogenic (Baums & Valentin-Weigand, 2009; Baums et al., 2009; Martin del Campo Sepúlveda et al., 1996; Wisselink et al., 2001).

Adherence of bacteria to host tissues is beneficial for colonization and infection. Fibronectin-binding proteins have been identified in different Gram-positive cocci as virulence factors involved in host-pathogen interaction (Schwarz-Linek et al., 2006). A fibronectin (FN) - and fibrinogen (FGN)-binding protein of *S. suis* (FBPS) was identified as a gene upregulated upon iron-restricted conditions *in vitro* and by experimental infection of piglets (Smith et al., 2001). FBPS is needed for infection of different inner organs, but not for colonization of the tonsils (de Greeff, 2002). Further, it is a surface-associated protein lacking a cell wall sorting signal (CWS) including an LPXTG-motif.

Similar to other streptococci *S. suis* expresses numerous surface proteins containing a LPXTG-motif. Some of these are likely involved in adhesion. As an example, SSU1889 is a protein with a CWS and a proposed function as adhesin and invasin to porcine brain microvascular endothelial cells (BMEC) (Vanier et al., 2009a). Muramidase-released protein (MRP) (Smith et al., 1992; Vecht et al., 1989, 1991a) and surface antigen one (SAO) (Li et al., 2006) are immunogenic proteins of *S. suis* with a CWS following repetitive sequences. The functions of MRP and SAO are not known. The CWS containing opacity factor of *S. suis* (OFS) is homologous to the serum opacity factor of *Streptococcus (S.) pyogenes*. OFS was demonstrated to be crucial for virulence of serotype 2 in a porcine infection model (Baums et al., 2006). Overall a total of thirty-three putative cell wall-anchored proteins with a LPXTG-motif where identified, but their functions in pathogenesis are not very well understood (Chen et al., 2007; Wang et al., 2009a).

Together with MRP the extracellular factor (EF, gene: *epf*), a secreted protein, was identified as a virulence-associated protein (Vecht et al., 1991a). However, *mrp*- and *epf*- deletion mutants were not attenuated in virulence in experimental infections of piglets (Smith et al., 1996). Both factors are used as virulence markers in various diagnostic laboratories in Europe, as in Europe *S. suis mrp+ epf+* serotype 2 strains are virulent in contrast to *mrp*- *epf*- serotype 2 isolates (Smith et al., 1996; Vecht et al., 1991b).

Many virulent *S. suis* strains secrete a pore-forming cholesterol-dependent cytotoxin named suilysin (SLY) (Jacobs et al., 1994). SLY is cytotoxic active against macrophages (Segura & Gottschalk, 1994).
2002), human BMECs (Charland et al., 2000), porcine BMECs (Vanier et al., 2004) and different epithelial cells like laryngeal epithelial cells (Norton et al., 1999). However, SLY is not crucial for full virulence of S. suis serotype 2 strains in piglets as a sly mutant was only slightly attenuated in systemic infection of piglets (Allen et al., 2001; Lun et al., 2003).

Three further putative virulence factors of S. suis are involved in the innate immunity escape. Peptidoglycan N-deacetylase (PgdA) is involved in the resistance to phagocytosis and the gene is highly upregulated after incubation with porcine neutrophils. The presence of PgdA leads to modifications of the cell wall peptidoglycan and in infection experiments with pigs a pgdA mutant was attenuated (Fittipaldi et al., 2008a). Further D-alanine-poly (phosphoribitol) ligase subunit 1 (DltA) protects S. suis against antimicrobial peptides and killing by porcine neutrophils by D-alanylation of lipoteichoic acid (Fittipaldi et al., 2008b). The detailed function is described in Chapter 1.3.3. A dltA mutant was attenuated in experimental infections in pigs and mice.

In addition to these two factors the serine protease (SpA) degrades interleukin 8 (IL-8) and therefore the recruitment of neutrophils is affected (Bonifait & Grenier, 2011; Fittipaldi et al., 2012; Vanier et al., 2009b).

Furthermore, factors of S. suis can cleave immunoglobulins (Ig). IgA1 protease of S. suis cleaves human IgA1 and is described as a virulence factor of S. suis (Zhang et al., 2010, 2011). In 2013 Seele et al. identified a novel host-specific immunoglobulin M-degrading enzyme of S. suis (IdeSsuis). This protease is a member of the IdeS family as it is homologous to IdeS of S. pyogenes. In contrast to the other members of this family, IdeSsuis does not cleave IgG but only IgM. Interestingly, IgM of various other species but pigs is not cleaved by IdeSsuis (Seele et al., 2013). Immunization of weaning piglets with recombinant IdeSsuis protected them efficient against an infection with S. suis serotype 2 (Seele et al., 2015).

Nevertheless, until now all of the known and characterized virulence factors are not protecting against a S. suis infection, when they were used for production of a cross protective vaccine. One reason could be that the pathogenesis represents a complex process between different pathogen-host-interactions and more than one factor leads to virulence.

1.2 S. suis meningitis

A tissue layer called meninges surrounds the brain and the spinal cord. These can be infected by some highly invasive pathogens, which lead to meningitis. In addition, meningitis can result from various non-infectious causes like cancer (Chamberlain, 2010; Van Horn & Chamberlain, 2012) or toxic chemicals (Moris & Garcia-Monco, 1999). Viruses are the most common causes of infectious meningitis, but bacterial meningitis is generally associated with a more severe, very often life-threatening clinical course. Important bacterial meningitis agents in humans are Neisseria (N.) meningitides, Streptococcus (S.) pneumoniae, Listeria (L.) monocytogenes, Haemophilus (H.) influenzae B, Group B streptococci and Escherichia (E.) coli K1 (Hacker & Heesemann, 2000).
Bacteria causing haematogenous meningitis must be able (1) to survive in the bloodstream and to escape from host defense mechanisms and (2) are thought to reach a high level of bacteremia prior to breaching the blood brain barrier (BBB) or blood cerebrospinal fluid barrier (BCSFB) (Kim, 2003). Accordingly virulent S. suis strains are able to survive in the bloodstream and reach high bacterial concentrations (Fittipaldi et al., 2012; Gottschalk, 2012).

In pigs as well as in humans the most important clinical signs associated with a S. suis infection are CNS disorders due to meningitis. Important clinical signs for bacterial meningitis in pigs are tremor, paddling movements, convulsions, opisthotonus, tetanic contractions, ataxia and nystagmus (Straw et al., 2006). In diseased humans, early unspecific symptoms include fever, vomiting, feeling unwell and headache. Subsequently, specific meningitis symptoms such as stiff neck, dislike of bright lights, confusion and seizures may develop (van de Beek et al., 2004). In humans the S. suis meningitis could be purulent or non-purulent and one late effect is deafness (Lütticken et al., 1986).

S. suis serotype 2 is one of the most common causes of meningitis in pigs. Animals at an age from 2 to 22 weeks might be affected (Madsen et al., 2002b; Windsor & Elliott, 1975).

Spreading of S. suis is suggested to be lymphogenously and haematogenously through palatine and nasopharyngeal tonsils and mandibular lymph nodes (Madsen et al., 2002a). After breaching the mucosal barriers S. suis is entering the blood stream by crossing the epithelial cell layer (Fittipaldi et al., 2012). S. suis may enter the CNS as free circulating bacteria or in association with monocytes (Gottschalk & Segura, 2000). The “Trojan horse” theory postulates that S. suis breaches the BBB or BCSFB inside monocytes (Williams, 1990; Williams & Blakemore, 1990). Noteworthy, a limited number of immune cells overcome the blood brain barriers even in healthy individuals. Based on the “modified” Trojan horse theory S. suis adheres to monocytes as these cells enter the CNS (Gottschalk & Segura, 2000).

For infection of the brain one of the three blood brain barriers (in detail described in chapter 1.5.1) must be crossed. S. suis serotype 2 invades porcine BMECs, which form an important part of the BBB (Vanier et al., 2004). SLY-positive strains were toxic for the porcine BMECs at high bacterial doses. Furthermore intracellular viable streptococci were detectable 7 h after an antibiotic treatment. The second barrier in the brain is the BCSFB, which is formed by the choroid plexus epithelia cells (CPECs) and a fenestrated endothelium. Supernatants of interferon gamma (IFN-γ) stimulated primary porcine CPECs are able to inhibit growth of S. suis. Accordingly, an active defense role of the choroid plexus against bacterial meningitis was hypothesized (Adam et al., 2004). However, S. suis may efficiently invade CPECs and translocate through this barrier as indicated by in vitro results using an inverted transwell filter system with primary porcine CPECs (Tenenbaum et al., 2009). Similar observations were made using a human BCSFB model (Schwerk et al., 2012). These findings indicate that the BCSFB is an important entry gate for S. suis.

After infection of the meninges, the disease may progresses rapidly. Without treatment many diseased animals must be euthanized or die. Typical pathological findings are congestion, edema and/or
purulent exudate in the meninges. A *S. suis* meningitis is typically characterized by a high influx of neutrophil granulocytes (neutrophilic meningitis [Figure 1-1]), a choroiditis and hyperaemic meningeal blood vessels (Reams *et al.*, 1994, 1996). After a disruption of the plexus brush border, deposition of fibrin and infiltration with inflammatory cells occurs in the CSF (Sanford, 1987; Staats *et al.*, 1997).

The high number of infiltrating neutrophils is a general feature described for bacterial meningitis demonstrated in a clinical study with bacterial meningitis. Pathogens detected in this study were for example *N. meningitides* or *H. influenzae* (Straussberg *et al.*, 2003). It was demonstrated in an *in vivo* infection of mice with *S. pneumoniae* that 12 h after infection neutrophils are recruited to the brain to control bacterial infection. The recruited neutrophils inhibit the growth of the streptococci in meningitis (Mildner *et al.*, 2008).

### 1.3 Neutrophil granulocytes

The immune system is divided in the innate and the adaptive part that are working individually and synergistically. Both systems include components of the humoral immunity and the cell-mediated immunity. Parts of the innate immune system are: the complement system, acute-phase-proteins, granulocytes, monocytes, macrophages, dendritic cells and natural killer cells. Neutrophil granulocytes are one of the main players in the regulation of the innate host defense. As all blood cells, they are originated from pluripotent hematopoietic stem cells from the bone marrow. They are differentiated white blood cells similar as eosinophil or basophil granulocytes. Around 90% of the granulocyte population is neutrophils. The nucleus of neutrophil granulocytes can vary in the shapes from lobed into segmented leading to the designation polymorphnuclear leukocytes (PMN) (von Engelhardt, 2015; Janeway *et al.*, 2009). The granulocytes are described as short living cells with a 6-8 h circulating half-life (Summers *et al.*, 2010). Based on a study with human neutrophils labeled with $^2$H$_2$O a lifespan of over 5 days was postulated (Pillay *et al.*, 2010), but this was called into question by other researchers (Tofts *et al.*, 2011). In mammals around one billion granulocytes per liter blood are produced on one day. After maturation (granulopoiesis) granulocytes are stored extravascularly or attached to the endothelium of small blood vessels (marginal granulocyte pool = MGP). Under stress granulocytes are released rapidly out of MGP (e.g. after release of adrenaline). It takes approximately 20 min after infection to release granulocytes out of the MGP. In addition, within a day granulocytes are released out of the extravascular pool. Upon infection the number of granulocytes in the blood is increased by release of immature granulocytes (von Engelhardt, 2015; Kolaczkowska & Kubes, 2013). As a response to invaded microorganisms, cytokines like tumor necrosis factor α (TNFα) or IL-8 are secreted by tissue macrophages and mast cells. TNFα activates endothelial cells leading to the extravasation of neutrophils, which is initiated by binding to receptors on the endothelium. IL-8 works as a chemoattractant and induces migration of neutrophils from the blood to the tissue. The
transmigration through an endothelium is named diapedesis. This process leads to the activation of PMNs including altered expression of surface antigens (Janeway et al., 2009). ICAM1, ICAM2 and PECAM1 are important molecules of the endothelium of postcapillary venules involved in recruitment of neutrophils. But for other tissues such as the brain the adhesion molecules on endothelium and neutrophils are unknown or only speculated on (Kolaczkowska & Kubes, 2013).

As part of the first immune defense, neutrophil granulocytes can send signals to other cells of the innate immune system by releasing for example TNFα or chemokines leading to activation and regulation of innate and adaptive immunity (Mantovani et al., 2011). Additionally, neutrophils can exhibit different antimicrobial mechanisms for elimination of pathogens [Figure 1-3]. The first mechanism is phagocytosis, described by Paul Ehrlich in 1880, which is characterized by an inclusion of microorganisms in phagosomes. Intracellular lysosomes fuse with the phagosomes to phagolysosomes. And afterwards inside the phagolysosome microorganisms are killed through acidification, reactive oxygen species (ROS) and antibacterial proteins like lysozyme (Nathan, 2006; Rada & Leto, 2008). For an effective phagocytosis the process of opsonization marks the microbes. Therefore antibodies or components of the complement system (e.g. C3b) bound on the microbe surface. This marking is recognized by fragment crystallisable (Fc)-receptors or complement receptor 1 (CR 1) of immune effector cells like neutrophils or monocytes. Sometimes neutrophils can bind directly on specific surface antigens of bacteria e.g. to lipopolysaccharide (LPS). Pattern recognition receptors (PRRs) recognize special conserved pathogen associated molecular patterns (PAMPs). An important group are the toll-like receptors (TLR), for example TLR-9 recognizes unmethylated CpG-rich sequences or TLR-4 reacts on cell wall components of Gram-positive bacteria (Janeway et al., 2009, page 4-139). The second antimicrobial mechanism of PMNs is degranulation, which is characterized by a release of neutrophil granules outward the cell or into phagosomes inside the cell. The granules contain antimicrobial peptides (AMPs) and proteases e.g. myeloperoxidase (MPO), lactoferrin and gelatinase or metalloproteinase 9 (MMP 9). Additionally the production of cytokines mediate the inflammation (Borregaard, 2010; Kolaczkowska & Kubes, 2013). The third mechanism is the formation of neutrophil extracellular traps (NETs) which is described in detail in the next part [Chapter 1.3.1]. Further neutrophils are also involved in resolution of inflammation by apoptosis. Apoptosis is characterized as an active programmed cell death that can occur in all biological cells. To start apoptosis cysteine-dependent aspartate-specific proteases (caspases) are identified as a main trigger (Fadeel et al., 1998). The activation of caspases leads to a condensation of nucleus and cytoplasm, DNA fragmentation and externalization of membrane-associated phosphatidylserine. Apoptosis of PMNs prevents release of toxic neutrophil contents and is therefore considered to be part of inflammatory regulation. Surface markers on apoptotic cells lead to clearance by macrophages and other phagocytes and thus to resolution of inflammation. Taken together neutrophils have versatile functions in innate immunity.
1.3.1 Neutrophil extracellular traps

In 2004 a novel defense mechanism of neutrophils was described: the formation of NETs (Brinkmann, 2004). Upon induction of NET formation, neutrophils release decondensed chromatin as extracellular fibers. Antimicrobial granule proteins as well as histones are bound to those fibers. NETs entrap and kill microorganisms (Brinkmann, 2004). The importance of NETs in different host pathogen interactions has been explored for parasites (Abdallah & Denkers, 2012; Muñoz Caro et al., 2014), viruses (Narasaraju et al., 2011; Saitoh et al., 2012; Wardini et al., 2010), fungi (Guimarães-Costa et al., 2012; McCormick et al., 2010; Urban et al., 2006) and mainly for bacteria (reviewed by Lu et al., 2012). In chapter 1.3.2 the interaction of NETs and bacteria is reviewed in detail and in chapter 1.3.3 the NET evasion mechanisms of bacteria are described.

The release of NETs, also referred as NETosis (Steinberg & Grinstein, 2007), is classically described as a novel cell death of PMNs besides apoptosis and necrosis. (Fox et al., 2010; Hallett et al., 2008; Leitch et al., 2008; Mocsai, 2013). The differences between apoptosis, necrosis and NETs were demonstrated by Fuchs et al. in 2007 and revealed that the nuclei of neutrophils decondensate and the nuclear envelope disintegrates, allowing the mixing of granule and nuclei components that form NETs. Finally, the NETs are released as the cell membrane breaks (Fuchs et al., 2007). PMNs that are
activated by Phorbol-12-myristate-13-acetate (PMA) or *Staphylococcus (Staph.) aureus* or IL-8, undergo typical features of NETosis and release NETs after two to three hours. By live video microscopy the process from activation to NETosis was monitored (Fuchs et al., 2007). It was suggested that this mechanism of NET release by cell lysis is going on after a direct neutrophil activation by pathogens (Papayannopoulos & Zychlinsky, 2009).

Publications are increasing that describe the cellular mechanisms leading to NETosis. After the stimulation of receptors on the PMN surface, the PMNs stick flattened to the substrate and a cascade of reactions is started (Brinkmann & Zychlinsky, 2012). With PMA stimulation NETosis occurs through activation of protein kinase C (PKC) and NADPH-oxidase (or phagocytic oxidase = PHOX) leading to generation of ROS (Fuchs et al., 2007; Papayannopoulos et al., 2010). The signaling cascades involved in PKC and NADPH-oxidase activation include the raf–mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway (raf-MEK-ERK) (Hakkim et al., 2011) and the Rac-related C3 botulinum toxin substrate 2 (Rac2) (Lim et al., 2011). In a following step H$_2$O$_2$ might become a substrate for MPO, an enzyme localized in azurophilic granules. Moreover, neutrophil elastase (NE) is stored in these granules and both enzymes are mobilized. NE enters the nucleus after ROS production and degrades the linker 1 histone. This promotes chromatin decondensation. After binding of MPO to chromatin, the decondensation is initiated. The nuclear membrane dissolves and the contents of granules, the nucleus and the cytosol mix. As chromatin decondensation is completed, the cell ruptures and releases NETs into the extracellular space [Fig. 1-4] (Fuchs et al., 2007; Papayannopoulos et al., 2010). Moreover histone citrullination and chromatin decondensation by peptidylarginine deiminase 4 (PAD4) after TNFα treatment was reported (Wang et al., 2009b). This is an important step for the nuclear DNA release and at the end the PMN is dead. This previously described mechanism is oxidant-dependent. Nevertheless, in 2012 Parker et al. tested different stimuli in presence of inhibitors of oxidant generation (e.g. diphenyleneiodonium chloride = DPI). As stimuli they used PMA, the calcium ionophore ionomycin, *Staph. aureus, E. coli* and *Pseudomonas aeruginosa*. They were able to demonstrate that NET release after ionomycin incubation is also possible via an oxidant-independent way. A NET release after ionomycin incubation was possible in the absence of NADPH-oxidase.

Most publications describe NET release as a form of pathogen-induced active cell death, which gives PMNs the possibility to fight against microbes beyond their life span. Interestingly, recently NET formation was explained by three different mechanisms in the literature (Brinkmann, 2004; Fuchs et al., 2007; Pilsczek et al., 2010; Yousefi et al., 2009): 1. Classical NET release through cell lysis (NETosis) as described above, 2. NET release by viable cells mediated by vesicular mechanism [see Chapter 4.2] and 3. NET release by viable cells formed of mitochondrial DNA. Importantly, the ‘vital’ NETosis via vesicular release of nuclear DNA is faster and oxygen independent, but the detailed cellular mechanisms that lead to NET formation by viable cells or by release of mitochondrial DNA is still not entirely clear.
General Introduction

Receptor stimulation on neutrophils (A) leads to an adhesion of the neutrophil and the start of the raf-MEK-ERK pathway (B). Oxygen dependent granule components like NE and MPO becoming mobile and histones are in the nucleus processed (C). The cytosol and the granule content mixes and at the end the cell membrane ruptures and NETs are released (D).

However, the hallmark of NET release independent of the above-mentioned three mechanism is the release of DNA associated with antimicrobial compounds. These antimicrobial components are MPO, NE, cathelicidin LL-37, histones, proteinase 3, cathepsin, lactoferrin or gelatinase (Brinkmann, 2004; Papayannopoulos & Zychlinsky, 2009). With those compounds, NETs are able to bind, disarm and occasionally kill bacteria. Beside its antimicrobial effects, Schauer et al. (2014) described a further function of NETs in a study about gout. The authors showed that aggregated NETs (aggNETs) degrade cytokines and chemokines. Thus, aggNETs constitute an anti-inflammatory mechanism reducing the recruitment and activation of PMNs. These aggNET structures are formed in the presence of a high neutrophil density. NET structures were detected in human tissue sections of gout patients. Further, the gout associated monosodium urate crystals induce NETosis and aggNETs. The authors hypothesized that aggNETs are involved in spontaneous resolution of acute inflammation in patients with gout.

However, besides a protective effect in the host, recent publications also demonstrate a detrimental effect for the host when NETs are accumulating and not eliminated by the host. As an example, NETs are involved in pathologic processes with inflammation where cytotoxic molecules from PMNs or lysed PMNs are involved. Some studies demonstrated a damage of endothelium and tissue by NETs (Clark et al., 2007; Marin-Esteban et al., 2012). By Papayannopoulos and colleagues in 2011 it was suggested that NET formation and the release of NE promotes chromatin decondensation in sputum of patients with cystic fibrosis, a chronic lung infection and inflammation, by proteolytic processing of...
histones and is therefore maybe a factor for sputum viscosity and tissue damage. In different autoimmune diseases for example systemic lupus erythematosus (SLE) and systemic vasculitis (SVV) the role of NETs has been characterized (Garcia-Romo et al., 2011; Hakkim et al., 2010; Knight & Kaplan, 2012; Knight et al., 2012; Pieterse & van der Vlag, 2014). Interestingly, from 25 proteins identified in NET structures by proteomic analysis, 84 % are reported in the literature as autoantigens in autoimmune diseases, cancer, or other disorders. From these identified proteins, 74 % are described to be the target of autoantibodies in systemic autoimmune diseases. The most reports are from patients with SVV, SLE or rheumatoid arthritis. Because cell death was considered as the main source of autoantibodies, Darrah and Andrade hypothesized that NETs are a link between cell death and autoimmune diseases. (Darrah & Andrade, 2013). Accordingly, it was demonstrated that an impairment of NET degradation is associated with autoimmune lupus nephritis: Nuclease-deficient individuals that are not able to eliminate NETs, have more SLE, as they are unable to regulate the beneficial versus detrimental effects of NET formation (Hakkim et al., 2010).

Taken together the formation of NETs is an important part of the innate immune defense protecting the body against invading pathogens. On the other hand, an overproduction or dysregulation can contribute various pathologies. Therefore, a regulation of NET production and a balance between NET formation and degradation is needed.
1.3.2 NET mediated binding and killing of bacteria

A number of publications demonstrated an entrapment of bacteria by NET structures with immunofluorescence microscopy, but specific binding partners are not entirely clear. It was published that nanoparticles stick to NETs through charge interactions (Bartneck et al., 2010), but it seems that NET binding is not only due to charge (Urban et al., 2009). It was in addition discussed if a special staining technique for electron microscopic analyses opens a new door to investigate the role of bacterial fimbriae-mediated adhesion to NETs (Krautgartner & Vitkov, 2008). Nevertheless, for many bacteria trapping by NETs is described. As mentioned in detail above, the NET structures contain histones, granule proteases and AMPs which might exhibit antimicrobial effects on trapped pathogens. A total of 24 neutrophil proteins were identified to be associated with NETs, among those antimicrobial factors as histones, calprotectin, elastase or myeloperoxidase (Urban et al., 2009). The direct antimicrobial activity of histones in NETs was demonstrated in the first description of NETs (Brinkmann, 2004). Whereas some bacteria are able to escape and / or survive in the presence of NETs, others are killed (Table 1). The possibility for NET escape is independent from the Gram classification, for example staphylococci and Pseudomonas aeruginosa can survive in presence of NETs, whereas L. monocytogenes or Shigella flexneri are reduced in bacterial numbers in the presence of NETs.

NETs are described to have bactericidal activity or a bacteriostatic antimicrobial effect. In case of bactericidal substances, 99.9 % of the bacteria are killed compared to the inoculum (Noviello et al., 2003). Bacteriostatic antimicrobial substances inhibit the reproduction of bacteria that leads to a bacterial growth inhibition. It was discussed that NETs only lead to a bacteriostatic antimicrobial effect as a result of entrapment and partial killing within NETs (Baums & von Köckritz-Blickwede, 2015).
### Table 1 Bacterial interaction described with PMNs and/or NETs

<table>
<thead>
<tr>
<th>Gram-negative</th>
<th>Bacterial interaction with PMNs / NETs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>NET induction</td>
<td>NET entrapment</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>-</td>
<td>- (in presence of PMNs)</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Borrelia burgdorferi Sensu Stricto</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Burkholderia pseudomallei</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannheimia haemolytica</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neisseria meningitides</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
### General Introduction

<table>
<thead>
<tr>
<th>Gram-positive</th>
<th>Bacterial interaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>NET induction</strong></td>
<td><strong>NET entrapment</strong></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>+</td>
<td>only unencapsulated +</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Group A Streptococci (GAS)</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Group B Streptococci</em></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Acid-fast bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium canettii</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Lu et al. 2012; adapted and supplemented
1.3.3 NET evasion by pathogens

As some pathogens are able to escape NETs and others are not, it is obvious that microorganisms produce factors involved in escape mechanisms. Four escape mechanisms are conceivable: 1. suppression of NET formation, 2. protection against NET-mediated entrapment, 3. degradation of NETs, 4. protection against antimicrobial activity of NETs. The hypothesis that microbes are able to suppress the formation of NETs, was initiated by Papayannopoulos & Zychlinsky (2009), who hypothesized that bacterial catalases consume H$_2$O$_2$, a key factor in formation of NETs. However, this has not been confirmed with experimental data. The protease SpyCEP of M1T1 Group A Streptococcus (GAS) reduces the production of NETs. SpyCEP cleaves IL-8, an inducer of NET formation (Zinkernagel et al., 2008). These findings strengthen the concept that pathogens are able to suppress NET formation.

As the charge of the surface of pathogens plays a role in the trapping process by NETs (Bartneck et al., 2010), modification of the bacterial surface charge might be used by pathogens to protect themselves against entrapment. Different streptococci such as GAS increase the positive surface charge by D-alanylation of the surface-exposed lipoteichoic acid (Kristian et al., 2005). Wartha and colleagues (2007) described protection of S. pneumoniae against NET-mediated killing due to D-alanylation. A similar result for evasion from neutrophil killing due to D-alanylation was found for Staph. aureus (Kraus et al., 2008) and S. suis (Fittipaldi et al., 2008b). Further a mechanism for pathogens to protect themselves against the AMP activity inside the NETs is the charge of the cell envelope (Epand & Vogel, 1999). Furthermore, it was demonstrated that the polysaccharide capsule of S. pneumoniae reduced the entrapment by NETs (Wartha et al., 2007), which has been proven as a general protection against phagocytosis.

As the antimicrobial activity of NETs is lost after DNase digestion (Brinkmann, 2004), it was assumed that DNase production is a benefit for pathogens. Indeed the role of a DNase as NET evasion factor was first demonstrated for GAS (Buchanan et al., 2006). Table 2 gives an overview about bacterial DNases as NET evasion factors. For viruses and parasites DNase activity as NET escape mechanism is not yet described, with the exception of Leishmania infantum (Table 2). Interestingly, a number of publications have presented data about streptococci and staphylococci. In chapter 1.4 a more detailed introduction about the function of DNases in streptococci and staphylococci is given. Another way of pathogens to evade NET-mediated antimicrobial activity is described for GAS. M1 protein protects GAS by deactivating the human cathelicidin LL-37 (Lauth et al., 2009). Since LL-37 protects NETs against degradation by bacterial nucleases (Neumann et al., 2014a, b), deactivation of LL-37 by the M1 protein results in efficient NET degradation by bacterial DNases.

1.4 Function of nucleases in microorganisms

As nucleases of microorganisms have been described to degrade NETs and mediate microbial escape from NETs, this chapter gives an overview about the classification of nucleases in the enzyme family
and their general function. Enzymes have a main function in the metabolism of organisms and they are classified by IUPAC in 6 groups. The enzyme group with commission number 3 (EC 3) are named as hydrolases. They are further divided into 13 subclasses depending on the special bounds they can cleave. In the group of esterases nucleases are classified with the EC-number 3.1.11 to 3.1.31. They can be divided into exonucleases and endonucleases (McNaught & Wilkinson, 2009). In 1968, Stuart Linn and Werner Arber isolated two enzymes in *E. coli*. The first enzyme was able to cleave unmethylated DNA and the second added a methyl group to DNA (Arber & Linn, 1969; Linn & Arber, 1968). This findings were one of the landmarks for the discovery of restriction enzymes and ten years later Werner Arber, Daniel Nathans and Hamilton O. Smith got the Nobel Prize in Physiology or Medicine "for the discovery of restriction enzymes and their application in molecular genetics" (Nobelprize.org, 2015). Since this time information on restriction enzymes and the function of endo- and exonucleases accumulated. An exonuclease (from Ancient Greek éksō, “outer, external”) removes nucleotides from the end of the DNA molecule. On the other hand an endonuclease (from Ancient Greek éndon, “within”) cuts DNA in the interior.

Before the identification of NETs occurred, researchers were interested in bacterial nucleases as factors in genetic transformation (Lacks et al., 1975) or the characterization of enzymatic activity of bacterial nucleases (Faustoferri et al., 2005). Interestingly new results demonstrated that the main function of Cas4, a 5’ to 3’ DNA exonuclease, is an antiviral defense mechanism of bacteria (Sorek et al., 2008; Zhang et al., 2012).

Furthermore, some researchers are working on methods to use DNases as a therapeutic target. For example NucB, a nuclease from *Bacillus licheniformis*, was tested for the effect on biofilm forming microorganisms (Shields et al., 2013). In this study microorganisms were isolated from patients with a chronic rhinosinusitis and most of them were *Staph. aureus* or *α-haemolytic streptococci*. NucB is small nuclease compared to other nucleases and in the study of Shields et al. (2013) more than 50 % of the isolated bacteria produced biofilms and a high number produced NucB-sensitive biofilms. Earlier studies discussed biofilms growing within paranasal sinuses as mayor factors in the pathogenesis of chronic rhinosinusitis (Foreman et al., 2012). Interestingly, nine of the staphylococci and streptococci tested in the NucB study are known to produce extracellular nuclease, but after treatment with NucB the biofilm formation was reduced (Shields et al., 2013).

In the last ten years in different studies nucleases were characterized to be involved in the escape of pathogens from NETs. DNases of bacteria were explored as factors against the innate immune system. Remarkable most DNases involved in NET escape were identified in Gram-positive cocci (Table 2). But also Gram-negative bacteria are able to escape NETs by production of a nuclease (Juneau et al., 2015b; Seper et al., 2013). EndA of *S. pneumoniae* is not only involved in NET escape but also in spreading of the pneumococci from the upper airways to the lungs and from there to the bloodstream (Beiter et al., 2006).
Many bacteria such as GAS produce more than one DNase. Sumby et al. (2005) discussed possible reasons. Firstly, different DNases may be produced at different growth phases and function therefore at different phases of infection. Secondly, in close correlation to point one, it is conceivable that different DNases might show differences in the biochemical conditions for optimal activity, e.g. pH or ion concentrations. This might be important for invasive bacteria as they are exposed to different body fluids or tissues. Thirdly, if one DNase is inactivated by the host, e.g. by antibodies, another DNase might still work and contribute to bacterial survival.

To counteract the innate immune system, bacterial DNases are not only degrading NETs. Unmethylated CpG-rich bacterial DNA is recognized by TLR-9. The degradation of bacterial DNA by Sda1 suppresses the TLR-9 innate immune response and macrophage bactericidal activity (Uchiyama et al., 2012). This constitutes an additional novel innate immune evasion mechanism.

Taken together DNases of microorganisms, especially of bacteria, may exhibit a wide spectrum of multiple different functions in metabolism and host immune evasion.
### Table 2 DNases in microorganisms

<table>
<thead>
<tr>
<th>Species</th>
<th>DNase</th>
<th>Location / detection</th>
<th>NET degradation</th>
<th>Other function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Leishmania infantum</em></td>
<td>3'NT/NU</td>
<td>membrane-anchored</td>
<td>yes</td>
<td></td>
<td>(Guimaraes-Costa <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>?</td>
<td>?</td>
<td>yes</td>
<td></td>
<td>(Brogden <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Xds</td>
<td>extracellular (supernatant)</td>
<td>yes</td>
<td></td>
<td>(Blokesch &amp; Schoolnik, 2008; Seper <em>et al.</em>, 2013)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Dns</td>
<td>extracellular nuclease</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>Nuc</td>
<td></td>
<td>yes</td>
<td></td>
<td>(Juneau <em>et al.</em>, 2015b)</td>
</tr>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Nuc</td>
<td>extracellular (supernatant)</td>
<td>yes</td>
<td></td>
<td>(Berends <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>GBS0661 (NucA)</td>
<td>transmembrane domain (supernatant)</td>
<td>yes</td>
<td></td>
<td>(Derré-Bobillot <em>et al.</em>, 2013)</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>SDC</td>
<td>cell wall-anchored, (supernatant)</td>
<td>not investigated</td>
<td></td>
<td>(Wolinowska <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>SmnA (Smx)</td>
<td></td>
<td>not investigated</td>
<td></td>
<td>(Faustoferri <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>EndA</td>
<td>membrane-bound, surface located</td>
<td>yes</td>
<td>DNA uptake during transformation; promote bacterial spreading</td>
<td>(Beiter <em>et al.</em>, 2006; Bergé <em>et al.</em>, 2013; Lacks &amp; Neuberger, 1975; Midon <em>et al.</em>, 2011; Moon <em>et al.</em>, 2011; Zhu <em>et al.</em>, 2013)</td>
</tr>
</tbody>
</table>
## General Introduction

<table>
<thead>
<tr>
<th>Species</th>
<th>DNase</th>
<th>Location / detection</th>
<th>NET degradation</th>
<th>Other function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> (M1/T1)</td>
<td>Sda1</td>
<td>(supernatant)</td>
<td>yes</td>
<td>degradation of NET; virulence factor in mice; provides switch to invasive GAS; prevent TLR9-dependent recognition;</td>
<td>(Aziz <em>et al.</em>, 2004; Buchanan <em>et al.</em>, 2006; Uchiyama <em>et al.</em>, 2012; Walker <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> (M1)</td>
<td>SpnA</td>
<td>cell wall-located</td>
<td>yes</td>
<td></td>
<td>(Chang <em>et al.</em>, 2011; Hasegawa <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> (M6)</td>
<td>Spd1 or MF2, supernatant</td>
<td></td>
<td>not investigated</td>
<td></td>
<td>(Broudy, 2002)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> (M1)</td>
<td>Spd3, SdaD2, Spd</td>
<td>extracellular</td>
<td>not investigated</td>
<td>enhance evasion of the innate immune response, major DNase is SdaD2</td>
<td>(Sumby <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td>SWAN</td>
<td>cell wall-anchored</td>
<td>not investigated</td>
<td>protection against NET killing</td>
<td>(Morita <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td>Don (DNase one)</td>
<td>exonuclease</td>
<td>not investigated</td>
<td>Repair of DNA damage</td>
<td>(Lindler &amp; Macrina, 1987)</td>
</tr>
<tr>
<td><em>Streptococcus suis</em></td>
<td>SsnA</td>
<td>cell wall-located, supernatant</td>
<td>not investigated</td>
<td><em>S. suis</em> vaccine candidate in mice</td>
<td>(Fontaine <em>et al.</em>, 2004; Gómez-Gascón <em>et al.</em>, 2014)</td>
</tr>
</tbody>
</table>
1.5 Role of brain barriers in bacterial infections

Around the brain and the spinal cord the subarachnoid space (outer CSF space) is located and the ventricular system is a set of four ventricles (inner CSF space) in the brain. Both spaces are connected and filled with CSF that represents a fluid protection of the brain. The dura mater and the arachnoid mater on the skull site and the pia mater and glia limitans on the brain site cover the subarachnoid space [Fig. 1-5].

1.5.1 Three brain barriers

Three brain barriers are formed to protect the brain against harmful substances and pathogens: 1. blood-leptomeningeal barrier (BLMB), 2. BBB and 3. BCSFB [Fig. 1-5]. These barriers also regulate the access of immune cells into the CNS. This is the reason why the brain is described as an immune-privileged site (Nickel et al., 2004; Shechter et al., 2013). The immune response against pathogens in the CNS is different to the response in other organs. As a high influx of extracellular fluid or immune cells leads to an inflammatory swelling and increased pressure, the body regulates the entrance of immune cells to the CNS strictly. Therefore, the CNS is better described as a site of “selective and modified immune reactivity” (Ransohoff et al., 2003).

The BLMB is localized at the surface of the brain and the spinal cord. This barrier is formed by endothelial cells of the leptomeningeal microvessels and these cells are connected with tight junctions (TJs) (Engelhardt & Ransohoff, 2012; Shechter et al., 2013). The second barrier is the BBB formed by the glia limitans perivascularis and tightly connected endothelia cells of the microvessels in the CNS parenchyma. One important function of this barrier is the regulation of moving agents from the blood to the CNS (Lossinsky & Shivers, 2004; Shechter et al., 2013).

Furthermore, the BBB and the BLMB are classified due to the endothelial cell formation as true barriers. In contrast to these true barriers, the third barrier is called an educational gate which allows immunosurveillance of the CSF (Shechter et al., 2013). This is the BCSFB, which is located in the ventricles. From the pia mater, blood capillaries extend like villi into the ventricles. This barrier is also called choroid plexus and is formed by an endothelium of the fenestrated blood vessels without TJs and by a single-layer of ependymal cells (specialized cuboidal epithelial cells), with microvilli on the apical surface. These epithelial cells are connected by TJs. Important TJ proteins are occludin (which is involved in neutrophil transmigration (Huber, 2000)), claudins or ZO-1, ZO-2 and ZO-3 (Matter & Balda, 2003, 2007). The CSF is produced by the ependymal cells of the choroid plexus (Nickel et al., 2004; Shechter et al., 2013). As high numbers of leukocytes and especially neutrophils in the CSF compartment characterizes bacterial meningitis, these cells must cross at least one of the three brain barriers. Different histopathological analyses were able to demonstrate that the BCSFB can be a main entry site for pathogens in case of bacterial meningitis. This was observed for E. coli (Zelmer et al., 2008), N. meningitidis (Guarner et al., 2004; Pron et al., 1997) and S. suis (Madsen et al., 2002b; Sanford, 1987; Williams & Blakemore, 1990).
Three brain barriers are formed in mammals and are located at different sites in the brain. The blood brain barrier (BBB) and the blood leptomeningeal barrier (BLMB) are true barriers as the endothelium (5) is connected by TJs (2). In contrast the blood cerebrospinal fluid barrier (BCSFB) is characterized by an epithelium (7) with TJs (2) but a fenestrated endothelium (8). 1 CSF, 2 TJs, 3 Glia limitans with astrocytes, 4 blood, 5 endothelial cell, 6 endothelial basement membrane, 7 epithelial cell, 8 choroidal endothelia cell.
1.5.2 Cell culture systems of the BCSFB

To understand the importance and function of the BCSFB, different cell culture systems have been established (Redzic, 2013). The first description of a functional human BCSFB model was published in 2012 (Schwerk et al., 2012). This model bases on a human choroid plexus papilloma cell line (HIBCPP) and is characterized by high transepithelial electrical resistance (TEER), formation of TJs and expression of junctional proteins. It was used to study transmigration of this barrier by *S. suis* and *N. meningitides*. Furthermore, transmigration of neutrophil granulocytes through this barrier after infection was demonstrated (Steinmann et al., 2013) [Fig. 1-6].

In addition to the HIBCPP a human choroid plexus carcinoma cell line (CPC-2) has been tested. However, these cells show irregular staining of TJ proteins indicating reduced barrier function (Redzic, 2013). Further, a commercially human choroid plexus epithelium primary culture (ScienCell laboratories, Carlsbad, CA, USA) is available. However up to now, these cell line was only used in studies without interest on the barrier features and some questions are unresolved like the origin of the cell line (Redzic, 2013). Taken together the best established human choroid plexus cell culture system is the BCSFB model published by Schwerk *et al.* in 2012. The HIBCPP cells have also been used in an inverted system. The inverted orientation is an important modification, which mimics the *in vivo* situation as infectious agents and immune cells might be applied to the basolateral (blood side). Bacteria and immune cells may breach the barrier and enter the apical side, which represents the “CSF compartment” with regard to the orientation of the plexus epithelial cells.

In addition to the models of the human BCSFB, there are also porcine BCSFB models. Until now infection and transmigration studies were conducted only with primary porcine choroid plexus cells (Adam *et al.*, 2004; Tenenbaum *et al.*, 2009; Wewer *et al.*, 2011), but in 2012 a novel porcine *in vitro* model of the BCSFB with a porcine choroid plexus epithelial cell line (PCP-R) was published (Schroten *et al.*, 2012). This new model is associated with strong barrier functions such as high TEER and low permeability of macromolecules.

The BCSFB models were used to answer questions dealing with bacteria and neutrophils or monocytes. In the human model of the BCSFB invasion of *S. suis* as well as *N. meningitides* was reported to be possible only from the physiologically relevant basolateral side (Schwerk *et al.*, 2012). Also in a porcine model of the BCSFB the same polar invasion of *S. suis* was demonstrated (Tenenbaum *et al.*, 2009). The neutrophil transmigration following infection of the cell layer was visualized via three-dimensional Apotome®-imaging and electron microscopy (Tenenbaum *et al.*, 2013; Wewer *et al.*, 2011). After an infection with *N. meningitidis* a paracellular as well as a transcellular transmigration of neutrophils through a HIBCPP cell layer was observed (Steinmann *et al.*, 2013). Furthermore, after a *S. suis* infection of the porcine blood-CSF barrier model a transcellular migration of neutrophils was detected (Wewer *et al.*, 2011).
The function of the BCSFB is lost in an *in vitro* model after *S. suis* infection, but until now the mechanisms for the barrier breakdown are not found (Tenenbaum *et al.*, 2005). The described *in vitro* models might also be used to explore the role of cytokines in the CSF compartment. For example IFN-γ and TNFα are detected in CSF of patients with meningitis and are key mediators for proinflammation (Glimåker *et al.*, 1994; Kornelisse *et al.*, 1997). Growth of *S. suis* was significantly reduced in the presence of IFN-γ stimulated porcine CPEC and TNFα enhanced this effect (Adam *et al.*, 2004). The function of PMN’s after transmigration through a model of IL-1 stimulated mouse brain epithelium has been studied. The transmigrated neutrophils were found to be neurotoxic after transmigration of this barrier, based on experiments with cultured neurons and released NETs (Allen *et al.*, 2012). Nevertheless, up to now no study has been published that focused on the interaction between *S. suis* or in general bacteria and neutrophil granulocytes after a transmigration through the BCSFB in this model and if for example NET formation is possible.

![Diagram](image)

**Figure 1-6 Overview of the *in vivo* BCSFB [A] and the inverted *in vitro* model [B]**

A. BCSFB, B inverted cell culture system of the BSCFB established by Schwerk *et al.* 2012

1 blood [A] / blood compartment [B], 2 CSF [A] / CSF compartment [B], 3 choroidal endothelium, 4 epithelial cells [A] / HIBCCP [B], 5 TJs, 6 filter membrane of insert, 7 *S. suis*, 8 neutrophil
2 Aims of the study

To date, nothing is known about the *S. suis* interaction with NETs and the role of NETs in an *S. suis* infection. As DNases have been described to be important NET evasion factors in streptococci and staphylococci, the first aim of the present study was to investigate putative DNase candidates of *S. suis* and their role in the interaction with NETs *in vitro* [Chapter 3.1 and 3.2]. Loss-of-function-mutations were used to reveal the function of the known DNase SsnA (Fontaine *et al.*, 2004) but also of a novel DNase identified during this work. Furthermore, comparative investigations included a double mutant to identify possible synergistic effects of both DNases [Chapter 3.2].

As *S. suis* infects piglets and humans, tests were conducted with neutrophils from both species. This experimental design offered the chance to identify differences in the two hosts [Chapter 3.1].

After the characterization of the function of both DNases in interaction with NETs *in vitro*, the second part of the present study focused on the detection of NETs in the *S. suis*-infected CSF compartment. For *in vitro* studies, a cell culture system of the choroid plexus was adapted from an established protocol (Schwerk *et al.*, 2012) to address two topics, a) NETosis in the *S. suis*-infected CSF compartment and b) examination of the functions of SsnA and EndAsuis in the CSF compartment *in vitro*. In addition, CSF drawn from *S. suis*-infected piglets was analyzed to screen for DNase activity as well as NET formation *in vivo* [Chapter 3.3]. The aims of the study are illustrated in figure 2-1.
Aims of the study

Part I

A

NET-induction?

B

NET-killing?

NET-evasion?

Part II

CSF
Choroid plexus

C

D

Figure 2-1 Illustration of the aims of the study

[A] Does S. suis induce NETs? [B] Is S. suis entrapped and killed in NETs or able to evade by degrading NETs with one or more DNases? [C] What is the function of S. suis DNase in the CSF compartment? [D] Are NETs formed in the CSF compartment?
3 Results

3.1 SsnA is a NET-evasion factor in the stationary growth

*Streptococcus suis* DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity

Nicole de Buhr¹, Ariane Neumann², Natalja Jerjomiceva², Maren von Köckritz-Blickwede²† and Christoph G. Baums¹†
Streptococcus suis DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity

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Contribution of Nicole de Buhr to this work:
Nicole de Buhr conducted mutagenesis of S. suis, the genotyping assays and most of the phenotyping assays including the immunofluorescence analysis. She carried out generation, expression and functional analysis of the recombinant protein. All DNase assays were done by Nicole de Buhr. Furthermore, she analyzed the data, conducted statistical analysis, designed the figures and participated in experimental design and drafting the manuscript.
ABSTRACT

*Streptococcus suis* is an important cause of different pathologies in pigs and humans, most importantly fibrinosuppurative meningitis. Tissue infected with this pathogen is substantially infiltrated with neutrophils, but the function of neutrophil extracellular traps (NETs) - a more recently discovered antimicrobial strategy of neutrophils - in host defense against *Strep. suis* has not been investigated. The objective of this work was to investigate the interaction of *Strep. suis* with NETs in vitro. *Strep. suis* induced NET formation in porcine neutrophils and was entrapped but not killed by those NETs. As the amount of NETs decreased over time, we hypothesized that a known extracellular DNase of *Strep. suis* degrades NETs. Though this nuclease was originally designated *Strep. suis*-secreted nuclease A (SsnA), this work demonstrated surface association in accordance with an LPXTG cell wall anchor motif and partial release into the supernatant. Confirming our hypothesis, an isogenic ssnA mutant was significantly attenuated in NET degradation and in protection against the antimicrobial activity of NETs as determined in assays with phorbol myristate acetate (PMA)-stimulated human neutrophils. Though assays with PMA-stimulated porcine neutrophils suggested that SsnA also degrades porcine NETs, phenotypic differences between wt and the isogenic ssnA mutant were less distinct. As SsnA expression was crucial for neither growth in vitro nor for survival in porcine or human blood, the results indicated that SsnA is the first specific NET evasion factor to be identified in *Strep. suis*.

Abbreviations:

MAP, murein-associated protein; NET, neutrophil extracellular trap; PMA, phorbol myristate acetate; SsnA, *Strep. suis* secreted nuclease A.
3.2 EndAsuis degrades NETs in exponential growth phase

Identification of a novel DNase of *Streptococcus suis* (EndAsuis) important for neutrophil extracellular trap degradation during exponential growth

Nicole de Buhr¹, Matthias Stehr¹², Ariane Neumann³, Hassan Y. Naim³, Peter Valentin-Weigand¹, Maren von Köckritz-Blickwede³† and Christoph G. Baums¹⁴†
Identification of a novel DNase of *Streptococcus suis* (EndAsuis) important for neutrophil extracellular trap degradation during exponential growth

Nicole de Buhr¹, Matthias Stehr¹,², Ariane Neumann³, Hassan Y. Naim³, Peter Valentin-Weigand¹, Maren von Köckritz-Blickwede³† and Christoph G. Baums¹,⁴†

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**Contribution of Nicole de Buhr to this work:**

Nicole de Buhr conducted mutagenesis of *S. suis*, the genotyping assays and most of the phenotyping assays including the immunofluorescence analysis. She carried out generation, expression, purification and functional analysis of the recombinant proteins. All DNase assays were done by Nicole de Buhr. In addition she supported the implementation of the 3D modeling part. Furthermore, she analyzed the data, conducted statistical analysis, drafted the figures and participated in experimental design and drafting the manuscript.
3.3 NETs detected in *S. suis*-infected CSF compartment

Neutrophil extracellular trap formation after transmigration of neutrophils through *Streptococcus suis* infected human choroid plexus epithelial cell barrier

Nicole de Buhr¹, Friederike Reuner², Ariane Neumann², Carolin Stump-Guthier³, Tobias Tenenbaum³, Horst Schroten³, Hiroshi Ishikawa⁴, Peter Valentin-Weigand¹, Christoph G. Baums¹,⁵† and Maren von Köckritz-Blickwede²†
Results

In preparation

Neutrophil extracellular trap formation after transmigration of neutrophils through *S. suis* infected human choroid plexus epithelial cell barrier

Nicole de Buhr¹, Friederike Reuner², Ariane Neumann², Carolin Stump-Guthier³, Tobias Tenenbaum³, Horst Schroten³, Hiroshi Ishikawa⁴, Peter Valentin-Weigand¹, Christoph G. Baums¹,⁵† and Maren von Köckritz-Blickwede²,⁶†

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Contribution of Nicole de Buhr to this work:

Nicole de Buhr conducted the cell culture experiments including the immunofluorescence analysis, RNA preparation and RT-PCR. All DNase assays were done by Nicole de Buhr. Furthermore, she analyzed the data, conducted statistical analysis, drafted the figures and participated in experimental design and drafting the manuscript.
ABSTRACT

*Streptococcus (S.) suis* is the most important meningitis causing pathogen in pigs and also an emerging zoonotic agent. Neutrophil extracellular traps (NETs) have been identified as host defense mechanism against different pathogens. Here, in *in vivo* experiments NETs could be detected in the cerebrospinal fluid (CSF) of piglets infected with *S. suis*. Thus, to study NETosis and NET-degradation after consecutive transmigration of *S. suis* and neutrophils through the human choroid plexus epithelial cell barrier, a previously described inverse *in vitro* model of the human blood-CSF barrier was used. Using confocal immunofluorescence microscopy NETosis and respective entrapment of streptococci in NETs was recorded in the lower compartment after transmigration of neutrophils through *S. suis*-infected human choroid plexus epithelial cell barrier. Comparative analysis of *S. suis* wildtype and different *S. suis* nuclease mutants did not reveal differences in NET-formation or bacterial survival. However, expression of antimicrobial peptide LL-37, which has been previously been shown to stabilize NETs increased after transmigration of neutrophils through the choroid plexus epithelial cell barrier, suggesting that LL-37 might protect NETs against bacterial DNase degradation in the lower ("CSF") compartment. In conclusion, neutrophils form NETs and entrap *S. suis* after breaching the infected choroid plexus epithelium.

Non-standard abbreviations: cerebrospinal fluid (CSF), human choroid plexus papilloma cells (HIBCPP), neutrophil extracellular trap (NET), phorbol-myristate-acetate (PMA), Todd Hewitt broth (THB)
INTRODUCTION

Streptococcus (S.) suis is worldwide one of the major porcine pathogens and characterized by a high diversity of serotypes (Gottschalk, 2012). Furthermore, it is also a very important zoonotic pathogen. Serotype 2 is most frequently isolated from morbid animals and humans (Staats et al., 1997; Wisselink, 2000; Wei et al., 2009). Central nervous system (CNS) disorders due to meningitis are frequently observed in S. suis-infected piglets and humans (Straw et al., 2006; Lun et al., 2007). S. suis meningitis is characterized by a high number of neutrophil granulocytes in the cerebrospinal fluid (CSF) (Williams and Blakemore, 1990; Lun et al., 2007; Beineke et al., 2008). In general neutrophils are part of the innate immune system and can act antimicrobial by several defence mechanisms like phagocytosis, apoptosis and NETosis after breaching a cell barrier and infiltrating infected tissue (Steinberg and Grinstein, 2007; Mocsai, 2013). NETosis is a term used for the formation of neutrophil extracellular traps (NETs) identified in 2004 as extracellular release of nuclear DNA and associated antimicrobial compounds (Brinkmann, 2004; Fuchs et al., 2007). However, many pathogens produce DNases to escape from the antimicrobial effects of NETs. Earlier we showed that S. suis is able to induce NETs and can also be trapped by NETs (de Buhr et al., 2014). The S. suis secreted nuclease A (SsnA) is a NET-evasion factor protecting against the antimicrobial activity of NETs (de Buhr et al., 2014). Furthermore, endonuclease A of S. suis (EndAsuis) is an additional NET-degrading DNase active in the exponential growth phase (de Buhr et al., 2015). The function of both DNases differs, since they are active at different pH and ion concentrations (de Buhr et al., 2015).

During the pathogenesis of S. suis meningitis streptococci and neutrophils meet in the CSF compartment after crossing the blood-CSF barrier (BCSFB) (Williams and Blakemore, 1990; Lun et al., 2007; Beineke et al., 2008). In an inverted transwell filter system with primary porcine choroid plexus epithelial cells the invasion and translocation of S. suis from the “blood” to the “CSF compartment” was analysed and the relevance of the BCSFB as an entry gate for S. suis into the CNS was indicated (Tenenbaum et al., 2009). Furthermore, an inverse model of the human BCSFB using a human choroid plexus papilloma (HIBCPP) cell line on transwell filters with constant barrier function was established by Schwerk et al. (2012). Neutrophils were shown to efficiently cross this barrier from the upper “blood” to the lower “CSF”-side after an infection with S. suis or N. meningitides (Steinmann et al., 2013). However, until now, nothing is known about the formation of NETs in CSF.

In this study we first analyzed CSF samples of piglets infected with S. suis to investigate NETosis in the fluid CSF compartment in vivo. Furthermore, an in vitro BCSFB model was used to study the formation of NETs and activity of S. suis nucleases after transmigration of neutrophils through S. suis-infected human choroid plexus epithelial cells.
**Results**

**RESULTS**

**Fig. 1:** Immunofluorescence microscopy of NETs and DNase activity is detectable in the *S. suis*-infected CSF compartment CSF of infected piglets *in vivo* and SsnA is active in porcine CSF.

Piglets were infected intranasally with *S. suis* and the CSF was analysed by cytospin. A. The CSFs of four piglets (9780, 9807, 9884, 9899) with central nervous system disorders were stained after cytospin. In all animals NET structures with fibres were detected (blue = DNA [DAPI], green =...
Results

histone 1). B. CSF samples of uninfected and *S. suis* wt infected piglets were analyzed for DNase activity by addition of eukaryotic DNA to the CSF samples and subsequent incubation at 37°C for 22 h. In the PBS control and the uninfected animals no DNase activity was detectable. The 7 infected animals are divided in two groups. Group 1 (9780, 9807, 9884) was characterized by CNS disorders, suppurative meningitis, altered CSF with pleocytosis and re-isolation of *S. suis* in CSF. Group 2 (9725, 9881, 9898, 9971) was characterized by no signs of central nervous system dysfunction or CSF alterations. C. DNase activity of the indicated *S. suis* strains in the presence of CSF from healthy piglets spiked with eukaryotic DNA. After 4 h incubation only SsnA dependent DNA degradation was detectable (ctr = control). D. Inhibition of DNase activity in CSF of meningitis piglet 9884 and a CSF sample pre-incubated with *S. suis* wildtype by incubation with an antiserum against rSsnA prior to incubation with eukaryotic DNA. CSF pre-incubated with the double mutant was used as negative control for DNA degradation. DNA was visualized by 1% agarose gel electrophoresis.
Results

Fig. 2: Modified inverse blood cerebrospinal fluid barrier model for monitoring NETosis in the „CSF compartment“.

A. An inverted system with human choroid plexus papilloma cells (HIBCPP) grown on filters was used to form a blood compartment and a CSF compartment. The latter contained cover slips for immunofluorescence microscopy. Two hours after addition of streptococci to the upper compartment the liquid in this part was removed and neutrophil granulocytes in fresh medium were added to this “blood” compartment. Four hours after addition of neutrophils NET-formation was monitored in the “CSF compartment” after cytospin. B. TEER analysis 2 and 6 h after infection with S. suis. As depicted in A neutrophils were added 2 h after infection. The percentage to the baseline was calculated based to the TEER before infection (442 ± 35 Ω cm²). No significant differences were detectable comparing wells infected with the different indicated S. suis strains. (one way ANOVA, Tukey’s test, SEM, n=5) and the TEER was stable in the experiment. C. DNase activity was determined in the supernatants of the „CSF compartments“ at the end of experiment. For this supernatants were incubated with eukaryotic DNA for 4 h at 37°C and DNA was visualized by 1% agarose gel electrophoresis.
Fig. 3. After breaching the infected choroid plexus epithelium in vitro human neutrophils form NETs entrapping S. suis.

A. Immunofluorescence microscopy analysis of NETosis in the “CSF compartment” 6 h post infection. Transmigrated neutrophils and bacteria in the “CSF compartment” were centrifuged down
on coverslips and stained (NETs = green, Hoechst [DNA] = blue, *S. suis* = red). Entrapped *S. suis* are marked with white arrows. Representative pictures of NET-formation are shown. B-C. Per sample 13 pictures were taken at predefined positions (Fig. S2) and analyzed regarding the number of neutrophils and the NET-releasing neutrophils. B. A significant difference in the number of neutrophils was found between bacterial infected filters and the control filters (*P* = 0.0004, ANOVA, Tukey’s test). C. Further a significant difference was found in the percentage of activated neutrophils. Using ANOVA, Dunnett’s multiple comparison test (*P* = 0.0389) a significant difference was between ΔendAsuis and uninfected control. D – F By PicoGreen analysis of NET-associated DNA in the „CSF compartment” revealed only a difference between bacterial infected and uninfected filters. G The percentage of NET-associated DNA to total DNA was calculated. No significant differences were observed. All graphs show the mean ± SEM of 5 independent experiments, except the filter “wt without PMN” in D-F, which was tested four times. A-C are one experiment series and D-F are a second experiment series. * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001 by ANOVA (B) or t-Test (C)
Fig. 4: Bacterial transmigration and survival in the „CSF compartment” is SsnA and EndAsuis independent in the presence and absence of neutrophils.

The bacterial number of the indicated S. suis strains in the „CSF compartment” was determined at 2 h and 6 h post infection of the choroid plexus epithelium in vitro. The assay was performed without neutrophils (A) and after addition of neutrophils to the upper (“blood”) compartment 2 hours post infection (B). Differences in the specific bacterial content in the „CSF compartment” were not recorded in both assays comparing the indicated S. suis strains at 2 or 6 hour post infection. All data are shown as mean ± SD of four (A) or five (B) independent experiments in duplicates each. Statistical differences were analyzed by one-way ANOVA using Tukey’s adjustment.
Fig. 5: LL-37 immunofluorescence signal in HIBCPPs after transmigration of neutrophils.

Immunofluorescence microscopy was conducted to detect LL-37 in HIBCPP and/or transmigrating neutrophils. A. Detection of LL-37 in filters 6 h post infection with S. suis wildtype and subsequent addition of neutrophils in the upper (“blood”) compartment to initiate transmigration (wt + PMN) in comparison to S. suis-infected filters not exposed to neutrophils (wt – PMN). In filters stimulated for 6 h with TNF α for PMN transmigration a LL-37 signal was also detectable (TNFα + PMN). White squares highlight the area of magnification. The isotype control (control) was negative for LL-37 signal. (blue = DNA [Hoechst], green = LL-37, grey = phalloidin). B. Analysis of LL-37 localization with PMNs by MPO co-staining. Results for the filters infected with the double mutant 10ΔssnAΔendAsuis (ΔΔ) including transmigrating PMNs (ΔΔ + PMN) are shown. (blue = DNA
Results

[Hoechst], green = LL-37, red = MPO, grey = phalloidin). All pictures show examples of representative pictures. In A only overlays presented.

Fig. 6: LL-37 expression analysis in transmigrating neutrophils and HIBCPPs after infection with S. suis.

A-C qRT-PCR was conducted with oligonucleotide primers listed in Table S1. Gapdh expression was used as reference (housekeeping gene). A. Relative transcript levels of transmigrated neutrophils were compared by calculation of \( \Delta \Delta C_T \) based to non-transmigrated neutrophils incubated in the „CSF compartment“. B. Relative transcript levels of non-transmigrated neutrophils were compared by calculation of \( \Delta \Delta C_T \) based to non-stimulated neutrophils incubated in 1% FBS cell culture medium. C Relative transcript levels of the wildtype infected or TNF\( \alpha \) stimulated HIBCPP cells and transmigrating neutrophils (HIBCPP + wt + PMN or HIBCPP + TNF\( \alpha \) + PMN) were compared. As after 40 cycles no signal for the control (HIBCPP + PMN non-transmigrated in „CSF compartment“) was measured, calculation of \( \Delta \Delta C_T \) was impossible. Therefore the determined \( C_T \) values are presented. Data in A and B are presented as means ± SD of \( \Delta \Delta C_T \) to control of three independent experiments with technical duplicates. No significant statistical differences were found analyzed by paired one-way Student’s t-Test. Data in C presented as single values of three independent experiments with 3 technical independent runs each.
Results

Formation of NETs in the CSF of S. suis-infected piglets.
As a first step, we screened CSF of piglets infected experimentally with S. suis serotype 2 (strain 10) for NET-formation immediately after collection of the samples. Therefore, cells in CSF were transferred to glass cover slides by cytopin and NETs were visualized by immunofluorescence microscopy with NET-specific antibodies against histone-DNA-complexes. In all four piglets with CNS dysfunctions like tremor, opisthotonus or convulsions and histologically confirmed meningitis, NET structures were detected (Fig. 1A). These four piglets exhibited pleocytosis in the CSF with leukocyte numbers ranging from $5.4 \times 10^5$ /ml to $1.2 \times 10^8$ /ml. S. suis serotype 2 was isolated in pure culture from the CSF of these piglets. The CSF sampling method did not induce NET-like structures as determined by control experiments (Fig. S1). Thus, detection of NETs in CSF samples of S. suis-infected piglets suggests occurrence of NETosis by granulocytes infiltrating the CSF compartment in the course of a S. suis meningitis.

Degradation of eukaryotic DNA in the CSF of S. suis-infected piglets.
S. suis has been shown to express at least two DNases involved in NET-degradation (de Buhr et al., 2014; de Buhr et al., 2015). Thus, as a next step, we analyzed CSF samples of S. suis-infected piglets for DNase activity to determine if DNase activity is detectable in this infected compartment in vivo. Interestingly, high DNase activity was detectable in CSF samples of S. suis-infected animals with meningitis, whereas moderate activity in infected animals with no detectable lesions was seen, and almost no activity was found in uninfected animals (Fig. 1B).
As DNase activity in meningitis lesions might be of pathogen and/or host origin, we performed experiments to investigate S. suis nuclease activity in CSF. First, we analyzed if S. suis wildtype and DNase mutants lead to degradation of DNA in CSF of healthy piglets. As shown in Fig. 1C, S. suis wildtype and 10ΔendAsuis but not 10ΔssnA incubated in CSF of healthy piglets spiked with eukaryotic DNA degraded this DNA indicating that the bacterial nuclease SsnA is expressed and active in CSF. Furthermore, pre-incubation of a meningitis CSF sample (No. 9884) with an antiserum directed against rSsnA led to a prominent inhibition of DNase activity compared to the respective control treated with the pre-immune serum (Fig. 1D). In conclusion, SsnA-specific DNase activity is detectable in S. suis-infected CSF.

Usage of a human BCSFB model to study NETosis in the “CSF compartment”.
We wanted to study NETosis in CSF in more detail. For this a previously described inverse model of a human BCSFB (Ishiwata et al., 2005; Schwerk et al., 2012) was adapted to investigate NETosis and NET-degradation after transmigration of streptococci and neutrophils through this barrier. The protocol was modified to ensure a functional epithelial barrier in the absence of antibiotics. An important modification was the exchange of the fluid in the upper (“blood”) compartment after two hours of bacterial infection and prior to addition of neutrophils (Fig. 2A). This step was conducted to
prevent lytic effects through suilysin accumulation (Norton et al., 1999; Charland et al., 2000). To monitor functionality of the choroid plexus epithelial barrier, the transepithelial electrical resistance (TEER) was recorded at different time points. As shown in Fig. 2B, the percentage baseline TEER referring to the start value changed only slightly over time and significant differences in TEER were not recorded between infected and uninfected filters during the course of experiments. Furthermore, dextran flux measurements confirmed that the modifications of the model did not lead to a reduced barrier function (data not shown).

After evaluation of the modified inverse model, we investigated if SsnA and EndAsuis dependent DNase activity is detectable in the lower „CSF compartment” after transmigration of streptococci. Supernatants of the lower „CSF compartment” infected with S. suis wildtype and S. suis ΔendAsuis exhibited distinct degradation of DNA in contrast to the compartments infected with S. suis ΔssnA or S. suis ΔendAsuis ΔssnA and the mock control (Fig. 2C).

**Neutrophils form NETs after transmigration through the infected choroid plexus epithelium in vitro.** Immunofluorescence microscopy and a PicoGreen assay were conducted to investigate NETosis in the lower compartment of the inverse trans-well system. Importantly, we confirmed that neutrophils formed NETs in the S. suis-infected lower (“CSF”) compartment in this modified model and streptococci were trapped in these NETs (Fig. 3A). For quantification of NETosis per sample, a total of 13 pictures were made with a blind target method (Fig. S2). A significantly higher number of neutrophils were found in infected compartments compared to the uninfected controls (Fig. 3B). This indicated a functional epithelial barrier, which is inducing transmigration of neutrophils upon infection with S. suis. Significant differences in the number of neutrophils were not found between the different infected groups (Fig. 3B). Furthermore, differences in the percentage of NET-releasing cells were not recorded between wildtype and S. suis DNase mutants (Fig. 3C) (de Buhr et al., 2014; de Buhr et al., 2015). To confirm these results, the amount of double stranded DNA was quantified in the lower (“CSF”) compartment by PicoGreen assay in five independent experiments. The DNA was quantified in three fractions: intracellular components, NET-associated components and free components. In all analyzed fractions a distinct difference in the amount of DNA was detectable between infected and uninfected samples (Fig. 3D-F). Importantly, the percentage of NET-associated DNA to the total DNA amount was nearly the same in the filters infected with the different strains: The wildtype and double mutant infected compartments had mean values for the NET-associated DNA of showing 47% and 51%, respectively, and no significant differences (Fig. 3G). In conclusion, no differences were recorded in the percentage of NET-releasing neutrophils and the NET-associated DNA after transmigration of neutrophils through the choroid plexus epithelium infected with wildtype or the different DNase mutants. These results suggest that neither SsnA nor EndAsuis are efficiently degrading NETs in the „CSF compartment”.
**Results**

**Bacterial transmigration into and survival in the lower „CSF compartment“ is independent of SsnA and EndAsuis.**

Determination of bacterial load in the lower „CSF compartment“ after 2 and 6 hours revealed comparable values for the wildtype and the DNase mutants in the experiment without neutrophils (Fig. 4A) and after transmigration of neutrophils (Fig. 4B). In addition, at the end of the experiment the CFU in the lower „CSF compartment“ was nearly the same with or without the addition of neutrophils (Fig. 4). These results suggest that the DNase mutants are neither attenuated in transmigration through the choroid plexus epithelium nor in survival (and growth) in the lower „CSF compartment“.

**LL-37 expression in human choroid plexus epithelial cells and transmigrating neutrophils.**

The host antimicrobial peptide LL-37 has recently been shown to stabilize NETs against bacterial nuclease degradation (Neumann *et al.*, 2014). Furthermore, it was demonstrated that choroid plexus epithelial cells in rats produce the rat-homologue of LL-37 (rCRAMP) after *S. pneumoniae* infection and that LL-37 is detectable in CSF and serum samples from patients with acute meningitis (Brandenburg *et al.*, 2008). Therefore, we hypothesized that LL-37 might protect NETs against degradation by the *S. suis* DNases in the described BCSFB model. Immunofluorescence microscopy using an anti-LL-37 antibody revealed a strong LL-37 signal associated with the transmigrating neutrophils (Fig. 5A): Co-staining of LL-37 and myeloperoxidase (MPO) demonstrated that LL-37 is located in close proximity to MPO-containing neutrophils (Fig. 5B). By real time PCR experiments we analyzed the transcript expression of LL-37 in the transmigrated neutrophils as well as the cell layer. LL-37 transcript expression was not significantly changed in neutrophils after transmigration upon infection with *S. suis* wt or TNFα stimulation (Fig. 6A). No change of LL-37 transcript expression was also observed in non-transmigrated neutrophils infected with *S. suis* wt or stimulated with TNFα (Fig. 6B). Besides neutrophils, it is also known that epithelial cells produce LL-37 as a chemo-attractant for neutrophils after bacterial invasion (Zanetti, 2005). Thus, we additionally analysed LL-37 transcript expression in HIBCPPs cells. As seen in Fig. 6C, HIBCPPs infected with *S. suis* wt or stimulated with TNFα and transmigrated neutrophils show a clear CT value indicating transcript expression of LL-37. It is reasonable to assume that this signal derived from HIBCPPs itself, but it cannot be excluded that this signal partially derives - despite washing the filters- from nesting neutrophils on the plexus epithelial cell layer. In contrast in the HIBCPP cells with non-transmigrated neutrophils, directly added to the „CSF compartment“, no CT value was measured after 40 cycles (Fig. 6C). In summary these data indicate that transmigration of neutrophils through the *S. suis*-infected choroid plexus epithelium results in higher expression of LL-37.
DISCUSSION

Our data show for the first time that NETs are formed in the CSF compartment of infected *S. suis* piglets (Fig. 1A). Until now, only little is known about NET-formation in body fluids with continued fluxes like CSF or blood. Interestingly, in septic blood of mice, NET-formation was detectable *in vivo* in the vasculature under flow conditions upon TLR-4-mediated activation of platelets that interact with neutrophils (Clark *et al.*, 2007). Next to the circulation in blood vessels and the lymphatic system, the cerebrospinal fluid compartment is described as the third circulation (Cushing, 1914; Cushing, 1925), but CSF exhibits only a pulsatile movement and a bulk flow is not detectable as seen in the blood (Yamada, 2014; de Lahunta *et al.*, 2015). This study includes to the best of our knowledge for the first time *in vivo* data depicting NETs in CSF drawn from animals with bacterial meningitis (Fig. 1A). Furthermore, we screened CSF samples of *S. suis*-infected piglets for DNase activity. Importantly, CSF from *S. suis*-infected piglets exhibited a strong DNase activity (Fig. 1B). As *S. suis* DNases SsnA and EndAsuis were shown to degrade NETs induced by PMA (de Buhr *et al.*, 2014; de Buhr *et al.*, 2015), we analysed the activity of both DNases in CSF. In accordance with loss of DNA degradation in *S. suis* ΔssnA incubated with CSF spiked with DNA, SsnA dependent DNase activity was also detected in the CSF of a *S. suis*-infected piglet (Fig. 1D). Nevertheless, the overall function of SsnA for the pathogenesis of *S. suis* meningitis is not well understood. Here we additionally found that *S. suis* grows independent of SsnA in the CSF (Fig. S3). However, we cannot exclude that besides SsnA also EndAsuis or host nucleases are present and contributing to the phenotype. It is known, that host DNases are found in the CSF in meningitis patients (Kovacs, 1954)

The choroid plexus is described as an entry gate for immune cells to the CNS (Wilson *et al.*, 2010; Meeker *et al.*, 2012) and histopathological examinations of experimentally infected piglets suggest the BCSFB as an entry gate for *S. suis* (Sanford, 1987; Williams and Blakemore, 1990; Madsen *et al.*, 2002). Here, we were interested whether neutrophils form NETs after transmigration through this barrier infected with *S. suis*. Thus, we investigated NETosis in a *S. suis*-infected CSF compartment using a modified inverse model of human BCSFB (Schwerk *et al.*, 2012). The model described by Schwerk *et al.* (2012) was modified removing the medium including non-transmigrated bacteria prior to addition of neutrophils. This modification was necessary for the establishment of a protocol without antibiotics. Furthermore, removal of the liquid in the upper compartment ensures that neutrophils interact mainly with streptococci after breaching the activated choroid plexus epithelial barrier.

Importantly, NETs with fibres were detectable in all *S. suis*-infected „CSF compartments“ of the BCSFB model and trapped bacteria were found inside these structures (Fig. 3A). Control experiments revealed that neutrophils incubated in 1% FBS medium alone do not exhibit increased NETosis level (Fig. S4). Thus, our results indicate that plexus epithelial cells and/or bacteria induce formation of NETs in our model. *S. suis* has already previously been shown by us to induce NETs (de Buhr *et al.*, 2015). Furthermore, HIBCPP might release IL-8 and TNFα during PMN transmigration and after
infection, which are both well known as NET-inducers (Brinkmann, 2004; Steinmann et al., 2013). Surprisingly, the NET-releasing cells and the percentage of NET-associated DNA in the „CSF compartment” did not significantly differ between cells infected with S. suis wildtype and the different nuclease mutants (Fig. 3). Despite the fact that a S. suis-mediated DNase activity in the used 1% FBS cell culture medium was detectable (Fig. S5). Furthermore, the quantitative recovery of streptococci from the „CSF compartment” was independent of the strain and the presence or absence of neutrophils (Fig. 4). This is in contrast to our previous results, showing SsnA-mediated evasion of antimicrobial NET activity by its degradation (de Buhr et al., 2014). Thus, we hypothesize that the NETs in the „CSF compartment” are somehow protected against degradation by streptococcal nuclease.

Recently, the antimicrobial peptide LL-37 was identified as a NET stabilizing factor preventing the degradation of NETs by bacterial nucleases (Neumann et al., 2014). Interestingly, we were able to detect LL-37 positive neutrophils nesting on the plexus epithelial barrier during the in vitro experiments (Fig. 5). Thus, it may be hypothesized, that LL-37 is highly produced by the transmigrating neutrophils or released by plexus epithelial cells. This might contribute to stabilization of NETs against S. suis nuclease. In good correlation to this hypothesis, increased transcript expression of epithelial cell barrier after transmigration of neutrophils was detected (Fig. 6). One important function of LL-37 in the S. suis-infected CSF compartment might be the protection of NETs against bacterial degradation. Such an overexpression of LL-37 might explain the described lack of phenotype of the DNase mutants in interaction with NETs in the lower compartment.

Besides LL-37, also other antimicrobial peptides like β-defensins are described to induce formation of NETs and/or to stabilize NETs against bacterial nucleases (Neumann, et al., 2014; Neumann et al., 2014). β -defensins are also expressed by the choroid plexus and may also contribute to the phenotype shown here (Nakayama et al., 1999; Kraemer et al., 2011; Williams et al., 2012).

In conclusion, we demonstrated for the first time the formation of NETs in neutrophils following S. suis through the choroid plexus epithelium in vitro. This “NETosis” in the „CSF compartment” was associated with entrapment of streptococci and in accordance with NET structures detected in the CSF of piglets with S. suis meningitis in vivo. Interestingly, SsnA activity in CSF was detectable, but attenuation of the isogenic ssnA mutant in survival in the “CSF compartment” was not recorded in the presence of NETs in the in vitro model.

Besides their antimicrobial effect NETs have been shown to also exhibit detrimental effects when released upon infections, especially when the host is not able to eliminate NETs by its own nucleases (Hakkim et al., 2010). As an example, neurotoxic effects for NETs are described (Allen et al., 2012). Further, in the course of sepsis the injury of endothelium and tissues by platelet TLR-4 activated NETs was demonstrated (Clark et al., 2007). However, our study shows for the first time that NETs are formed in CSF upon infection and provides evidence for the formation of NETs by neutrophils breaching the choroid plexus epithelium when encountering S. suis in the CSF compartment. Still,
future studies are needed to evaluate the overall role of NET-formation in the CSF for bacterial survival and/or immobilization and finally its contribution to the pathogenesis of meningitis in vivo.

METHODS

**Bacterial strains and growth conditions.**

*S. suis* strain 10 is a virulent serotype 2 strain that has been used by different groups for mutagenesis and experimental infections of pigs (Smith *et al.*, 1999; Baums *et al.*, 2006; Baums *et al.*, 2009; Baums and Valentin-Weigand, 2009). In addition to the wildtype, three knock out mutants were used: *S. suis* strain 10ΔssnA (de Buhr *et al.*, 2014), strain 10ΔendAsuis and strain 10ΔendAsuisΔssnA (de Buhr *et al.*, 2015), designated as ΔssnA, ΔendAsuis and ΔendAsuisΔssnA or ΔΔ in the figures of this paper, respectively. Streptococci were grown on Columbia agar plates with 6% sheep blood or in Bacto™ Todd Hewitt broth (THB).

**Animal experiments.**

The analyzed samples (cerebrospinal fluid) drawn from experimentally infected piglets immediately after euthanasia were collected within a study conducted for other reasons (Seele *et al.*, 2015). The protocol for this animal experiment was approved by the Committee on Animal Experiments of the Lower Saxonian State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit [LAVES]; permit no. 33.14-42502-04-12/0965). This study was conducted in strict accordance with the principles outlined in the EU Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/labanimals/legislation_en.htm) and the German Animal Protection Law (Tierschutzgesetz).

**Purification of porcine neutrophils.**

In our institute the blood collection from healthy piglets is registered at the Lower Saxonian State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under no. 33.9-42502-05-11A137, and was performed in line with the recommendations of the German Society for Laboratory Animal Science (Gesellschaft für Versuchstierkunde) and the German Veterinary Association for the Protection of Animals (Tierärztliche Vereinigung für Tierschutz e.V.) (http://www.gv-solas.de). Purification of porcine neutrophils was conducted from heparinized blood as previously described with Ficoll Hypaque 1077 (Biochrom) and hypotonic lysis of erythrocytes (Benga *et al.*, 2008).
Purification of human neutrophils.
Neutrophils were isolated from fresh blood of healthy donors with the PolymorphPrep system (Axis Shield) as previously described (Köckritz-Blickwede et al., 2010) and resuspended in RPMI 1640 (without phenolred, PAA).

DNase activity in CSF.
The DNase activities of CSF from healthy and diseased pigs were tested by mixing 1 µg calf thymus DNA (Sigma) as substrate and 30 µl of CSF. This suspension was incubated for 22 h at 37°C. Visual examination of DNA was conducted after 1 % agarose gel electrophoresis and staining of DNA with Roti®Safe (Gelstain ready-to-use, Roth).

Nuclease assay with calf thymus DNA in CSF.
Bacterial nuclease activity leading to degradation of eukaryotic DNA in CSF was determined using calf thymus DNA as substrate. Therefore, 2 µg calf thymus DNA (Sigma) was added to 35µl CSF of healthy piglets and mixed with 35 µl of a bacteria suspension. This suspension was made of bacteria grown to an OD_{600nm} of 0.6, centrifuged (2600 x g, 5 minutes), washed twice with PBS and adjusted to an OD_{600nm} of 1.0 with PBS. Samples were incubated for 4 h at 37°C. Visual examination of DNA was conducted as described before.

DNase activity inhibition in CSF with an antiserum against rSsnA.
The inhibition of DNase activity in 35 µl CSF of piglet 9884 was conducted by a 1 h pre incubation on a rotator with 3.5 µl polyclonal rabbit serum raised against rSsnA (Gómez-Gascón et al., 2012). As control CSF was incubated with rabbit pre-immune serum. Furthermore, CSF samples inoculated with S. suis wt or 10ΔendAsuisΔssnA to monitor bacterial growth (see below) were used as control. All samples were supplemented with 1 µg calf thymus DNA (Sigma) after the pre-incubation. This suspension was incubated for 24 h at 37°C. Visual examination of DNA was conducted as described above.

Determination of bacterial growth in CSF of piglets.
To analyze bacterial growth in CSF, bacteria from overnight cultures were pelleted via centrifugation (2600 x g, 5 min) and washed twice with PBS. Bacteria were adjusted to OD_{600} 0.2 and 10 µl of this suspension were incubated in 1 ml CSF of healthy piglets at 37°C. The CFU was determined by plating on blood agar plates.
Results

Cell culture.
We used a model of the BCSFB with human choroid plexus papilloma cells (HIBCPP), described and characterized previously with modifications (Ishiwata et al., 2005; Schwerk et al., 2012). HIBCPP were cultured in DMEM/HAM’s F12 1:1 (Invitrogen) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 4 mM L-glutamin, 5 µg/ml insulin and 15 % of heat inactivated fetal calf serum. Cells were seeded with a density of 8 x 10^4 / filter and cultured for two days at 37°C and 8% CO_2 on flipped over trans-well filter inserts (pore diameter 3.0 μm, pore density 2.0 x 10^6 pores per cm^2, growth area 0.33 cm^2, Greiner Bio-one) in a medium-flooded 12-well plate. The filters were turned into a 24-well plate and the cells were cultured for three further days. After reaching a transepithelial electrical resistance (TEER) of 200Ω x cm², measured with volt-ohm meter using the Millicell-ERS system (Millipore), the filters were washed in DMEM/F12 1:1 without phenolred (Invitrogen) and antibiotics but containing 5 µg/ml insulin and 1% heat inactivated fetal calf serum (1% FBS media) and cultured for up to two additional days before infection. At the day of infection the TEER was between 330Ω x cm², and 530Ω x cm², and filters were put into fresh 1 % FBS media.

Determination of the specific bacterial load and DNase activity in the „CSF compartment“ of the BCSFB model.
For the establishment of a stable cell culture system after infection with bacteria the TEER was measured at different time points. In addition paracellular permeability was measured with dextran-TexasRed (MW 3000, Sigma) as previously described (Steinmann et al., 2013). The CFU in the lower compartment was determined by plating dilutions on blood agar plates at different time points. For the assessment of nuclease activity 2 µg calf thymus DNA (Sigma) was added to 50 µl supernatants and incubated for 4 h at 37°C. Visual examination was conducted as described above.

Nuclease activity in cell culture medium.
To determine nuclease activity in the cell culture medium 2 µg calf thymus DNA (Sigma) was added to 35 µl 1% FBS cell culture medium mixed with 35 µl of bacteria suspension. This suspension was made of bacteria grown to an OD₆₀₀nm of 0.6 and used as described previously. Samples were incubated for 4 h at 37°C. Visual examination was conducted as described above.

NET induction in cell culture medium.
The NET induction of freshly isolated human neutrophils in presence of 1 % FBS cell culture medium or RPMI was verified as previously described (de Buhr et al., 2014). Briefly, neutrophils were seeded on poly-L-lysine-coated coverslips (12 mm, 1.5 thickness, neuVitro) and stimulated with 25 nM PMA for 4 h at 37°C and 5 % CO₂. As control neutrophils were incubated without PMA. Afterwards samples were fixed with 4 % paraformaldehyde and NET-formation was visualized as described below.
Results

Cell culture infection and transmigration assay.
For infection of the HIBCPP cell culture all filters were put into 1 ml fresh 1 % FBS medium and infected with \( S. \) \( suis \) wt or one of the three mutants (MOI = 10) in 500µl 1 % FBS medium in the blood (upper) compartment for 2 h at 37°C and 8 % CO\(_2\). Then, the blood compartment was emptied and refilled with 500 µl 1 % FBS medium containing pure human neutrophils (MOI = 4) or only medium and the plate was incubated for further 4 h. As indicated at different time points the CFU was determined in the lower compartment and TEER was measured. For additional immunofluorescence assays, poly-L-lysine coated glass coverslips (12 mm, 1.5 thickness, neuVitro) were inserted before the infection into the lower compartment and the plate was centrifuged (250 x g, 8 min) at the end of experiment. The supernatant from the CSF (lower) compartment was collected and the coverslips were fixed with 4 % paraformaldehyde for later NET staining as described below. Furthermore, the complete CSF compartment was harvested and the content was directly used for DNase activity tests or immediately put into liquid nitrogen and stored at -80°C for later analysis. To distinguish between NET / cell released, NET bound and intracellular components (Pico Green assay) the content of the CSF compartment was centrifuged (500 x g, 10 min) and the supernatant was used untreated (released components). The pellet was resuspended in 125µl HBSS and mixed with micrococcal nuclease (0.5 U/ml, Worthington) and incubated for 10 min at 37°C. The reaction was stopped with 5 mM EDTA (pH = 8) and the sample again centrifuged (500 x g, 5 min). The supernatant was used in the assays (NET bound components) and the pellet was resuspended in 125 µl HBSS and used in the subsequent Picogreen assay (intracellular components). At the end of the experiment the filters were fixed 10 min in 4% paraformaldehyde and washed in PBS three times.

Immunofluorescence in vivo and in vitro of NETs.
For the co-staining of NETs and bacteria (cell culture) a previously described protocol was used (de Buhr \textit{et al.}, 2015). Briefly, samples were permeabilized and blocked. Then, the samples were incubated with a mouse monoclonal-antibody against DNA/histone 1 (1:5000 in PBS containing 1 % BSA and 0.05 % Tween-20, MAB3864, Millipore) for 1 h to visualize the NETs and a rabbit anti-\( S. \) \( suis \) antibody (1:500) (Beineke \textit{et al.}, 2008). The secondary staining was performed using a goat anti-rabbit Alexa 633-conjugated antibody (1: 500; Invitrogen) or a goat anti-mouse Alexa 488-conjugated antibody (1: 500; Invitrogen). After washing, all coverslips were embedded in ProLong\textsuperscript{®} Gold antifade reagent with DAPI (Invitrogen). For the NET staining, \textit{in vivo} CSF samples were stained with a monoclonal antibody against histone H2A-H2B-DNA complex as previously described (Berends \textit{et al.}, 2010). Briefly, after blocking and permeabilization, neutrophils were incubated over night at 4°C with a mouse monoclonal antibody against DNA/histone 1 (1:5000 in PBS containing 2% BSA, 0.2% Triton X-100; MAB3864, Millipore). An Alexa 488-conjugated goat anti-mouse antibody (diluted 1:1000 in PBS containing 2 % BSA, 0.2 % Triton X-100; Thermo Scientific) was used as secondary
Results

Samples were recorded using a Leica TCS SP5 confocal inverted-base fluorescence microscope with a HCX PL APO 40× 0.75-1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each sample in the cell culture assay, 13 randomly selected images per independent experiment were acquired and used for quantification of the neutrophil number and the induced neutrophils (Fig. S2). For each sample in the in vivo detection of NETs in CSF all slides were screened completely for NET-releasing neutrophils.

Pico Green assay.
For quantification of NETs a spectrofluorometric method was conducted using the Quant-iT™ Picogreen® dsDNA kit (Invitrogen) as previously described (von Köckritz-Blickwede et al., 2010).

Filter immunostaining of LL-37.
After washing the filters the membrane was removed and stained for LL-37. In general, the filters were stained in 250 µl and washed in 500 µl liquid floating. The filters were blocked and permeabilized for 60 min in PBS containing 10% goat serum and 0.5% TritonX-100. Incubation of antibodies was conducted in PBS containing 2% BSA. The filters were incubated with a polyclonal rabbit-anti-LL-37 antibody (1:300, Richard Gallo) or a polyclonal mouse-anti-LL-37 antibody (1:50, Hycult biotech) combined with a polyclonal rabbit anti-MPO antibody (1:300, Dako) as indicated for 1 h at room temperature. After washing steps as secondary antibody AlexaFluor 488 goat anti-rabbit (1:1000, Invitrogen), AlexaFluor 488 goat anti-mouse (1:500, Molecular Probes) and AlexaFluor 633 goat anti-rabbit (1:500, Invitrogen) was used as indicated. Together with the secondary antibodies a staining of the cytoskeleton with phalloidin Alexa 546 (1:100, Invitrogen) and the DNA with Hoechst (1:250000, Invitrogen) was conducted for 45 min at room temperature. After staining, the filters were washed and embedded in ProLong Gold antifade without DAPI (Invitrogen). Samples were recorded as described above.

In vivo detection of NETs in CSF.
Experimental infected piglets showing clinical signs of meningitis or healthy piglets as control were anesthetized with 2 mg azaperon (Stresnil; Janssen, Neuss, Germany)/kg intramuscularly and 10 mg ketamine-hydrochloride (Ursotamin; Serumwerk, Bernburg, Germany)/kg intramuscularly. CSF samples were drawn by puncture of the subarachnoidal space (lumbosacral or occipital) with a 21 G x 2” Ø 0.8 x 50 mm needle (FINE-JECT®, Henke Sass Wolf) and collection in a 5 ml syringe (Norm-Ject, Henke Sass Wolf). Immediately after harvest the cells were counted by using trypan blue and a Neubauer chamber. The cells were seeded with an adjusted cell number of 2 x 10⁵ cells / 100 µl on poly-L-lysine-coated (0.01% solution for 20 min; Sigma Aldrich) coverslips (8 mm; Thermo Scientific) in a 48 well plate (Nunc, Thermo Scientific) or in case of low cell numbers in a 96 well
Results

plate with glass bottom (Falcon, MatTek Corporation). If needed cells were diluted in Hank’s Balanced Salt Solution (HBSS) without calcium and magnesium (GIBCO®). After seeding, the plate was centrifuged at room temperature for 5 min (370 x g) followed by a fixation with 4 % paraformaldehyde (PFA).

Real-time PCR from reverse transcribed RNA extracted from neutrophils and HIBCPPs.

RNA was extracted from neutrophils and HIBCPPs with the RNeasy Micro Kit (Quiagen) as described in the user’s manual. For the neutrophil RNA preparation three samples were pooled per experiment. Real-time PCR of reverse transcribed RNA (qRT-PCR) was designed to analyze expression of *Il-37* gene and the housekeeping gene *gapdh*. The respective primers are listed in Table S1. The qRT-PCR was conducted as previously described (Willenborg et al., 2011) with the following modified program: initial denaturation at 95 °C for 20 min and at least 45 cycles of denaturation at 95 °C for 25 s, annealing at 55 °C for 30 s, and amplification at 72 °C for 20 s. Products were verified by melting curve analysis and 1.5% agarose gel electrophoresis. Data were normalized to a non-regulated housekeeping gene (*gapdh*). The relative ΔCT values were determined for expression of the *Il-37* gene. CT is the cycle number at the chosen amplification threshold, ΔCT = C_T gene (Il-37) − C_T Reference (gapdh) and ΔΔCT = ΔC_T sample − ΔC_T Calibrator. The fold change in expression (2^-ΔΔCT) was calculated as the readout parameter. In case of the PMNs the calibrator was non-transmigrated neutrophils with contact to the cell layer. For the analysis of the HIBCPPs the calibrator is a cell layer with 4 h contact to non-transmigrated neutrophils in the "CSF compartment”.

Statistical analysis.

Data were analyzed by using Excel 2010 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Normal distribution of data was verified by Kolmogorov Smirnov normality test (GraphPad software) prior to statistical analysis. Differences between two groups were analyzed by using a one-tailed paired Student’s t-test in case of normal distributed data. Analysis of more than one group was conducted by one-way ANOVA using Dunnett’s or Tukey’s multiple comparison test. Probabilities lower than 0.05 were considered significant, lower or equal than 0.1 as statistically noticeable.

ACKNOWLEDGEMENTS

Both senior authors (Maren von Köckritz-Blickwede and Christoph G. Baums) contributed equally to this work. We thank H. Smith (DLO-Lelystad, Netherlands) for *S. suis* strain 10. We thank Kristin Laarmann (University of Veterinary Medicine Hannover, Germany) for her technical assistance and Tina Basler (University of Veterinary Medicine Hannover, Germany) for supporting qRT-PCR analysis. We also acknowledge Lydia Gómez-Gascón and Immaculada Luque (both Universidad de
Córdoba, Spain) for providing the rabbit serum against SsnA. We thank Richard Gallo (University of California San Diego, USA) for providing the rabbit serum against LL-37.

Nicole de Buhr was funded by a fellowship of the Ministry of Science and Culture of Lower Saxony (Georg-Christoph-Lichtenberg Scholarship) within the framework of the PhD program "EWI-Zoonosen" of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGNI). Ariane Neumann was funded by a fellowship of the Akademie für Tiergesundheit (AfT) and a fellowship from the PhD-program "Animal and Zoonotic Infections" of the HGNI. This project was supported by the Niedersachsen-Research Network on Neuroinfectiology (N-RENNT) of the Ministry of Science and Culture of Lower Saxony.

REFERENCES


Results


Results


The influence on NET-induction by the CSF sampling method in the animal experiment was imitated with an *in vitro* sampling of blood derived porcine neutrophils. This experiment was conducted to exclude, that the sampling method of CSF induce NETosis. Firstly neutrophils were adjusted in CSF of healthy piglets to $2 \times 10^5$ cells/ ml. Secondly the neutrophils were seeded on glasslips directly or after drawing up into a syringe with two different needles used in the animal experiment. The activation of neutrophils was analysed by immunofluorescence microscopy. In two independent experiments per sample 4 coverslips were analysed with 3 pictures each. In total per run 12 pictures were analysed. 12 to 18 % of the neutrophils were NET releasing in all sampling methods. The results of A and B were generated from two independent experiments.

**Fig. S1: CSF sampling does not induce NETosis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5´-3´) forward</th>
<th>Primer sequence (5´-3´) reverse</th>
<th>Amplicon length (bp)</th>
</tr>
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<tr>
<td>gapdh</td>
<td>GGTCATCCATGACAAACTTTGG</td>
<td>CCAAATTCGTGTCATACCAGG</td>
<td>471</td>
</tr>
<tr>
<td>ll-37</td>
<td>TGGTTGAGGGTCACTGTCCCC</td>
<td>GCCCAGGTCTCAGCTACAAG</td>
<td>260</td>
</tr>
</tbody>
</table>
Fig. S2: Target-method and sample pictures.
The black circle with the red dots represents a coverslip and the spots where pictures were generated by the target-method to get representative pictures per one coverslip. For each coverslip 13 pictures were made spread over the full size of the coverslip. One example result of 13 pictures demonstrates the wide range of neutrophils and the area of NETs or the NET releasing neutrophils.
Fig. S3: The isogenic *S. suis* mutants 10ΔsstA, 10ΔendAsuis and 10ΔendAsuisΔsstA are not attenuated in growth in porcine CSF *in vitro*.

To determine if the growth of *S. suis* in CSF is influenced by the mutations, growth experiments were conducted. The specific bacterial contents (CFU/µl) were determined at the indicated time points. All strains show similar growth behavior. All data are shown as mean ± SD of four independent experiments.
Fig. S4: NET formation in presence of 1% FBS cell culture medium is possible.
As a control, that neutrophils could be activated and release NETs in presence of the cell culture medium (DMEM/F12 1:1 without phenolred, Invitrogen) containing 1% FBS, human neutrophils were stimulated with PMA in presence of different media. Compared to RPMI and cell culture media without FBS the neutrophils were activated in a same amount after 4h of PMA stimulation. In addition in the absence of PMA the number of activated neutrophils was low (Fig. S3 A, B). The NET
formation in presence of 1% FBS cell culture medium was quantified by immunofluorescence microscopy in comparison to RPMI and 0% FBS cell culture medium. In all three media a PMA stimulation was conducted and compared to an unstimulated group. Per experiment 2 coverslips with 3 pictures each were analysed. A and B showing the results of two independent experiments and the legend in A also applies to B. C and D are representative pictures of neutrophils incubated in 1% FBS cell culture medium without PMA stimulation (C) and with PMA stimulation (D).

**Fig. S5: Bacterial associated DNase activity is detectable in cell culture medium.**

DNase activity of whole washed bacteria harvested at exponential growth phase (OD600nm of 0.6) in presence of 1% FBS cell culture medium (DMEM/F12 1:1 without phenolred, Invitrogen). The indicated *S. suis* strains were analysed by 1% agarose gel electrophoresis after incubation of calf thymus DNA for 4 h at 37°C. Bacteria were adjusted to the same OD in PBS and then mixed with the medium. In all DNase assays, controls (ctr) were incubated with PBS. Panels show representative examples of two independent experiments that all gave similar results.
4 General Discussion

*S. suis* can infect piglets as well as humans and the infection is often accompanied with CNS disorders: severe meningitis is a typical diagnosed pathology. The *S. suis* meningitis is characterized by a high number of neutrophils and pleocytosis is regarded as an indication for bacterial infection of the CSF (Deisenhammer et al., 2006). In an acute bacterial meningitis up to 20000 cells per microliter could be found and most of them are granulocytes (Dörner, 2001). Nevertheless, these high numbers of neutrophils in the CNS together with the whole immune system are not always able to cope with the infection and an antibiotic treatment is usually required. As neutrophils are described to undergo different mechanisms to fight against pathogens, the question arises how *S. suis* is able to survive in the presence of all these host defense mechanisms. As mentioned in the introduction the capsule protects against phagocytosis (Charland et al., 1998; Smith et al., 1999) and lipoteichoic acid D-alanylation helps the bacteria to evade neutrophil killing (Fittipaldi et al., 2008b). The survival of *S. suis* in the CSF is not only influenced by factors of the pathogen but is also determined by the host. Noteworthy, the opsonic and bactericidal activity of neutrophils is described to be suboptimal in the CSF and phagocytosis seems to be inefficient (Tunkel & Scheld, 1993). However, besides phagocytosis an additional fundamental innate immune defense mechanism of neutrophils has been discovered, namely the formation of NETs. NETs are extracellular trap-like fibers of DNA and histones which are able to entrap and occasionally kill various pathogens (Brinkmann, 2004; Fuchs et al., 2007). Some pathogens were identified to degrade NETs by DNases and thus escape from NETs. Especially in streptococci, numerous DNases have been identified and their role in degradation of NETs was demonstrated [Chapter 1, Table 2]. Here, in the first two parts of the study *S. suis* DNases were identified and their role in evasion of NETs analyzed [Chapter 3.1 and 3.2]. Comparing isogenic mutants to the respective wildtype strain, the interaction of *S. suis* nuclease and NETs was investigated. Further, the conditions for activity were defined [Chapter 3.1 and 3.2]. After the geno- and phenotypical characterization of the isogenic mutants, this thesis focused on host-pathogen interaction in the CSF compartment. Thus, general features of DNases as well as the role of the DNases in this particular compartment were investigated [Chapter 3.3].

4.1 Investigation of NET evasion factors in *S. suis*

Two candidates of *S. suis* DNases were analyzed in the present study [Chapter 3.1 and 3.2]. The first DNase SsnA was already previously described in the literature to exhibit magnesium and calcium dependent activity (Fontaine et al., 2004). The second DNase analyzed in this study was identified through functional analysis of an isogenic mutant in this study [Chapter 3.2]. This DNase, designated EndAsuis, shows high homology to EndA of *S. pneumoniae* (Beiter et al., 2006). The functions of DNases were tested first individually with respective isogenic constructed mutants and secondly by construction of a double mutant to unravel possible synergistic effects [Chapter 3.1 and 3.2].
DNases were able to degrade eukaryotic and bacterial DNA as well as NETs. However importantly, both DNases are active under different and specific conditions and a synergistic effect was not detectable. Thus, the respective growth dependent activity as well as biochemical conditions with different pH and ion concentrations were analyzed. Interestingly, SsnA is upregulated and more active in the stationary growth phase, whereas EndAsuis activity was only detectable in the exponential growth phase. In addition, only SsnA is released and active in the supernatant. Depending on the ions and pH value, differences in activity were recorded. The optimal condition for SsnA is in the presence of 1.5 mM calcium and 1.5 mM magnesium at neutral pH. Conversely, EndAsuis is active in the presence of just 1.5 mM magnesium at an acidic pH and is a membrane-bound DNase. The activity of both DNases under different growth and biochemical conditions might be beneficial for S. suis, as this invasive pathogen is confronted with different conditions in the course of infection in the different body compartments. S. suis enters different environmental conditions upon breaching different barriers of the host, as outlined in figure 4-1. One possible infection route of S. suis is from the upper respiratory tract through the blood to the CNS (Gottschalk & Segura, 2000). Accordingly, expression of the capsule is thought to be regulated dependent on the environmental conditions during the infection. It was discussed that S. suis downregulates capsule expression at the beginning of an infection and therefore the adhesion on epithelial cells is increased. After entering the bloodstream S. suis upregulates capsule expression and the capsule protects effectively against phagocytosis (Gottschalk & Segura, 2000). Regarding the nuclease activity, it may also be hypothesized that the different nucleases might act at different sites of the body to evade the host innate immune defense against the infection. As an example, the in vivo function of EndA of S. pneumoniae was demonstrated in murine lung tissue after intranasal infection. Thus, the authors suggested, that EndA promotes the bacterial spreading from local sites to the lung and from there to the bloodstream by degrading NETs (Beiter et al., 2006). Interestingly, in this present work [Chapter 3.2] it was demonstrated that EndAsuis is also able to efficiently degrade NETs. The findings about the biochemical conditions and growth phase for activity of EndAsuis leads to the hypothesis that EndAsuis is involved in the early part of S. suis infection in the upper respiratory tract. The upper respiratory tract is described as a possible entry for S. suis in the course of an infection. As mentioned above, the downregulation of capsule for enhanced adhesion to the epithelium, the low pH (< 7.0) of the nasal mucus and the airway surface liquid of the upper respiratory tract may constitute conditions for good EndAsuis-mediated NET escape. As the investigation of the respiratory tract was not part of this study, further studies are needed to confirm this hypothesis. In addition, only little is known about how S. suis interacts with the epithelial cell layer of the upper respiratory tract and invades the host during the course of an infection.
The upper respiratory tract is covered in the nose with nasal mucus and then with an airway surface liquid. *S. suis* is thought to cross the mucosal barrier, enter the bloodstream, proliferate in the blood and breach the blood-CSF barrier in the pathogenesis of *S. suis* meningitis. \(^a^\)(Beule, 2010), \(^b^\)(Bodem *et al.*, 1983), \(^c^\) (Moini, 2015), \(^d^\)(Andrews *et al.*, 1994), \(^e^\)(Kandel *et al.*, 2000), \(^f^\)(Heinritzi & Schillinger, 1996).

After crossing the barriers of the upper respiratory tract, *S. suis* enters the blood and is exposed to different environmental conditions in the course of tissue invasion. The nuclease deletion mutants survived with similar efficiency compared to the wildtype in the blood of humans and piglets (additional unpublished Figure 4-2). Therefore, it can be assumed that both investigated DNases play no or only a subordinate role during bacteremia. However, Clark and colleagues first published the detection of NETs in blood. This formation was detected in the course of sepsis after an infection with *E. coli* (Clark *et al.*, 2007) and *S. suis* can also lead to sepsis. Furthermore, circulation and replication inside the blood is one-step in the pathogenesis of meningitis. Nevertheless, if NETs are involved in the host defense against *S. suis* bacteremia is not known. Nevertheless, *S. suis* is able to cross the brain barriers and to survive and replicate further in the CSF. In accordance with the pH in CSF, we were only able to demonstrate an SsnA-dependent degradation in the presence of porcine CSF [Chapter 3.3]. However, as mentioned above this is maybe a reason why it is a benefit for *S. suis* to express at least two different DNases. It can be hypothesized that *S. suis* is well equipped in different body compartments with two DNases active under different conditions, which are both able to degrade NETs.
In addition to NET degradation, an SsnA-mediated protection against neutrophil and NET-mediated antimicrobial activity was demonstrated [Chapter 3.1]. This mediated protection was not observed for EndAsuis. As DNases are not only involved in degradation of NETs, both DNases that were investigated might exhibit additional functions. For example Sda1 of *S. pyogenes* M1T1 was identified to degrade bacterial DNA and to alter TLR-9-mediated recognition of *S. pyogenes* by immune cells (Uchiyama et al., 2012). In the present study it was also shown that both *S. suis* DNases are able to degrade bacterial DNA and therefore might play a role in modulating TLR-9 recognition. However, it was demonstrated for nuclease A of *Streptococcus agalactiae*, that the availability of DNase does not automatically negatively affect TLR-9-mediated activation of neutrophils (Derré-Bobillot et al., 2013). Possible reasons for this are the fact that maybe additional cofactors are needed, or that the degradation of bacterial DNA was not efficient enough. Currently, we can only speculate if SsnA or EndAsuis are able to influence the TLR-9 dependent recognition of *S. suis* as further experiments need to be conducted.

Figure 4-2 Expression of the two extracellular nucleases SsnA and EndAsuis by *S. suis* is not crucial for survival in porcine and human blood.

Survival factors of *S. suis* wt and mutants in human (a) and porcine (b) blood *ex vivo* (freshly drawn heparinized blood). C.f.u. was determined at t = 0 min, t = 60 min and t = 120 min. No significant differences between survival factors of *S. suis* wt and mutants from stationary growth phase were observed. All data are shown as mean ± SEM of three independent experiments; one-way ANOVA, Tukey’s multiple comparison test.
Multilocus sequence typing (MLST) analysis revealed that *S. suis* serotype 2 strains might belong to different clonal complexes (King *et al.*, 2002). The DNase activity in *S. suis* serotype 2 was investigated in different sequence types (STs) by Haas and colleagues. Three major STs were part of that study: ST 1, ST 25 and ST 28 (Haas *et al.*, 2014). *S. suis* P1/7 used in the present study is ST 1. The ST 25, which exhibits moderate virulence in mice, showed no DNase activity. Conversely, the highly virulent (in mice and humans) ST 1 and the non-virulent (in mice) ST 28 showed DNase activity (Fittipaldi *et al.*, 2011; Haas *et al.*, 2014). A comparative analysis of *ssnA* gene in the three tested STs identified the loss of a 14 bp region in ST 25 resulting in an inactive truncated protein. Furthermore, a mutant (M2D) derived from a bank of mutants was DNase-deficient and less virulent in an amoeba model. By using a plasmid rescue procedure, it was found that the mutation was in the *ssnA* gene. Macrophages stimulated with this mutant showed a decreased secretion of pro-inflammatory cytokines and MMP9 in comparison to the wildtype. Therefore, DNase activity seems to be beneficial for *S. suis* in the course of an infection, but not necessary since ST 25 shows moderate virulence in the absence of DNase activity. It was hypothesized that SsnA may contribute to the virulence of *S. suis* by increasing the inflammatory response (Haas *et al.*, 2014). The results from Chapter 3.1 demonstrated that SsnA is a NET evasion factor and protects against the antimicrobial activity of NETs. Nevertheless, it can be speculated that SsnA is involved in other parts of the host-pathogen interaction.

Induction of NETs by *S. suis* was for the first time demonstrated within the study described in Chapter 3.1 of this thesis (de Buhr *et al.*, 2014). After this NET induction by three different *S. suis* strains was also demonstrated by Zhao *et al.* 2015. They claimed that the capsule structure of *S. suis* serotype 2 strains plays a role in escaping NETs and the NET-associated killing. They based this statement on experiments with an isogenic capsule mutant compared to different virulent strains with a capsule. A non-phagocytic killing as well as a phagocytosis killing was significantly higher in the capsule mutant compared to the wildtype strains. Furthermore, they showed that a significantly higher number of the capsule mutant was trapped in NETs (Zhao *et al.*, 2015). However, the authors did not prove if an indirect effect of the capsule deletion could an explanation for these phenotypes. For example, the expression and activity of SsnA or EndAsuis might be influenced. Therefore, further experiments investigating DNase activity in the capsule mutant and the wildtype strains are crucial. Analysis of gene regulation of *ssnA* and *endAsuis* in the mutant compared to the wildtype strains would also be important. Furthermore, a determination of the c.f.u. in a classical NET-killing assay as described in chapter 3.1 and 3.2 was not conducted. This assay is crucial for conclusions drawn on NET-killing or NET-mediated antimicrobial activity. As these experiments are missing, it appears unjustified to state that the capsule is a NET evading factor. However, it was demonstrated that the capsule of *S. pneumoniae* protects against NET trapping in accordance with the study by Zhao and colleagues. However, in contrast to the hypothesis of Zhao and colleagues, it was experimentally clearly
demonstrated that the pneumococcal capsule is not required for resistance to NET-mediated killing (Wartha et al., 2007).

Additional to investigate whether the capsule of *S. suis* is a NET evading factor and required for resistance to NET-mediated killing, in this thesis two factors involved in the host-pathogen interaction have been identified. Taken together, the present study demonstrates that *S. suis* features at least two DNases (SsnA and EndAsuis) active under different conditions. Considering CSF, only SsnA activity was detectable. Further, both DNases degrade NETs, but only SsnA is active in the supernatant and required for resistance to NET-mediated killing. This differential activity of the two nucleases could be a benefit for *S. suis* and help to spread in the host during the course of an infection.

### 4.2 NETosis in the *S. suis*-infected CSF compartment

In the third part of the study NETosis was studied in a model of the *S. suis*-infected CSF compartment. To investigate the *in vitro* situation in the CSF compartment a modified model of the BCSFB was used. Using cell culture models to identify host cell–pathogen interaction is a reproducible and valid method, which helps to reduce numbers of experimental infected animals. This model facilitated the analysis of the formation of NETs and the function of both *S. suis* DNases in a *S. suis*-infected CSF compartment in more detail. Furthermore, experiments were designed without externally added NET inducers to read out NETosis in neutrophils entering the infected CSF compartment. The roles of the two DNases were investigated in an environment comprised by choroid plexus epithelium similar to the CSF compartment of the host. Importantly, this study demonstrated NETosis in neutrophils following *S. suis* through the choroid plexus epithelium for the first time [Chapter 3.3]. This NETosis might be determined by induced cytokines and chemokines levels. The release of pro-inflammatory cytokines and chemokines from human BMEC is induced after *S. suis* serotype 2 infection: IL-6, IL-8 and MCP-1 are released from BMEC after *S. suis* infection, whereas human umbilical vein endothelial cells (HUVEC) were not affected by *S. suis* (Vadeboncoeur et al., 2003). Another group analyzed the release of pro-inflammatory cytokines and chemokines of the porcine CPEC after *S. suis* infection in an *in vitro* model (Schwerk et al., 2011). In this analyses Leukemia inhibitory factor (LIF), TNFα, IL-6 and IL-8 were identified to be induced during the course of an *S. suis* infection. TNFα and IL-8 are known as NET inducers (Brinkmann, 2004; Fuchs et al., 2007; Gupta et al., 2010; Keshari et al., 2012), whereas nothing is known about LIF which was identified by microarray analyses of *S. suis* infected porcine CPEC cells as highly upregulated. Interestingly, LIF is known to activate the Janus kinase/signal transducer which is an activator of transcription (JAK/STAT) and the mitogen activated protein kinase (MAPK) cascade (Suman et al., 2013). Furthermore the raf-MEK-ERK pathway, as part of the MAPK cascade, is described to be involved in the NET release after IL-8 activation (Hakkim et al., 2011). Therefore, LIF might also be involved in NET induction in the CSF compartment, but further studies are needed to confirm this hypothesis. During the course of a CSF
compartment infection, the changing conditions in cytokine and chemokine profiles might play a decisive factor regarding NET induction.

As demonstrated in Chapter 3.1 and 3.2 both DNases are able to degrade NETs. SsnA is crucial for bacterial survival during NETosis in vitro. Nevertheless in the cell culture experiments, neither differences in the amount of NETs nor in bacterial loads were detectable when comparing wildtype and mutant infected CSF compartments. One explanation could be that factors produced by the transmigrated neutrophils or from the choroid plexus epithelium protect NETs against bacterial DNases. Possible candidates are AMPs known as effector molecules of the innate immune system and located in granules of phagocytes and on epithelial surfaces (Ganz, 2003; Zasloff, 2002). Recently, the cathelicidin peptide LL-37 was described to induce NETs and protect them against degradation by bacterial nucleases (Neumann et al., 2014a, b). LL-37 is stored as a pre-pro-peptide in the granula of neutrophils and in other cells of the innate immune system (Agerberth et al., 1995; Cowland et al., 1995; Guani-Guerra et al., 2010; Di Nardo et al., 2003). Additionally, LL-37 is produced by other host cells for example in epithelial cells of the urinary and respiratory tract, the choroid plexus epithelial cells, colon enterocytes and keratinocytes (Bals et al., 1998; Brandenburg et al., 2008; Frohm et al., 1997; Kim et al., 2003; Zasloff, 2006). After the enzymatic cleavage of the inactive propetide by elastase or other proteases, a cathelin part and the C-terminal AMP LL-37, which is the active peptide, is formed [Fig. 4-3].

During inflammation LL-37 is described to be induced in epithelial cells of the skin and the lung (Frohm et al., 1997; Mendez-Samperio et al., 2008). Further, LL-37 activity was significantly higher in CSF of patients with bacterial meningitis compared to the serum samples and CSF samples of healthy patients or patients with virus infected CSF (Brandenburg et al., 2008). In the same study rat cathelin-releated AMP was detected in choroid plexus, glia and ependymal cells by immunohistochemistry in a meningitis model with infant rats. As discussed in Chapter 3.3, one role of
LL-37 in the *S. suis* infected CSF compartment could be a protection of NETs against bacterial DNases. LL-37 protects NETs against degradation by bacterial nuclease (Neumann *et al.*, 2014b). Immunofluorescence microscopy and realtime PCR analyses of the HIBCPP cells gave indications that *S. suis* infection followed by neutrophil transmigration increased the LL-37 signal on protein and mRNA level. Nevertheless, if the neutrophils and / or the HIBCPP cells produce LL-37 was not clearly differentiated. Despite washing the filters with the HIBCPP cells, neutrophils are nesting on the filter and might be responsible for the respective signal. Neumann and colleagues demonstrated further that human β-defensin 3 (hBD-3), besides LL-37, protects host DNA against degradation by *Staph. aureus* nuclease (Neumann *et al.*, 2014b). These findings underline a possible neuroimmune function of AMPs in the CNS. In a review in 2012 it was already discussed that β-defensins and other AMPs are involved in neuroimmune function and neurodegeneration (Williams *et al.*, 2012). Interestingly, human β-defensin 1 and 2 (hBD-1 and hBD-2) are expressed in cells of the choroid plexus epithelium and other cells of the brain in the course of inflammation (Hao *et al.*, 2001; Nakayama *et al.*, 1999). It was suggested that hBD-1 is secreted into the CSF. Furthermore, Kraemer *et al.* showed that hBD-1 is a NET inducer (Kraemer *et al.*, 2011).

In another study, the bactericidal activity of porcine neutrophils was tested after PMA stimulation in the supernatant *in vitro*. Porcine cathelicidin myeloid antimicrobial peptide 36 (PMAP-36) and lactotransferrin were identified by mass spectrometry. However, in these supernatants only a non-pathogenic *E.coli* K-12 strain was reduced in number. Other swine pathogens like *S. suis* were not efficiently killed. The authors discussed that this is possibly due to the absence of neutrophils in this experiment. (Scapinello *et al.*, 2011).

Importantly, NETs with fibers were detected in CSF from piglets with a *S. suis* meningitis, but only at a low level. The time point chosen for NET detection is crucial as the formation and degradation is a flowing process. A suitable fixation in tissue and/ or body fluids and an intravital documentation needs special techniques like the multiphoton microscopy. Numerous publications present data on NETosis *in vitro*. For the understanding of the physiological and pathophysiological relevance of NETs *in vivo* studies need to be conducted. An intravital record of NETs in atherosclerosis was described using two-photon microscopy. NETs were visualized in this study after an intravenous injection of propidium iodide, a viable cell impermeable DNA-binding dye, in monocyte-depleted Lysm<sup>egfp<sub>e</sub>egfp<sub>f</sub></sup> Apoe<sup>-/-</sup> mice (Doring *et al.*, 2012; Megens *et al.*, 2012). In another study an *in vivo* documentation and characterization of NETs was conducted using multiphoton microscopy in a mouse model (Tanaka *et al.*, 2014). Taking the two studies, an intravital NET detection was successful inside the carotid bifurcation and in hepatic sinusoids of the liver, postcapillary venules of the cecum and pulmonary capillaries of the lung. In the literature, data are also available about *in vivo* NET determination during infection. The first *in vivo* detection was published in 2004 after analysis of tissue sections from experimental shigellosis in rabbits and spontaneous appendicitis in humans by immunofluorescence microscopy (Brinkmann, 2004). Furthermore, *in vivo* staining of NETs from mouse skin biopsies was
achieved after an infection with an *sdal* deletion mutant of *S. pyogenes* M1, but not after infection with the wildtype (Buchanan *et al.*, 2006). After infection with *S. pneumoniae* a NET detection in murine pneumococcal pneumonia was successful (Beiter *et al.*, 2006). Another study investigated NETs and *Staph. aureus* nuclease and identified the presence of NETs decorated with AMPs in *Staph. aureus*-infected lungs (Berends *et al.*, 2010). In all aforementioned publications, NET detection was possible, but NET fibers as found in *in vitro* experiments have not been demonstrated.

Yipp *et al.* made a groundbreaking study on the *in vivo* relevance of NETs in 2012. The authors showed live imaging techniques of NET formation and revealed that neutrophils, after an acute infection, induced NET release, are viable and are able to phagocytose and crawl. It was discussed that a population of neutrophils in the early stage of infection retain the ability to multitask (Yipp *et al.*, 2012). Based on this and some other publications one year later, Yipp and Kubes discussed how vital NETosis is. They named the two different mechanisms that lead to NETs the ‘suicidal’ and the ‘vital’ NETosis. The ‘suicidal’ NETosis is a synonym for the lytic NET release described in the introduction [Chapter 1.3.1]. The ‘vital’ NETosis is in contrast characterized by the rapid release of NETs and the possibility for the PMNs afterwards to phagocyte or degranulate (Yipp *et al.*, 2012). A study by Clark *et al.* (2007) showed that after LPS-stimulation, TLR-4-activated platelets induced NETs and after this NET release the PMNs remain intact because the SYTOX Green access to the PMNs was restricted. Independent of the study by Clark *et al.* a fast NET release was identified in an additional further study. This NET release was TLR-2 and complement receptor 3 (CR 3) dependent. It was described as a rapid (5-60 min) NET release by a vesicular mechanism. In contrast to the described lytic pathway, the plasma membrane was not disrupted. Prior to NET release, the multilobular nucleus first became quickly rounded and condensed. This process is followed by a degradation of the nuclear envelope and after this only chromatin is visible in the cell center. Vesicles containing DNA concentrate near the plasma membrane and then the NET release occurs. This mechanism is oxidant-independent, which is in contrast to NET release by the cell lysis mechanism. Interestingly, this rapid NET release was demonstrated after an induction by *S. aureus* and supernatants of overnight cultures containing factors like Panton-Valentine leukocidin (Pilszczek *et al.*, 2010; Yipp & Kubes, 2013; Yipp *et al.*, 2012). In addition a third mechanism of NET release is only poorly understood and described in one study as a NET release by mitochondrial DNA (Yousefi *et al.*, 2009). A schematic diagram illustrating cell lysis (‘suicidal’) and vesicular (‘vital’) mechanism is given in Figure 4-4. No further distinctions were made, for the *in vitro* as well as *in vivo* NETs detected in Chapter 3.3 in the CSF compartments. NET inducers in the *S. suis*-infected CSF compartment could be the bacteria or interleukins and therefore stimuli for ‘suicidal’ and ‘vital’ NETosis are present. The NET induction by *S. suis* was demonstrated in Chapter 3.1 and 3.2 *in vitro*. Additionally, IL-8 is well described as a NET inducer (Brinkmann, 2004) and in two studies IL-8 was identified as a marker for an acute bacterial meningitis (Ostergaard *et al.*, 1996; Pinto Junior *et al.*, 2011). In both studies, IL-8 was measured in patients with and without bacterial meningitis and the values were compared to control groups higher. In the study by
Ostergaard et al., the mean IL-8 concentration was between 6-10 µg/l in septic meningitis. In aseptic meningitis the IL-8 concentration was around 1.7 µg/l and in non-meningitis patients 0.03 µg/l. Human neutrophils stimulated with 10 µg/l IL-8 for 30 minutes released the same amount of NETs as neutrophils stimulated with 25 nm PMA in vitro (Gupta et al., 2005).

Figure 4-4 Steps in formation of NETs: ‘suicidal’ and ‘vital’ NETosis

(a) IL-8
(b) MPO
(c) PAD4
(d) NE
(e) PMA
(f) NADPH
(g) PKC
(h) Raf
(i) MEK
(j) ERK
(k) O₂
(l) O₂⁻
(m) H₂O₂
(n) E. coli
(o) TLR4
(p) CR3
(q) TLR2
(r) S. aureus
(s) CD11a

‘suicidal’ NETosis
‘vital’ NETosis

Figure 4-4 Steps in formation of NETs: ‘suicidal’ and ‘vital’ NETosis

a - e The ‘suicidal’ NETosis starts after a stimulation by e.g. PMA or IL-8. At the end of the oxygen dependent protein kinase C (PKC) – raf-MEK-ERK pathway hydrogen peroxide is present (b). An oxygen independent pathway via PAD4 is also possible (c). In both pathways the cytosol and the content of the nucleus mixes together and after 3-4 h the outer membrane ruptures and a NET release into the extracellular space occurs. f - j The ‘vital’ NETosis has been described to be rapid (5-60 min). It can be induced by a platelet TLR-4 activation after interaction with CD11a on neutrophils or over complement receptor 3 (CR 3) and TLR-2 for Gram-positive bacteria. The nucleus becomes rounded and decondensed (g) Vesicles with DNA are formed (h-i) and via nuclear budding NETs are released (j). The outer membrane is intact at the end and an intact anuclear neutrophil remains after NET release. MPO and NE are localized in the NETs structures.
Prior to this work, only little was known about NETs from porcine neutrophils, but here we have proven the release of NETs by porcine neutrophils in Chapter 3.1. To compare our results with human cells, we stimulated the porcine PMNs with the well-known stimulator PMA. Nevertheless, in our hands different concentrations of PMA led only to NET release from a few porcine PMNs compared to human PMNs. However as demonstrated in other animals, PMA is not always the best NET inducer (Muñoz Caro et al., 2014; Silva et al., 2014). The reasons why neutrophils of different species might be good or poorly stimulated by PMA are not clear. The present study includes in vivo detection of NETs with NET fibers in S. suis-infected CSF of piglets, although DNase activity was detectable in this compartment [Chapter 3.3]. An in vivo detection of NETs after infection of the CSF, which is a fluid body compartment, was never described before. It is known from some studies that NETs are also formed during the course of sepsis under fluid conditions in the blood (Clark et al., 2007).

A further interesting aspect is that the CSF compartment is part of an immune-privileged site in the body (Shechter et al., 2013). The entry of immune cells to this site is strictly regulated by the three brain barriers, and the function of neutrophils after crossing a barrier like the BCSFB is still poorly understood. Taken together, the detection of NETs with fibers in vivo demonstrates that in general porcine neutrophils can produce NETs in the immune-privileged CSF compartment during the course of a S. suis infection. However, the mechanism leading to NET formation in this special compartment was not investigated. Interestingly, blood derived neutrophils in the presence of CSF from healthy piglets are blocked from forming in NETs [Fig. 4-5]. One explanation could be that components in the CSF of healthy piglets suppress NET formation in general, because NETs are described to be toxic and especially neurotoxic (Allen et al., 2012). The injury of endothelium and tissues was moreover demonstrated by platelet TLR-4 activated NETs in the course of sepsis (Clark et al., 2007), and it was suggested that epithelial cell damage is due to NET-bound proteases (Marin-Esteban et al., 2012). The brain barriers might protect the CNS against these detrimental effects in the absence of infection (Engelhardt & Coisne, 2011) by a limited entrance of immune cells. The few numbers of PMNs in CSF of healthy animals are maybe guardians under control. However, one can only speculate if transmigration of neutrophils through the BCSFB triggers the cells to form an extracellular trap. A further aspect in the course of an infection of the CSF compartment is that the barriers break down and infiltration occurs. In addition, chemokines and cytokines increase in the course of infection and some are known as NET inducers. On the other hand factors that might block NET release in CSF of healthy piglets are unknown. However, it is known that human immunodeficiency Virus-1 (HIV) blocks NET release over CD 209 on dendritic cells. This leads to an IL-10 release and suggests suppression of the NET release by certain interleukins (Saitoh et al., 2012). Interestingly in one study about cytokines in CSF of humans, IL-10 was measured. The IL-10 value is around 10 pg/ml in CSF of healthy humans (Peterson et al., 2015). Furthermore it was noted that IL-10 limits inflammation in the brain (Strle et al., 2001). Therefore, it can be hypothesized, that IL-10 blocks NETosis in CSF of healthy individuals.
Taken together the published data lead to the hypothesis that the levels of different interleukins can modulate neutrophils in the CSF compartment. Specific interleukins such as IL-10 might block NET release in the uninfected CSF compartment and others such as IL-8 might induce NET release in the course of septic meningitis. The amount of cytokines in the CSF compartment inducing NET could be determined with analyses of cell culture used in chapter 3.3 as well as with CSF of S. suis infected piglets. The established cell culture model could be used to understand the detailed function of the detected NETs in the CSF compartment and the relevance for the host-pathogen interaction in the course of CNS infections. One possible experiment to clarify this open question could be a combination of cell culture with live cell immunofluorescence imaging in the CSF compartment. With this combination, the time kinetics and interaction of neutrophils and S. suis in the CSF compartment could be investigated after crossing the BCSFB. As such documentation is impossible in vivo, the model established in this thesis allows questions about the specific host cell-pathogen interaction in the infected CSF compartment to be answered. However, in vivo experiments are needed to clarify the function and the role of both DNases in pathogenesis.

Figure 4-5 CSF of healthy piglets blocks NET release
The NET releasing cells of porcine blood derived neutrophils were determined by immunofluorescence microscopy in presence of RPMI or CSF of healthy piglets. A stimulation was conducted with PMA, S. suis wildtype (WT) or S. suis ΔendAΔsuisΔssnA (ΔΔ) as indicated. A significant difference in NET releasing cells was identified in RPMI by stimulation with PMA after 1 and 3 h (Fig. 6-3 a) and after stimulation with bacteria comparing WT and mutant after 3 h (Fig. 6-3 b). In presence of CSF the percentage of NET releasing cells was lower than in RPMI incubated samples and independent of the stimulus. All data are shown as mean ± SD of 3 independent experiments. Per experiment 2 coverslips with 3 pictures each were analysed. Statistical differences were analysed by one-way paired Student’s t-Test (* p < 0.05, ** p < 0.005).
4.3 Concluding remarks

In this study the interaction of S. suis with NETs was investigated. S. suis induces NETs in porcine and human neutrophils and expresses at least two DNases characterized in this study: SsnA and EndAsuis [Chapter 3.1 and 3.2]. Both DNases degrade NETs, but only SsnA is active in the supernatant and is crucial for a resistance against antimicrobial activity of NETs under the chosen experimental conditions [Chapter 3.1]. The two characterized S. suis DNases are active under different pH and ion conditions [Chapter 3.2]. The differences in DNase specific activity under variable conditions might be important for this pathogen in order to cope with different environmental conditions in the course of an infection. S. suis expresses SsnA and EndAsuis in the exponential growth phase and mainly SsnA in the stationary growth phase.

EndAsuis functions similar to EndA of S. pneumoniae and the activity depends on the active center (DRGH-motif). An analysis of rEndAsuis_H165G rescued through imidazol treatment and a 3D structure modeling of EndAsuis demonstrated that the high amino acid identity and homology between EndAsuis and EndA leads to structural similarity and similar functions [Chapter 3.2].

Expression of SsnA and EndAsuis is not crucial for growth in human and porcine blood as well as in the CSF [Chapter 3.3 and 4.1]. Importantly, SsnA is released and active in the CSF during S. suis infection of the meninges in piglets [Chapter 3.3].

Interestingly, NET formation with fibers occurs in S. suis-infected CSF compartments despite nuclease activity [Chapter 3.3]. Accordingly, neutrophils form NETs after breaching the S. suis infected BCSFB barrier in vitro. These NETs were shown to trap S. suis. Therefore, it was hypothesized that NET formation functions as a host-defense mechanism against S. suis [Chapter 3.3].

The NET stabilizing factor LL-37 was visualized in close proximity to MPO-containing neutrophils nesting on HIBCPP cells, which represent the BCSFB in the in vitro model. Realtime PCR analysis showed an increase of LL-37 transcript expression in S. suis-infected HIBCPP cells compared to uninfected cells [Chapter 3.3]. Thus, it is hypothesized that LL-37, produced by the CPECs and/ or the transmigrating neutrophils, protect NETs in the S. suis-infected CSF compartment against S. suis DNases.

Taken together this study revealed phenotypes of S. suis elicited by the expression of two DNases. Degradation of NETs and protection against the antimicrobial activity of NETs are important SsnA-mediated phenotypes involved in the interaction with human and porcine neutrophils. NETs are formed in the S. suis infected CSF compartment and these NETs are able to trap bacteria. The role of these NETs in protective immunity needs further investigation. The final concluding remarks are illustrated in figure 4-6 and summarize the main findings of this project.
Figure 4-6 Illustration of the concluding remarks

[A] SsnA and EndAsuis are expressed by S. suis [B]. Distributing via the blood S. suis reaches the CSF by crossing the choroid plexus [C]. In porcine CSF a SsnA activity was detectable. Further in vivo analysis demonstrated NET formation in the S. suis infected CSF compartment of piglets in presence of DNase activity [D]. Using an in vitro model of the BCSFB NET formation was detectable in the S. suis infected CSF compartment, but both DNases do not lead to a benefit for the invading bacteria. However, in vitro experiments with human and porcine neutrophils demonstrated a NET induction by S. suis, an entrapment of S. suis and a degradation of NETs and eukaryotic DNA by SsnA as well as EndAsuis [F]. Green scissors = EndAsuis, pink scissors = SsnA; D. BCSFB, E inverted cell culture system of the BSCFB: 1 blood [D] / blood compartment [E], 2 CSF [D] / CSF compartment [E], 3 choroidal endothelium, 4 epithelial cells [D] / human choroid plexus papilloma cells [E], 5 TJs, 6 filter membrane of insert, 7 S. suis, 8 neutrophil granulocyte
Summary

5 Summary

Nicole de Buhr

Interaction of *Streptococcus suis* with neutrophil extracellular traps (NETs)

*Streptococcus* (*S.*) *suis* causes as infectious agent suppurative meningitis in humans and piglets, which is characterized by a pleocytosis in the cerebrospinal fluid (CSF). In neutrophils, a novel fundamental innate immune defense mechanism against different pathogens has been identified: the formation of neutrophil extracellular traps (NETs). NETs are formed by neutrophils following extracellular ejection of DNA in combination with antimicrobial molecules and bind, disarm and kill pathogens. Different pathogens express NET evasion factors like DNases. NETs might play different roles in diseases and the mechanisms leading to NET release are only poorly understood.

In this study, the interaction of *S. suis* and NETs was investigated using immunofluorescence microscopy. Especially the function of two DNases was investigated. Phenotypic analysis was conducted using *in frame* deletion mutants, which were investigated comparatively to the wildtype under various conditions. Important phenotypes in the pathogen-host interaction were identified. In the first part of this thesis [Chapter 3.1] it was demonstrated for the first time that *S. suis* induces NETs, is entrapped in NETs and degrades NETs. Furthermore, this work revealed that the known secreted nuclease A (*SsnA*) is involved in NET degradation and that this nuclease protects *S. suis* against the antimicrobial activity of NETs. The second part [Chapter 3.2] includes the identification of a novel endonuclease A of *S. suis* (*EndAsuis*), which is involved in NET degradation during the exponential growth phase. *EndAsuis* was shown to require Mg\(^{2+}\) for activity whereas *SsnA* needed Mg\(^{2+}\) and Ca\(^{2+}\).

In the third part [Chapter 3.3] NET formation in the *S. suis*-infected CSF compartment was examined. In an inverse transwell model, human neutrophils followed *S. suis* by transmigration through the epithelium formed by choroid plexus papilloma cells in the lower compartment, which represents the CSF-compartment. Importantly, NETs were detected in the CSF compartment by immunofluorescence microscopy and biochemical determination of dsDNA. Furthermore, it was visualized that streptococci were entrapped in NETs. However, the number of detectable NETs and the bacterial survival did not depend on the expression of the bacterial nucleases *SsnA* and *EndAsuis*. The detected induction of the NET protecting factor LL-37 might explain this result. These *in vitro* results were corroborated by the detection of NETs in CSF samples of *S. suis* infected piglets.

In conclusion, the results suggest that NET formation and degradation might play a role in the pathogenesis of *S. suis* meningitis.
6 Zusammenfassung

Nicole de Buhr

**Interaktion von Streptococcus suis mit neutrophilen extrazellulären Netzen (NETs)**


7 Literature


8 Appendix

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Acknowledgment

First of all I would like to thank my supervisors Prof. Dr. Christoph G. Baums and Prof. Dr. Maren von Kückritz-Blickwede for giving me the opportunity to work on an extremely interesting and very exciting project. The combination of both experts on the one hand for *S. suis* and on the other hand for NETs was the best I could ever dream of. All rising questions were discussed and solved together. Thank you Christoph for the directly support in Hannover and the remote support from Leipzig. Thank you Maren, that you always had an open ear and our fruitful discussions via email at night. This combination was a key factor for the success of this awesome project.

I would particularly like to thank Prof. Dr. Peter Valentin-Weigand for giving me the opportunity to perform my PhD study at the Institute for Microbiology, for his constructive discussions and the relaxed working atmosphere.

Further, I would like to thank the external member of my supervisory group Prof. Dr. Horst Schrotten (Department of Pediatrics, Pediatric Infectious Diseases, Medical Faculty Mannheim, Heidelberg University) for his interest in my work and the helpful comments and suggestions. Furthermore, I want to thank him for the fruitful cooperation with his lab in Mannheim. A special thank goes to the very helpful email and telephone help of Carolin Stump-Guthier for answering all questions around the cell culture system.

I would like to thank Prof. Dr. Hassan Y. Naim for his interest in my project and giving me the opportunity to perform parts of my study at the Department of Physiological Chemistry.

A special thank goes to the members of the “Streptoconga” office at the Institute of Microbiology: Daniela Willms, Tina Basler and Jana Seele. Daniela you are the best good mood propagator! Thank you for all jokes we made together, for all wise sayings of you, the unforgettable discussions about the serious things in the lab and everyday life. Tina you are the best telephone joker for all lab questions! I thank you a lot for all your helpful comments, ideas, answers and discussions about lab and life. Jana you are the good soul of the Streptoconga’s! Thank you very much for a wonderful time, I was lucky to have you as my office neighbor and colleague. All discussions about science were fruitful and helpful independent of the question.

Furthermore, I would like to especially thank Nantaporn Ruangkiattikul, Kristin Laarmann and Matthias Stehr. Bo you are the best Thai teacher: ทีวานนีหืดหู่ติ่ง (Tum one nee hai dee te sud). Thank you very much for numerous burst of laughter I got and the training of my sixth sense. Kristin thank you for all your helping hands in the lab and especially for conducting the RRRR in the cell culture. Every time when I was searching for help, you were there. Matthias thank you for all the philosophical and scientific discussions and your always-helpful nature. You are the Master of the ÄKTA and you helped me lot to get one key element in the second manuscript: the purified rEndAsuis_H165G. Unforgotten is our discovery of the 90-degree language and the first word: BALIMALAM.
Many thanks go to the present and former members of the Institute of Microbiology for the excellent working atmosphere and the helping hands and discussions: Ketema Merga Abdissa, Yenehiwot Berhanu Weldearegay, Elke Eckelt, Marcus Fulde, Anika Glenz, Ralph Goethe, Katrin Hail, Charlotte Heede, Lena-Maria Hillermann, Nina Janze, Anna Koczula, Aline Kostka, Jochen Meens, Andreas Nerlich, Dennis Päglow, Silke Schiewe, Anja Schulze, Maren Seitz, Anna Seydel, Kira van Vorst and Jörg Willenborg.

Antonio Eramo and Werner Scharnhorst thank you very much for all your help in the lab everyday life. Furthermore, I would like to thank Claudia Otto and Jörg Henstorf for preparing the media and autoclaving lab stuff.

I also want to give thanks to Sabine Baumert and Jörg Merkel for their organization and technical support.

Particularly I would like to thank two members of the Department of Physiological Chemistry: Ariane Neumann and Friederike Reuner. Ariane thank you a lot for your helping hands in my project, your helpful comments and the time we spend together in the lab. For answering all questions at the microscope, in the lab and in all general things. Your help strongly supported my work. Rike thank you very much for organizing everything in the Department of Physiological Chemistry in searching for blood donors, blood collection, evaluation of experiments, ordering of lab stuff and always be there for answering questions.

In addition, I would like to thank all blood donors of the Department of Physiological Chemistry, without you this work would not have been impossible! Thank you very much for all your blood! And I thank Graham Brogden for the proofreading.

Also, I would like to thank Prof. Dr. Andreas Beineke (Department of Pathology, University of Veterinary Medicine Hannover) for histopathological examination of porcine samples and Prof. Dr. Karl-Heinz Waldmann for collaboration as well as Klaus Schlotter and colleagues for drawing blood samples from pigs (Clinic of Swine and Small Ruminants, Forensic Medicine and Ambulatory Service, University of Veterinary Medicine Hannover).

Furthermore I have to thank the Ministry of Science and Culture of Lower Saxony for financial support of my work with a Georg-Christoph-Lichtenberg Scholarship within the framework of the PhD program ‘EWI-Zoonosen’ of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology and Translational Medicine (HGNI) the Niedersachsen-Research Network on Neuroinfectiology (N-RENNT).

Finally and most importantly I want to thank the center of my life: my family and friends. I thank especially my parents, that they were always there, giving me advice and supporting me and encouraging me when I was feeling sad. I would also like to thank Eugen for his love, every time believing in me, calming me down, joking with me and always being on my side.

Moreover, I thank my unforgotten brother Oliver somewhere in the sky as a twinkling star. I dedicate this work to you!

BALIMALAM