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Interaction of *Streptococcus suis* with blood immune cells: Influence of the complement system and modification of lipoteichoic acids

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To my wife
Isabell

Contents

List of abbreviations

Adr	Attenuator of drug resistance
Ag	Antigen
AMP	Antimicrobial peptide
ApdS	Aminopeptidase
ApuA	Amylopullulanase
BBB	Blood brain barrier
BCR	B cell receptor
B-CSFB	Blood-cerebrospinal fluid barrier
BMEC	Brain microvascular endothelial cells
CcpA	Catabolite control protein A
CD	Cluster of differentiation
CFU	Colony forming units
CNS	Central nervous system
CPEC	Choroid plexus epithelial cell
CPS	Capsular polysaccharide
CR	Complement receptor
CSF	Cerebrospinal fluid
DC	Dendritic cell
dltA	Gene encoding for a ligase to D-alanylate lipoteichoic acids
DNA	Deoxyribonuclease
e.g.	*Exempli gratia*
ELISA	Enzyme-linked immunosorbent assay
EndASuis	Endonuclease A of *Streptococcus suis*
FBPS	Fibrinogen-binding protein
Fc	Fragment crystallizable (region of antibodies)
Fg	Fibrinogen
Fhb/Fhbp	Factor H-binding proteins
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
h	Hour
H_2O_2	Hydrogen peroxide
hFg	Human fibrinogen
HP	Hypothetical protein
Ide_{Ssuis}	Immunoglobulin M-degrading enzyme of *Streptococcus suis*
Ig	Immunoglobulin
IgdE	Immunoglobulin G protease of *Streptococcus suis*
IL	Interleukin
LL-37	Human cathelicidin, 37 amino acids with two leucines at N-terminus
LPS	Lipopolysaccharide
LPXTG	Leucine-proline-X(variable)-threonine-glycine
LTA	Lipoteichoic acid
MHC	Major histocompatibility complex

mg	milligram
ml	milliliter
MLST	Multilocus sequence typing
mm	millimeter
MRP	Muramidase-released protein
MS	Mass spectrometry
MurM/MurN	Aminoacyl-tRNA Ligases (peptidoglycan synthesis)
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAG	N-acetyl-D-glucosamine
NAM	N-acetyl muramic acid
NET	Neutrophil extracellular trap
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain containing 3
NOX	NADH oxidase
OatA	O-acetyltransferase
OFS	Serum opacity factor of *Streptococcus suis*
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PCV-2	Porcine Circo Virus 2
PG	Peptidoglycan
PgdA	Peptidoglycan N-acetylglucosamine deacetylase
pH	Potential of hydrogen
PR-39	proline-arginine rich antibacterial peptide 39
PRR	Pattern Recognition Receptor
PRRSV	Porcine reproductive and respiratory syndrome virus
PrsA	Parvulin-like peptidyl-prolyl isomerases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.	*Streptococcus*
SAO	Surface antigen one
SIV	Swine influenza virus
SLA	Swine leukocyte antigen
SLY	Suilysin
SntA	*Streptococcus suis* cell surface protein
SOD	Superoxide dismutase
SodA	Superoxide dismutase A of *Streptococcus suis*
SOF	Serum opacity factor of *Streptococcus pyogenes*
Srt	Sortase
Ssads	Adenosine synthase
SsnA	*Streptococcus suis* secreted nuclease A
ST	Sequence type
STK	Serine/threonine kinase

StkP	Serine/threonine kinase of *Streptococcus pneumoniae*
STP	Serine/threonine phosphatase
STSLS	Streptococcal toxic shock-like syndrome
T4SS	Type IV-like secretion system
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VCP	Vaccinia virus complement control protein
VirD4	Protein component of type IV-like secretion system
WTA	Wall teichoic acid
wt	Wild type

1 Introduction

Streptococcus (S.) suis is an important pathobiont distributed worldwide in commercial pig farms (GOYETTE-DESJARDINS et al. 2014, VÖTSCH et al. 2018). It frequently colonizes the upper respiratory tract of pigs, but can also cause severe diseases like meningitis, septicemia, arthritis, serositis, endocarditis and pneumonia. Piglets at the age of 5 to 10 weeks are most susceptible to disease (GOTTSCHALK and SEGURA 2019). *S. suis* is a zoonotic pathogen with special relevance in Asia, as it caused large outbreaks in China (LUN et al. 2007) and is a very common cause for adult meningitis in Vietnam, Thailand and Hong Kong (HO et al. 2011, GOYETTE-DESJARDINS et al. 2014). Bacteremia is a hallmark of invasive *S. suis* infections and an adequate immune response in blood is crucial for the hosts well fare, while the aim of the pathogen is to overcome this response, survive and disseminate in different tissues to find new habitats for proliferation. Therefore, *S. suis* has developed numerous virulence-associated factors to avoid mechanisms of the immune response (BAUMS and VALENTIN-WEIGAND 2009, FITTIPALDI et al. 2012). On the other side the immune system is also comprised of a huge array of innate and adaptive mechanism involving cellular and humoral components. In porcine blood neutrophil granulocytes are the most numerous phagocytes (NERBAS 2008) and build the first line of defense against *S. suis* infection (BLEUZÉ et al. 2021). They are capable of phagocytosis and the production of reactive oxygen species and they possess granules containing antibacterial peptides and enzymes. Their major role lies in either killing pathogens inside a phagolysosome or by releasing antimicrobial substances into the extracellular space to target pathogens that were not phagocytosed, but they also contribute to communication with other cells of the immune system (MAYADAS et al. 2014). Another important leukocyte subset in blood are monocytes. Also capable of phagocytosis, but to a lesser extent, their main role lies in the secretion of cytokines and chemokines to orchestrate the communication between immune cells in case of disease and homeostasis (AUSTERMANN et al. 2022). Importantly, the cellular immune response is completed by humoral factors like the complement system, which builds a bridge between innate and adaptive immunity. It is composed of more than 50 proteins and among others, has the function to recognize and opsonize pathogens (HAJISHENGALLIS et al. 2017). This thesis investigates the ability of porcine blood immune cells to kill *S. suis* by phagocytosis and oxidative burst and the importance of IgM and the complement system in the process. In this context, the ability of *S. suis* to avoid certain aspects of the innate immune system, especially the complement cascade, by modifying the alanine content of lipoteichoic acids in its cell wall and the resulting influence on the interaction of *S. suis* with blood leukocytes is focused.

2 Literature

2.1 Streptococcus suis

2.1.1 Characteristics and classification

Streptococci belong to the family of *Streptococcaceae*, which is placed within the order *Lactobacillales*. They are non-sporulating Gram-positive cocci with a size of up to 2 µm and are considered homofermentative lactic acid bacteria, that ferment sugars to lactic acid as sole fermentation product. As all lactic acid bacteria streptococci grow under anaerobic conditions but are not sensitive to the presence of oxygen, making them aerotolerant anaerobic bacteria (MADIGAN et al. 2013). Most streptococci physiologically colonize the skin and mucous membranes, but some pathogenic species can cause disease in animals and humans. Differentiation of species is based on biochemical reactions, hemolytic activity and serologic specificity (PATTERSON 1996, SELBITZ et al. 2015). Carbohydrates of the cell wall with antigenic potential were used by Rebecca Lancefield to classify streptococci into Lancefield groups in the nineteen-twenties and thirties (LANCEFIELD 1933). This classification is still frequently used, while hemolytic ability allows a more general classification of the streptococci into α-hemolytic species, showing incomplete hemolysis with the formation of methemoglobin, β-hemolytic species, showing complete hemolysis and non-hemolytic species (SELBITZ et al. 2015).

The species *Streptococcus suis* (*S. suis*) was named after its main host the pig. It grows under aerobic as well as anaerobic conditions and is commonly cultivated on sheep blood agar plates, where it forms grey glossy colonies of up to 1 mm size and shows α-hemolysis. *S. suis* is a frequent colonizer of the tonsils and the upper respiratory tract (ARENDS et al. 1984, KATAOKA et al. 1991, BAELE et al. 2001, LUQUE et al. 2010, ARAI et al. 2018) and can also be found in the gastro-intestinal (DEVRIESE et al. 1994) and the genital tract (AMASS et al. 1996) of pigs worldwide . On the one hand this leads to a large number of healthy carrier pigs, while on the other hand, *S. suis* also causes invasive infections including meningitis, septicemia, endocarditis, (poly-)arthritis and bronchopneumonia (VÖTSCH et al. 2018, GOTTSCHALK and SEGURA 2019). Especially young piglets, from 5 to 10 weeks of age, are at risk to develop the disease, when maternal immunity declines (CLOUTIER et al. 2003, GOTTSCHALK and SEGURA 2019). Therefore, *S. suis* can be considered an important pathobiont (VÖTSCH et al. 2018) and is found in up to 100% of commercial pig farms (GOYETTE-DESJARDINS et al. 2014). It causes high economic losses in the swine industry worldwide and is one of the most important porcine pathogens (SELBITZ et al. 2015, NEILA-IBÁÑEZ et al. 2021). As a zoonotic pathogen, *S. suis* also poses a threat to humans working or living in close contact to pigs (ARENDS and ZANEN 1988, YU et al. 2005, DUTKIEWICZ et al. 2017). Especially in Asia numerous infections of humans have been recorded with two major outbreaks in 1998 and 2005 in China, associated with a partly fatal streptococcal toxic shock-like syndrome (STSLS) (YU et al. 2006, LUN et al. 2007). In Vietnam, *S. suis* is the main cause of adult meningitis (HO et al. 2011, FITTIPALDI et al. 2012).

S. suis is a very heterogenic species that is classified by serotyping (ELLIOTT 1966, PERCH et al. 1983) and multilocus sequence typing (MLST) (KING et al. 2002). Classifying *S. suis* into Lancefield groups is not reliable and is not considered useful (GOTTSCHALK et al. 2010). Serotyping relies on differences in the capsular polysaccharide and 29 serotypes are presently known (SEGURA et al. 2017), whereby serotype 2 is worldwide the most frequently isolated from diseased pigs (GOYETTE-DESJARDINS et al. 2014). In Europe serotype 9 is even more frequently found in association with disease in pigs (GOYETTE-DESJARDINS et al. 2014). By sequencing of seven household genes in MLST, *S. suis* is also classified into more than 900 sequence types (ST) (KING et al. 2002), demonstrating the extremely high genetic diversity of this species. In the consequence serotype 2 strains can be divided into numerous sequence types with the predominant types being ST1, ST7, ST20, ST25 and ST28 (GOYETTE-DESJARDINS et al. 2014). Of these, ST1 consists of many virulent strains and is often associated with diseases in Europe (SCHULTSZ et al. 2012, GOYETTE-DESJARDINS et al. 2014), while a highly virulent single-locus variant of ST1, classified as ST7, was found to be responsible for the two large Chinese outbreaks in 1998 and 2005 (YE et al. 2006). In North America ST25 and ST28 are frequently isolated from diseased pigs or humans (FITTIPALDI et al. 2011).

2.1.2 Epidemiology and Pathology

In addition to pigs, *S. suis* isolates have been found in horses, ruminants, cats, dogs, and deer (STAATS et al. 1997, DEVRIESE et al. 1992). It is suspected to be an intestinal commensal in these species, but few cases of disease have also been described (DEVRIESE and HAESEBROUCK 1992, DEVRIESE et al. 1993, ROELS et al. 2009, OKURA et al. 2019, MUCKLE et al. 2010, MUCKLE et al. 2014, OKWUMABUA et al. 2017).

While transmission in the pig population is thought to take place horizontally via the airways by aerosols or nasal contact and vertically during birth due to vaginal colonization of the sows (AMASS et al. 1997, CLOUTIER et al. 2003), in human cases the entry through skin lesions or the consumption of raw pork products are likely infection routes (HO et al. 2011, FONGCOM et al. 2001). Therefore, in porcine but also in human disease, carrier pigs, colonized with potentially virulent strains, are an important source of *S. suis* infection (LUQUE et al. 2010). But also environmental contamination was confirmed and live vectors like flies and fomites like water, dust and feces can take part in the transmission of the pathogen (STAATS et al. 1997, CLIFTON-HADLEY and ENRIGHT 1984, ENRIGHT et al. 1987, ROBERTSON et al. 1991).

In 2015 FERRANDO et al. investigated the ability of *S. suis* to infect piglets after oral application with the result that 2 out of 15 piglets developed clinical signs and in 40% of challenged piglets *S. suis* was found in the mesenteric lymph nodes. However, a gastric-acid resistant capsule was used to apply *S. suis* in this experiment (FERRANDO et al. 2015). In the field the oral route of infection does not seem to play an important role in pigs and another study suggested, that *S. suis* is not able to survive in feed or the porcine stomach (WARNEBOLDT et al. 2016).

In the porcine population putative predisposing risk factors for the outbreak of disease are stress factors like crowding, bad ventilation, transport, vaccination and mixing of animals with

an age gap of more than two weeks (STAATS et al. 1997, GOTTSCHALK and SEGURA 2019). As reviewed by OBRADOVIC et al. (2021), co-infections with porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV-2) or *Bordetella bronchiseptica* can lead to a higher susceptibility of piglets to *S. suis* disease (OBRADOVIC et al. 2021, GALINA et al. 1994, LIN et al. 2015, WANG et al. 2020, VÖTSCH et al. 2021). The first expression of a clinical disease is usually an increase in body temperature up to 42.5 °C, likely caused by bacteremia which is followed by different possible clinical signs depending on the affected body sites. Central nervous system (CNS) dysfunctions, such as convulsions or opisthotonos, are caused by meningitis. Arthritis goes along with swollen joints, lameness and sometimes the incapability to rise from a lying position. Less specific signs like depression and inappetence often accompany the disease (CLIFTON-HADLEY and ALEXANDER 1988). Less frequently *S. suis* infection also leads to pneumonia, often associated with one of the mentioned co-infections (REAMS et al. 1995, OBRADOVIC et al. 2021). In severe cases, peracute death without particular clinical signs can also occur (WINDSOR and ELLIOTT 1975, CLIFTON-HADLEY and ALEXANDER 1988). Histopathology of affected organs mainly shows fibrinosuppurative lesions (WILLIAMS and BLAKEMORE 1990, REAMS et al. 1994, MADSEN et al. 2002). In piglets surviving acute *S. suis* disease, chronic disease or a healthy carrier status may develop (STAATS et al. 1997).

In human patients, meningitis is most frequently observed leading to clinical signs like neck stiffness, headache and confusion. Sepsis and less frequently arthritis, endocarditis and pneumonia can also occur (DUTKIEWICZ et al. 2017). Possible complications include renal and liver impairment, septic shock, acute respiratory distress syndrome, endophthalmitis and development of spinal abscess (WERTHEIM et al. 2009). Even when meningitis is resolved and patients survive, hearing loss is a common sequela which affects about 50% of patients (HUANG et al. 2005, LUN et al. 2007). Remaining loss of vision, paralysis, dysarthria with gait ataxia and residual renal impairment was also described (WERTHEIM et al. 2009). The peracute STSLS was mainly described during the two Chinese outbreaks (TANG et al. 2006) and is associated with sepsis, petechiae, erythematous rash and multi-organ failure. In the relatively frequent Asian cases of *S. suis* disease in humans, but also in the more sporadic cases in the rest of the world, especially people are affected with close contact to pigs, wild boars or raw pork products, either by their profession, cultural background or diet (ARENDS and ZANEN 1988, DUTKIEWICZ et al. 2017). A transmission from human to human was not described so far.

2.1.3 Pathogenesis of *S. suis* infection

Knowledge about the pathogenesis of *S. suis* infection improved in recent years, especially since outbreaks in Asian countries in the human population caused extended research in the field. An important question is, how this successful colonizer, living in healthy carrier piglets, can become an invasive pathogen causing severe inflammation and disease. Adhering to epithelial cells and breeching the mucosal barrier of the respiratory tract are the first steps necessary for invasion of the host, followed by survival and dissemination in blood, entering of body compartments, like the joints or the cerebrospinal fluid (CSF), and proliferation of the bacteria in body tissues. Many virulence-associated factors were suggested to aid *S. suis* in

this process and have been extensively reviewed (BAUMS and VALENTIN-WEIGAND 2009, FITTIPALDI et al. 2012). When reviewing the question if proposed virulence factors are "critical" SEGURA et al. (2017) are facing many controversies. Even the major virulence factor capsular polysaccharide (CPS), is not sufficient for full virulence, with other factors having important functions in pathogenesis as well. Glyceraldehyde-3-phoshate dehydrogenase GAPDH (BRASSARD et al. 2004), fibronectin- and fibrinogen-binding protein FBPS (DE GREEFF et al. 2002, LI et al. 2017), plasminogen- and fibronectin-binding α-enolase (ESGLEAS et al. 2008) and fibrinogen-binding muramidase released protein MRP (PIAN et al. 2015) have been described to play a role in adhesion to epithelial cells and extracellular matrix, but their contribution to virulence requires further investigation (FITTIPALDI et al. 2012, LI et al. 2017). A mutant deficient in the surface-located amylopullulanase (ApuA) proofed significantly less adherent to porcine mucous and a tracheal epithelial cell line *in vitro* and thus ApuA is supposed to play a role in colonization *in vivo* (FERRANDO et al. 2010). More recently it was discovered that *S. suis* strains express genes encoding the multimodal adhesion proteins antigen I/II (AgI/II) which lead to aggregation of *S. suis*, biofilm formation and adherence in the upper respiratory tract of pigs (CHUZEVILLE et al. 2017). Non-encapsulated strains adhere to and invade epithelial cells better than encapsulated strains (BENGA et al. 2004), possibly due to the capsule partially masking adhesins and integrins (LALONDE et al. 2000). It was suggested that *S. suis* has the ability to up- and downregulate its capsule depending on the stage of infection (GOTTSCHALK and SEGURA 2000, FITTIPALDI et al. 2012). While all these adhesion factors have been identified, it is still not completely clear, how *S. suis* actually breeches the epithelial barrier. The cytolysin suilysin (SLY) was shown to be cytotoxic to epithelial cells (NORTON et al. 1999) and was proposed to play a role in breeching the barrier by its destruction rather than by invasion of epithelial cells (LALONDE et al. 2000). Nevertheless, SLY negative strains are also able to cause invasive infections (ALLEN et al. 2001), so other factors must play a role in breeching the barrier as well.

WILLIAMS and BLAKEMORE (1990) suggested a "trojan horse theory", with *S. suis* being taken up into macrophages and monocytes and using these as vehicles to cross barriers, disseminate in the blood stream and invade inner organs. Nevertheless, the number of monocytes carrying intracellular *S. suis* was below 2% in this study and the capsule was shown to protect *S. suis* from phagocytosis, while non-encapsulated mutants were taken up but destroyed in the phagolysosome (CHARLAND et al. 1998, SMITH et al. 1999). This led to the suggestion of a "modified trojan horse theory", whereby the bacteria are bound to the surface of phagocytes, but are not taken up into the cells (GOTTSCHALK and SEGURA 2000). Supporting this suggestion, SEGURA and GOTTSCHALK (2002) could demonstrate significant numbers of *S. suis* adhering to macrophages and BENGA et al. (2008) found, that even encapsulated *S. suis* were killed rapidly by neutrophils and monocytes, once they were internalized. Despite the question if *S. suis* is transported by cells or moves freely in the blood stream, it encounters a prominent immune response and has developed numerous evasion strategies. This will be detailed in Chapters 2.2. and 2.3.

The main pathology associated with *S. suis* disease is meningitis. To reach the meninges, *S. suis* needs to cross the blood-cerebrospinal fluid-barrier (B-CSFB) or the blood-brain-barrier

(BBB). Which one of those barriers plays a bigger role as entry port is controversial. In pigs presenting meningitis after experimental *S. suis* infection, necrotic endothelial cells and immune cell invasion was observed directly below the meninges (SANFORD 1987), speaking for the BBB as possible entry port for *S. suis* to the brain. The BBB is composed of a monolayer of brain microvascular endothelial cells (BMEC) and *S. suis* was shown to be able to adhere to and invade porcine BMECs in vitro (VANIER et al. 2004) while it did not show invasion in a model of human BMECs (CHARLAND et al. 2000). In contrast, the *S. suis* surface protein MRP was proposed to improve the traversal ability of *S. suis* across human BMECs due to binding of human fibrinogen (hFg), as the MRP-hFg interaction destroyed the cell adherence junction protein p120-catenin (WANG et al. 2015). Sortases (Srt) were identified in *S. suis* in 2002 and the function of cleaving Leu-Pro-X-Thr-Gly (LPXTG, where X is any amino acid) motif carrying proteins and anchoring them to the peptidoglycan in the cell wall of *S. suis* was described for SrtA (OSAKI et al. 2002). As many of the already mentioned factors are LPXTG motif containing proteins (e.g. MRP), that require processing via SrtA, it is plausible that a *srtA* mutant showed reduced binding to extracellular matrix proteins and diminished adherence and invasion of porcine BMECs in vitro (VANIER et al. 2008). Another member of the sortase family, SrtF, is responsible for the elongation of polymeric pili structures on the cell surface. Its deletion mutant did not show impaired interaction with porcine BMECs or attenuation in the sepsis mouse model (FITTIPALDI et al. 2010), but proved highly attenuated in a pig model of intranasal infection where it supposedly not even managed to breach the epithelial barrier and reach inner body compartments at all (FAULDS-PAIN et al. 2019). When it comes to breaching the BBB, again the cytotoxin SLY might play a role by damaging BMECs after adhesion and disrupting the integrity of the BBB (GOTTSCHALK and SEGURA 2000). Additionally, it is described that SLY stimulates the inflammatory response in different cell types (VADEBONCOEUR et al. 2003, LUN et al. 2003, VANIER et al. 2009, LECOURS et al. 2011, SONG et al. 2020, XU et al. 2021) and this local increase in cytokines can influence the permeability of barriers. Also, D-alanylation of lipoteichoic acids (LTAs) plays a role in adherence to and invasion of porcine BMECs (FITTIPALDI et al. 2008a). A more recent study found that factor H-binding protein (Fhb) of *S. suis* binds to a receptor expressed on BMECs and leads to paracellular crossing of the BBB (KONG et al. 2017). The frequent isolation of *S. suis* from CSF of severely infected pigs and the fact that the most common histopathological findings in *S. suis* meningitis are within the choroidal plexus (WILLIAMS and BLAKEMORE 1990, BEINEKE et al. 2008) point to the B-CSFB as an important entry port for *S. suis* to the brain. Therefore, the interaction of *S. suis* with the B-CSFB has also widely been studied. TENENBAUM et al. (2009) developed an inverted transwell filter system of porcine choroid plexus epithelial cells to allow the investigation of bacterial translocation from the basolateral ‘blood side’ to the apical ‘CSF’ side, taking the *in vivo* situation at the B-CSFB into account. The invasion and translocation of *S. suis* across the B-CSFB could be shown in this model (TENENBAUM et al. 2009). This, as well as a model of human choroid plexus epithelial cells have since been used and modified to investigate the interaction of *S. suis* with the B-CSFB, focusing on different questions (WEWER et al. 2011, SCHWERK et al. 2011, DE BUHR et al. 2017, LAUER et al. 2021, MEURER et al. 2020, MARTENS et al. 2022).

2.1.4 Cell wall modifications of *S. suis*

The bacterial cell wall plays an important role in the interaction with the host immune system. Also in *S. suis* cell wall components have been proposed to be important virulence factors contributing to an increased inflammatory response of the host (GOTTSCHALK and SEGURA 2019). The main components of the Gram-positive cell wall are peptidoglycan, lipoproteins and teichoic acids. Table 1 summarizes cell wall modifications and their function in defense against the host immune response for different streptococci.

Peptidoglycan (PG), also called murein, consists of glycan strands made of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acids (NAM) connected with cross-linking peptide chains (VOLLMER et al. 2008, FITTIPALDI et al. 2008b). Some Gram-positive bacteria are capable of modifying their PG by N-deacetylation which provides resistance to lysozyme and prevents recognition of the bacteria by host receptors (VOLLMER and TOMASZ 2000, BONECA et al. 2007, DAVIS et al. 2008). The gene *pgdA*, responsible for this N-deacetylation of residues of NAG or to a lesser extent of NAM, has been identified in *S. suis* as well (FITTIPALDI et al. 2007). Although only a low quantity of muropeptides were found to be deacetylated and lysozyme resistance was not shown to be increased (FITTIPALDI et al. 2008b), the action of *pgdA* in *S. suis* provided resistance to killing by neutrophils and dendritic cells (DCs) (FITTIPALDI et al. 2008b, LECOURS et al. 2011). The virulence of a *S. suis pgdA* deletion mutant was severely impaired in a mouse model of infection (FITTIPALDI et al. 2008b). A few pathogens are capable of a second modification of PG - the O-acetylation of the C-6 atom of NAM by the O-acetyl transferase (OatA). In *S. pneumoniae* this modification increases lysozyme resistance as well as penicillin resistance (CRISÓSTOMO et al. 2006, DAVIS et al. 2008). In *S. suis* it was also shown to improve lysozyme resistance (WICHGERS SCHREUR et al. 2012). Another way of increasing the resistance to lysozyme is the introduction of extra peptide cross-linking between the NAG and NAM strands. WICHGERS SCHREUR et al. (2012) demonstrated this for a *S. suis* serotype 9 strain.

Teichoic acids represent another major cell wall component of Gram-positive bacteria. The presence of wall teichoic acids (WTAs) in *S. suis* was not proven so far. Two out of three members of the gene family responsible for anchoring WTAs to the cell wall were identified in *S. suis* (HUANG et al. 2021), rendering it very likely that *S. suis* also possess WTAs. However, the structure of *S. suis* LTAs has been studied intensely using high-resolution nuclear magnetic resonance spectroscopy and MS analysis (GISCH et al. 2018). It is controversially discussed, whether LTA itself possess immunomodulatory potency, as the demonstrated stimulation of toll-like receptor (TLR) 2 might also be due to lipoproteins accidentally isolated alongside LTA-preparations (HASHIMOTO et al. 2006, GISCH et al. 2018). Nevertheless, LTA modification by introduction of D-alanine was proofed to play a role in immune defense in multiple streptococcal species, as summarized in Table 1. In *S. suis* FITTIPALDI at al. (2008a) found an increased resistance towards antimicrobial peptides (AMPs) and towards killing by neutrophils, and LECOURS et al. (2011) showed that *S. suis* resistance to complement dependent killing by mouse DCs and the induction of a number of cytokines was increased due to D-alanylation of LTAs. In a mouse model of infection, a *S. suis* mutant incapable of D-alanylating its LTAs was found to be reduced in virulence and an attenuation was suggested for the same mutant in a porcine model of infection (FITTIPALDI et al. 2008a).

Table 1 Cell wall modifications of *Streptococcus sp.* and their function in defense against the host immune system

modification	defense mechanism	*Streptococcus sp.*	reference
N-de-acetylation of peptidoglycan (PgdA)	resistance to lysozyme	*S. pneumoniae*	(VOLLMER and TOMASZ 2000, BLAIR et al. 2005, DAVIS et al. 2008)
	resistance to killing by porcine neutrophils	*S. suis*	(FITTIPALDI et al. 2008b)
	resistance to complement dependent killing by murine dendritic cells	*S. suis*	(LECOURS et al. 2011)
O-acetyl transferase of peptidoglycan (OatA or Adr)	resistance to lysozyme	*S. pneumoniae* *S. suis*	(DAVIS et al. 2008) (WICHGERS SCHREUR et al. 2012)
	penicillin resistance	*S. pneumoniae*	(CRISÓSTOMO et al. 2006)
Extra peptide cross-linking in peptidoglycan layer (MurM, MurN)	increased resistance to lysozyme	*S. suis*	(WICHGERS SCHREUR et al. 2012)
	penicillin resistance antibiotic tolerance	*S. pneumoniae*	(FILIPE et al. 2001) (FILIPE et al. 2002)
	protection against acid induced growth defects	*S. pneumoniae*	(AGGARWAL et al. 2021)
D-alanylation of lipoteichoic acids	resistance to antimicrobial peptides	*S. agalactiae* *S. pyogenes* *S. pneumoniae* *S. gordonii* *S. suis*	(POYART et al. 2003, SAAR-DOVER et al. 2012) (KRISTIAN et al. 2005) (KOVÁCS et al. 2006) (CHAN et al. 2007) (FITTIPALDI et al. 2008a)
	resistance to lysozyme	*S. pyogenes* *S. pneumoniae*	(KRISTIAN et al. 2005) (ZAFAR et al. 2019)

modification	**defense mechanism**	***Streptococcus sp.***	**reference**
D-alanylation of lipoteichoic acids (continued)	resistance to complement dependent killing by murine dendritic cells	*S. suis*	(LECOURS et al. 2011)
	resistance to killing by neutrophils	*S. agalactiae* *S. pyogenes* *S. suis*	(POYART et al. 2003) (KRISTIAN et al. 2005) (FITTIPALDI et al. 2008a)
	resistance to killing by macrophages	*S. agalactiae*	(POYART et al. 2003)
	increased induction of a number of cytokines	*S. suis* *S. pneumoniae* *S. gordonii*	(LECOURS et al. 2011) (ZAFAR et al. 2019) (CHAN et al. 2007)
	adherence to (and invasion of) epithelial cells	*S. pyogenes*	(KRISTIAN et al. 2005)
	adherence to brain microvascular endothelial cells	*S. suis*	(FITTIPALDI et al. 2008a)
	acid tolerance	*S. mutants* *S. agalactiae* *S. pyogenes*	(BOYD et al. 2000) (POYART et al. 2003) (KRISTIAN et al. 2005)
	intrageneric coaggregations	*S. gordonii*	(CLEMANS et al. 1999)

2.2 Selected aspects of the porcine blood immune system

2.2.1 Characteristics and components of porcine blood

Blood is an indispensable body fluid that transports oxygen, nutrients and hormones to cells and tissues and cleans up waste products of the cellular metabolism. The composition of blood is variable, as this dynamic tissue adapts and reacts quickly to varying body conditions (MURPHY et al. 2017). In pigs it also depends on the race, age and reproduction status, resulting in a wide range for reference values of physiological blood conditions (THORN et al. 2022). For example, there is a remarkable shift within the leukocyte population with increasing numbers of T cells and a reduction of B cells and monocytes in adult sows compared to weaned piglets (SIPOS 2019). For a more detailed analysis of reference values for porcine blood parameters, the dissertation of EVA NERBAS (University of Veterinary Medicine Hannover, 2008) is recommended (NERBAS 2008). In “Schalm’s Veterinary Hematology” cellular components of porcine blood are described in more detail (BROOKS et al. 2022): Erythrocytes of grown up pigs have a diameter of 4 to 8 µm and are described as relatively fragile, which often leads to hemolysis due to the blood taking or handling of blood samples. Compared to other domestic animals, sedimentation is faster. Porcine thrombocytes are comparable with those of other species with a size of 1 to 3 µm and a volume of 6,9 to 8,9 fl. Lymphocytes are either small (7 to 10 µm) with a round to oval nucleus and a small rim of cytoplasm, or large (11 to 15 µm) with nuclei showing a more diffuse chromatin pattern surrounded by slightly more cytoplasm that may contain azurophilic granules. Monocytes have a diameter of 14 to 18 µm with a bend core with lose chromatin and local condensations. Their cytoplasm can contain vacuoles or granules. Porcine eosinophil granulocytes have round to oval granules that fill the entire cytoplasm and a hardly segmented core appearing immature, while basophil granulocytes show granules staining in a similar dark color like the core. Neutrophil granulocytes have a diameter of 12 to 15 µm and a clear segmentation or a u-shaped non-segmented core (THORN et al. 2022). Porcine neutrophils were found to be smaller in size then human neutrophils, had lower granularity and a higher activation threshold (BRÉA et al. 2012). Compared to other mammals, pigs have high blood lymphocyte counts of approximately 10^7/ml (CHASE and LUNNEY 2019).

2.2.2 Innate immunity

Innate immunity makes up an important part of the blood immune system. As in other animals, the porcine innate immunity provides an immediate defense against potentially harmful invaders. It does not require previous exposure to antigens and has no “memory” (CHASE and LUNNEY 2019). The main clue of innate immunity is to recognize pathogens by pattern-recognition receptors (PRRs) making use of the fact, that there are essential molecules which tend to be structurally conserved in large groups of pathogens like PG and LTAs in Gram-positive or lipopolysaccharides (LPS) in Gram-negative bacteria as well as viral RNA or bacterial DNA. These structures provide a pathogen-associated molecular pattern (PAMP) to be recognized by the PRRs. After recognition the innate immune system can rely on a collection of subsystems, including cellular and humoral mechanisms (MURPHY et al. 2017).

2.2.2.1 Cellular innate immunity in blood

An important function of the cells in innate immunity is phagocytosis - to engulf, kill and digest invading pathogens or altered body cells (like tumor cells or those infected by virus). Two major cell types, capable of phagocytosis form the pillars of cellular innate immunity in blood: granulocytes (neutrophils, basophils, mast cells, eosinophils) and monocytes (MAIR et al. 2014). While the lymphoid cell population is rather associated with an adaptive immune response, a few lymphoid cells also play a role in innate immunity, namely natural killer cells and γδ T cells (MAIR et al. 2014). This dissertation focuses on neutrophils and monocytes.

2.2.2.1.1 Neutrophils

In blood especially polymorphonuclear neutrophils are present in high numbers. They derive from myeloid progenitor cells in the bone marrow and have a half-life of approximately 12 hours (BASU et al. 2002, PRICE et al. 1996) to 5 days (PILLAY et al. 2010) after being released into the blood stream. In tissue neutrophil lifespan is prolonged by cytokines, microbial compounds and the local environment (KENNEDY and DELEO 2009). Especially growth factors, present in inflamed tissue, were shown to have an anti-apoptotic effect (DIBBERT et al. 1999). In case of an assault, chemotactic factors lead neutrophils to the vicinity of invading microorganisms and inflamed tissues resulting in a fast mobilization of high neutrophil numbers to the site of infection. Even under non-opsonizing conditions these cells are capable of recognizing pathogens, as well as damage-associated molecular patterns released by necrotic cells, due to PRRs. Neutrophils express a large number of PRRs including TLRs, Nod-like receptors, Dectin-1 and mannose receptors (NORDENFELT and TAPPER 2011). The last two are C-type lectin receptors, recognizing ß-glucan and mannan, respectively, and are important for the uptake of microorganisms (MORENO-MENDIETA et al. 2022). Opsonization of the pathogens with antibodies and/or complement components results in a strong increase of their uptake, as it improves the recognition of these organisms by Fc -receptors and complement receptors, also expressed on neutrophils (MORENO-MENDIETA et al. 2022). Neutrophils are very efficient phagocytes that can internalize an IgG-opsonized target within 20 seconds (SEGAL et al. 1980). The uptake into a phagosome is usually followed by the fusion of this phagosome with primary lysosomes and granules containing hydrolytic enzymes and NADPH oxidase subunits. Activation of this oxidase by assembly of cytosolic and membrane bound components leads to the generation of reactive oxygen species (ROS), starting with hydrogen peroxide which is subsequently transformed into hypochlorous acid through reaction with chloride by the granule protein myeloperoxidase (NORDENFELT and TAPPER 2011). This respiratory or oxidative burst is associated with a consumption of protons and a rise in pH in the phagolysosome (SEGAL et al. 1981). Compared to macrophages, neutrophils generate far more ROS and they can recruit additional NADPH oxidase to the phagosome, presumably by fusion with granules (NORDENFELT and TAPPER 2011). In neutrophils, immediately after formation, the phagolysosome alkalizes up to a mean pH value of 9 due to the action of NADPH oxidase and then decreases gradually until it reaches neutral values again (ARNHOLD 2020), while in macrophages progressive acidification of the phagolysosome to pH 4-5 was reported (HUYNH and GRINSTEIN 2007). The assembly of NADPH oxidase and the generation of ROS in neutrophils is not only restricted to the phagolysosome compartment, but ROS can also be

released extracellularly (NGUYEN et al. 2017). They additionally play a role in triggering degranulation, neutrophil extracellular trap (NET)-release and stimulating the formation of cytokines like tumor necrosis factor (TNF)-α (FUCHS et al. 2007, NAIK and DIXIT 2011). The generation of ROS often requires multiple signals – binding of ligands to TLRs, G-protein-coupled receptors, and cytokine receptors will prime neutrophils and prepare them for an additional signal to induce the production, while Fc and integrin receptors directly induce high levels of ROS generation (NGUYEN et al. 2017).

The above mentioned granules are a key feature of granulocytes. There are different types of granules, namely primary (also termed azurophilic), secondary (specific) and tertiary (gelatinase) granules and they contain numerous hydrolytic enzymes and AMPs (CHASE and LUNNEY 2019). Among other molecules, azurophilic granules contain myeloperoxidase and elastase, specific granules contain high amounts of lactoferrin and gelatinase granules contain matrix metalloproteinases. There is also a fourth granule population called secretory vesicles that can be formed in mature neutrophils and contains cytokines synthesized during immune activation (SHESHACHALAM et al. 2014). Besides their role during phagocytosis in the formation of phagolysosomes, these granules can also be released extracellular by degranulation during neutrophil activation. Due to a gradient in sensitivity to intracellular calcium, extracellular release of secretory and tertiary vesicles happens with modest neutrophil activation while secondary and primary granules need a stronger signal (SENGELØV et al. 1993). The release of primary and secondary granules can also be due to leakage from the phagosome, e.g. if the fusion of granules and phagosome takes place before the target is completely internalized. It can also occur in absence of a target fitting for phagocytosis, when neutrophils are “frustrated” due to activation by immune complexes or complement deposited along a host surface (MAYADAS et al. 2014).

Another defense mechanism of neutrophils is the formation of NETs, first described by BRINKMANN et al. (2004). During this so called NETosis neutrophils extrude decondensed chromatin and associated granule products into the extracellular space. NETs are defined by the colocalization of chromatin and granule proteins such as myeloperoxidase and elastase, visualized by immunostaining outside the cell. The ability to trap and kill microbes was shown for bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella enterica* (BRINKMANN et al. 2004). A number of different stimuli can induce the formation of NETs (VON KÖCKRITZ-BLICKWEDE and NIZET 2009) and neutrophils are not the only cells capable of this extracellular trap formation (YOUSEFI et al. 2008, VON KÖCKRITZ-BLICKWEDE et al. 2008). *S. suis* can also trigger neutrophils to form NETs as demonstrated *in vitro* (DE BUHR et al. 2014, ZHAO et al. 2015) and *in vivo* (DE BUHR et al. 2017). It is entrapped but not killed in those NETs (DE BUHR et al. 2014).

2.2.2.1.2 Monocytes

A second main cellular component of innate immunity in blood are monocytes. Like neutrophils they develop in the bone marrow and belong to the myeloid cell line, but survive far longer in blood and tissue. Granule-associated proteins are the same as found in neutrophils but in contrast to those monocytes preserve the ability to produce new granule proteins. Monocytes accumulate more slowly than neutrophils to the site of infection and persist longer. Their

oxidative burst is less extreme, but they can kill a large range of microbes. Finally they have a greater ability to synthesize cytokines (DALE et al. 2008). Formerly, monocyte function was mainly seen in migrating into the tissues to replenish local macrophages and DCs, but lately their role was found to be more divers. In humans three subsets based on specific surface markers (Cluster of differentiation (CD)14 and CD16) have been identified and where shown to have different functions. Classical monocytes make up 80 – 95% of circulating monocytes. They express TLRs and scavenger receptors that recognize PAMPs and enable phagocytosis as well as production of cytokines, myeloperoxidase and ROS. Intermediate monocytes are less abundant in human blood with 2 – 11%. They have pro-inflammatory functions. The non-classical monocytes, that make up 2 – 8% of the human monocyte population are also called “patrolling” monocytes as they roll along the endothelium rather then moving free in the blood stream (MURPHY et al. 2017, YANG et al. 2014). The cytokine panel of classical monocytes is not necessarily pro-inflammatory, as they produce interleukin (IL)-8 and IL-6 but also high levels of anti-inflammatory IL-10 and only low levels of TNF-α in response to LPS. In contrast, the pro-inflammatory intermediate monocytes produce high amounts of IL-1β and TNF-α in response to LPS (CROS et al. 2010).

Porcine studies of monocyte subsets are based on CD163 expression. CD163-positive cells show functions comparable to CD16 positive human monocytes corresponding to intermediate or non-classical monocytes. These monocytes make up for 5 – 50% of circulating monocytes in pigs. CD163-negative porcine monocytes can be considered closely related to classical human monocytes (ZIEGLER-HEITBROCK 2014).

In general, defense mechanisms of monocytes are comparable to those described for neutrophils, including ROS production and phagocytosis. However, their focus lies on regulation of inflammation, communication with other cells of the immune system, replenishing tissue macrophages and DCs, as well as repairing damaged host tissues (AUSTERMANN et al. 2022).

2.2.2.2 Humoral innate immunity in blood

Soluble plasma components including complement, AMPs and enzymes (e.g. lysozyme), natural antibodies and acute phase proteins, make up the humoral innate immune response in blood (MAIR et al. 2014). Also, inflammatory mediators like cytokines and chemokines, produced by cells of the innate immune response, can be seen as part of the humoral immune system.

2.2.2.2.1 Complement System

A major humoral component of innate immunity is the complement system. It’s a collection of soluble PRRs and effector proteins that detect and destroy pathogens and are designated by the letter C followed by a number. The complement system of mammals is made up of more than 50 soluble and membrane-associated proteins (HAJISHENGALLIS et al. 2017). There are three complement-activation pathways, namely the lectin pathway, the classical and the alternative pathway. The lectin pathway is initiated, when mannose-binding lectin and ficolins recognize carbohydrates in microbial cell walls or capsules and bind to the pathogen surface. In the classical pathway complement component C1q binds to the pathogen or to antibodies

already bound to the pathogens surface and in the alternative pathway C3 undergoes spontaneous hydrolysis. The activation of the alternative pathway does not require antibodies or antigens, but it will be enhanced by the presence of some antibodies, repeating polysaccharides, LPS, gas bubbles, heme and the phagocyte-derived properdin (VANDENDRIESSCHE et al. 2021). It provides an “amplification loop” for full activation of complement in all pathways, since deposited C3b continuously forms new C3 convertase by binding to Factor B (HOLERS 2014, HAJISHENGALLIS et al. 2017). In fact, all three pathways lead to the formation of a C3 convertase which cleaves complement component C3 into the small C3a and the larger C3b. C3b acts as opsonin, enabling phagocytosis by phagocytes expressing receptors for C3b. C3b can also bind to the C3 convertase to form a C5 convertase cleaving C5 into C5a and C5b. C5b then triggers the formation of a membrane-attack complex, that can directly disrupt membranes of certain pathogens and cause lysis. The smaller molecules C3a and C5a are inflammatory peptides that recruit phagocytic cells to the site of infection and promote inflammation (MURPHY et al. 2017).

2.2.2.2.2 Antimicrobial peptides

Further factors of innate immunity are AMPs. They represent an evolutionarily ancient weapon, found within all kinds of living organisms including plants. The antimicrobial mechanism is best understood for bacteria and relies on a fundamental difference in the membranes of microbes and multicellular organism: bacterial membranes display a large number of negatively charged phospholipid headgroups in the outer leaflet of their membrane in contrast to plant and animal cell membranes (ZASLOFF 2002). According to the Shai-Matsuzaki-Huang model interaction of the usually positively charged, amphiphilic AMPs with the bacterial membrane leads to pore formation, scrambling of the usual distribution of lipids and rupture (SHAI 1999, MATSUZAKI 1999, YANG et al. 2000b). However, entering the microbe and damaging of critical intracellular targets has also been suggested as antimicrobial mechanism (KRAGOL et al. 2001). AMPs are expressed in epithelial cells and phagocytes and play an important role at mucosal barriers but also in the blood system. In blood they can be produced by neutrophils, monocytes and even lymphoid cells (AGERBERTH et al. 2000). As already mentioned, their antibacterial activity is part of intracellular killing mechanisms in the phagolysosome, but they can also be released into the blood plasma, where they additionally perform important immunomodulatory functions, for example recruiting cells (YANG et al. 2000a), neutralizing LTA-induced TLR2 activation and LPS-induced TLR4 activation (HIRATA et al. 1994, SCOTT et al. 2002, MOOKHERJEE et al. 2006), promoting phagocytosis (WAN et al. 2014) and wound healing (CARRETERO et al. 2008). These functions cannot be generalized to all AMPs, as there are wide structural and functional differences in this group of peptides (ZASLOFF 2002, COORENS et al. 2017). In mammals AMPs consist of two families: cathelicidins and defensins. Cathelicidins are highly heterogenous, but are grouped into one family, based on the fact that they are all synthesized with a conserved cathelin pro-peptide sequence, which is cleaved off for the peptide to become active. Defensins are cationic, amphipathic peptides, rich of cystine and consist of three sub-classes: α, β and θ-defensins. Their structure is based on a common beta sheet core that is stabilized by three disulfide bonds (ZANETTI 2004). While in the human species only one cathelicidin has been identified so far, namely LL-37, there is a variety of porcine cathelicidins, that can be sorted into four categories: proline-arginine (PR) -rich PR-

39, protegrins, prophenins and porcine myeloid antimicrobial peptides (SANG and BLECHA 2009). Investigations of the porcine immunome have demonstrated, that the pig genome does not contain identifiable α-defensins (CHOI et al. 2012).

2.2.3 Adaptive immunity

Like innate immunity, adaptive or acquired immunity is a complex system made up of cellular and humoral components - mainly B and T lymphocytes and their products. It relies on a learning process, that leads to a specialized immune response to specific antigens. It cannot stand alone but needs certain components of innate immunity to bridge between the two systems. For example, antigen presenting innate cells like macrophages and DCs phagocytose microbes, digest them and bind fragments to surface molecules, called major histocompatibility complex (MHC) or swine leukocyte antigen (SLA), to present these so called, antigens to T cells. These have surface receptors that recognize appropriately presented molecules and learn to distinguish foreign, potentially dangerous antigens from those that are harmless or even belonging to the hosts own body. Therefore, lymphocytes that respond to self-antigens are eliminated during their maturation process. Also, cytokines produced by innate immune cells are necessary for an effective stimulation of the adaptive response and on the other hand lymphocytes and their products strongly improve innate mechanisms like complement opsonization and phagocytosis (CHASE and LUNNEY 2019).

2.2.3.1 Cellular adaptive immunity in blood

T cells can be divided into two subpopulations by their T cell receptors – αβT cells and γδT cells, whereby pigs have a larger γδT cell population in blood than most other mammals. αβT cells are usually CD8+ cytotoxic T cells or CD4+ T helper (Th) and regulatory T (Treg) cells, whereby in swine a population of peripheral blood T cells express both CD4 and CD8 antigens on their surface and is increasing with age from less than 2% at one week to 30-55% at 3 years of age (ZUCKERMANN and HUSMANN 1996). The functional significance of this population is still under investigation (PAREL and CHIZZOLINI 2004, CHASE and LUNNEY 2019). CD8+ cytotoxic T cells kill the cells they recognize, therefore they play an important role in combatting intracellular pathogens and cancer cells. CD4+ cells comprise several subsets. Best characterized are Th1, helping in immune response against intracellular pathogens, Th2 promoting humoral responses, Th17 contributing to elimination of extracellular pathogens and various Treg cells, preventing autoimmunity and resolving inflammation by IL-10 production (MCKINSTRY et al. 2010). γδ T cells can secrete pro-inflammatory and regulatory cytokines, show memory or recall responses and exhibit innate-like antigen recognition properties (LE PAGE et al. 2022). Usually, T cells do not respond to soluble antigen or whole microbes, therefore presentation of antigens via SLA (porcine MHC) molecules is essential to activate them (CHASE and LUNNEY 2019). Th cells are critical for initiating the B cells response. Their cytokines in combination with an antigen corresponding to the respective B cell receptor (BCR), activate B cells to undergo asymmetric division into antibody-producing plasma cells on the one hand and memory cells on the other hand (TIZARD 2018). Th cells help further in clonal expansion of B cells and switching of the BCR and the produced antibody classes (CHASE and LUNNEY 2019). Porcine B cells start their development in the bone marrow even before

birth. The fetal B cells already secret pre-immune antibodies including mainly IgM, IgA and IgG3 (BUTLER et al. 2017), providing natural antibodies for the above mentioned innate humoral immunity.

2.2.3.2 Humoral adaptive immunity in blood

The major part of humoral adaptive immunity in blood is B cell derived antibodies, also called immunoglobulins (Ig). Five different classes are known in mammals: IgM, IgD, IgG, IgA and IgE. All antibodies comprise of paired heavy and light polypeptide chains (MURPHY et al. 2017). Among mammals, there are differences in the number of genes encoding constant regions of the different antibody types. Humans, for example, have four genes encoding IgG, rabbits only one and swine have six genes (SINKORA and BUTLER 2016). In pigs, as in other species, IgG usually accounts for more than 80% of serum Ig, followed by IgM with 5-10%. IgA is mainly found on mucosal surfaces, whereby swine produce far more IgA than other species (CHASE and LUNNEY 2019). IgM and IgD are part of the mature BCR. Despite a long history of research the function of IgD is still not clear (BUTLER et al. 2017, MURPHY et al. 2017) and references on the presence of IgD in swine are controversial (PREUD'HOMME et al. 2000, BUTLER et al. 2006). IgE is mainly bound to tissue mast cells and plays a role in the defense against parasites and in allergic reactions (MURPHY et al. 2017).

2.2.3.2.1 IgM

While IgG is the most important neutralizing and opsonizing antibody class, IgM plays a major role in complement activation (MURPHY et al. 2017). Secretory IgM usually occurs as pentamer, occasionally as hexamer (KLIMOVICH 2011). This structure and a C1q binding motif present on the constant domain 3 of the heavy chain allows IgM to activate the classic complement pathway more efficiently than IgG (MURPHY et al. 2017). Hexamer IgM is twenty times more efficient in complement activation, then pentamer IgM, but only makes up for maximum 5% of circulating IgM in the human species (RANDALL et al. 1990). As all B cells first express IgM as BCR, IgM is also the first antibody class produced during primary immune response. With the help of Th cells, the corresponding B cell than switches to producing IgG, IgA or IgE, within a few days, without losing the specificity of the produced antibody. Therefore, during later immune responses to the same antigen, IgG is usually predominant (TIZARD 2018). Besides complement activation secreted IgM is also able to neutralize certain virus and toxins, opsonize pathogens and perform agglutination (KLIMOVICH 2011, MURPHY et al. 2017). IgM constitutes the main antibody class produced against CPS-antigens of *S. suis* after infection of mice and piglets, albeit CPS-specific antibody response was shown to be week compared to the *S. suis* protein-specific antibody response (CALZAS et al. 2015).

2.3 Interaction of *S. suis* with the blood immune system

2.3.1 Immune evasion strategies against blood leukocytes

S. suis has developed several strategies to avoid killing by blood phagocytes but also to influence the communication between cells of the immune system and the extent of inflammation (see Table 2).

The CPS provides direct protection from phagocytosis by increasing the hydrophilic character of the bacterial surface (ABSOLOM 1988, MOXON and KROLL 1990, CHARLAND et al. 1998) and indirectly protect *S. suis* by masking potential antigens and PAMPs. Even molecular mimicry was suggested with sialic acids, present in the capsules of serotype 1, 1/2, 2, 14, 15, 16 and 27 (CHARLAND et al. 1995, SMITH et al. 2000), mimicking sugar epitopes of mammalian cells (FITTIPALDI et al. 2012). Several studies have demonstrated a significant increase in phagocytosis and killing of non-encapsulated *S. suis* mutants by phagocytes and a strong attenuation of these mutants in experimental infection models (CHARLAND et al. 1998, CHABOT-ROY et al. 2006, BENGA et al. 2008, ZHAO et al. 2015). SEGURA et al (2004) partly elucidated the phagocytosis signaling pathway for *S. suis* in macrophages under non-opsonizing conditions and showed that the active (phosphorylated) phosphatidylinositol 3-kinase signaling pathways are needed for the uptake of the bacteria. CPS alone and an encapsulated *S. suis* strain showed reduced phosphorylation of the kinases involved in this pathway, probably due to activation of tyrosine phosphatases, leading to an antiphagocytic effect (SEGURA et al. 2004).

This is in accordance with the suggestive claim that *S. suis* is capable to up regulate its capsule expression in blood (GOTTSCHALK and SEGURA 2000, FITTIPALDI et al. 2012). WILLENBORG et al. (2011) had a closer look into the regulatory mechanism and found that transcriptional regulator catabolite control protein A (CcpA) is important for the capsule formation in *S. suis*. A *ccpA* mutant showed reduced capsule thickness (WILLENBORG et al. 2011). The surface protein HP0197 indirectly influences CPS synthesis by enhancing CcpA activity (ZHANG et al. 2012). Concerning extracellular killing and entrapment in NETs, DE BUHR et al. (2014) found *S. suis* to be protected from NET-mediated killing independently from its encapsulation, while ZHAO et al. (2015) described a protective effect of CPS in NET-entrapment and killing.

Streptococci do not produce catalases, but other factors have been suggested to aid *S. suis* in surviving the formation of ROS by phagocytes. Superoxide dismutase (SOD) and NADH oxidase (NOX) of *S. suis* were described to confer resistance to oxidative stress in growth experiments (TANG et al. 2012, ZHENG et al. 2017). SOD is encoded by *sodA* in *S. suis* and enzymatically catalyzes the reduction of superoxide anions to hydrogen peroxide and oxygen. A Δ*sodA* deletion mutant was found to be attenuated in a mouse model of infection and showed reduced survival in murine macrophages (TANG et al. 2012). NOX was found to reduce superoxide anions to water via the oxidation of NADH to NAD+ in *S. pneumoniae* and *S. mutans* (AUZAT et al. 1999, BAKER et al. 2014). A *S. suis* Δ*nox* mutant showed reduced growth in murine blood and a significant attenuation in murine and porcine infection experiments leading to the suggestion that evasion of killing by blood phagocytes is impaired in this mutant (ZHENG et al. 2017). Regulatory proteins like serine/threonine kinase (STK) and phosphatase (STP) as well as a type IV-like secretion system (T4SS) of *S. suis,* have also been proposed to play a role in oxidative stress tolerance (ZHU et al. 2014, FANG et al. 2017, JIANG et al. 2016). The serine/threonine kinase StkP of *S. pneumoniae* is conserved in *S. suis*. It is a global regulator and influences transcription of approximately 4% of the genome including genes involved in oxidative stress metabolism (SASKOVÁ et al. 2007). In *S. suis* a

Δ*stk* mutant as well as a mutant deficient in the gene encoding for the serine/threonine phosphatase, Δ*stp1*, were both found to be reduced in oxidative stress tolerance and showed diminished growth in porcine blood or in the presence of murine macrophages, respectively (ZHU et al. 2014, FANG et al. 2017).

Another important factor of *S. suis* that influences interaction with blood immune cells, is the production of SLY. Its cytotoxic effect attacks immune cells directly, it is proposed to help avoiding phagocytosis by an adherence dependent action (BENGA 2008) and to help avoiding opsonophagocytosis by activating complement at a distance apart from the bacteria. It has also been described that SLY induces degranulation (CHEN et al. 2016) and activates the NLRP3 inflammasome causing high levels of cytokine IL-1β to be secreted into the blood (SONG et al. 2020, XU et al. 2021) which can result in vascular leakage and STSLS (LIN et al. 2019).

The induction of cytokines and chemokines strongly influences the development of the pathology and the outcome of the infection. Surface components, exposed to the recognition of immune receptors are the main bacterial stimuli. In whole blood live and heat-killed *S. suis*, were shown to trigger a variety of cytokines (SEGURA et al. 2006, HOHNSTEIN et al. 2020). Monocytes were proposed to be the main producers of pro-inflammatory cytokines in response to *S. suis* in blood (HOHNSTEIN et al. 2020, AUGER et al. 2020), while neutrophils were also found to contribute to the inflammatory response, especially by chemokine production (AUGER et al. 2020). When investigating human monocytes in more detail, the CD14 receptor was found to play an important role in recognition of *S. suis* and signaling leading to the production of a number of cytokines, although not all investigated cytokines were CD14-dependent (SEGURA et al. 2002). Comparing several *S. suis* strains of porcine and human origin, SEGURA et al. (2002) found a very heterogenous cytokine pattern and saw no association between cytokine release and virulence of tested strains.

A number of other *S. suis* factors and the proposed underlying defense mechanisms can be found in Table 2 in relation to the respective blood leukocyte. Literature on *S. suis* interaction with monocytes is limited, as most studies are performed with macrophages or DCs. Even less can be found on direct interaction of *S. suis* with lymphocytes in blood. Therefore *S. suis* response to antibodies derived from B lymphocytes and the influence on T lymphocytes via antigen-presenting DCs in the spleen, were taken into account.

Table 2. Host-pathogen interaction between blood leukocytes and *S. suis*

cell type	host immune mechanism	*S. suis* immune evasion factor	proposed mechanism of *S. suis* immune evasion factor	reference
blood phagocytes	phagocytosis	CPS	- increases hydrophilic character of bacterial surface → reduces adherence and phagocytosis	(ABSOLOM 1988, MOXON and KROLL 1990, CHARLAND et al. 1998)
			- adherence to murine macrophages without phagocytosis was attributed to sialic acid	(SEGURA and GOTTSCHALK 2002)
			- inhibits phosphorylation necessary for phagocytosis signaling pathway	(SEGURA et al. 2004)
			- more evidence for CPS related protection from phagocytosis by blood phagocytes without additional explanation of the effect	(CHABOT-ROY et al. 2006, BENGA et al. 2008, ZHAO et al. 2015)
			- sialic acid mimic mammalian sugar epitopes	(FITTIPALDI et al. 2012)
			- CPS upregulation in blood suggested	(WILLENBORG et al. 2011)
		SLY	- "an adherence-dependent action […]"	(BENGA et al. 2008)
			- Impairing function and viability of cells	(HE et al. 2014)
		MRP and enolase	- Interaction with human fibrinogen	(PIAN et al. 2015)
		biofilm	- too large to be taken up	(MA et al. 2017)
		HP0487	- mediates adhesion to neutrophils instead of phagocytosis	(HUI et al. 2021)
	C3b deposition followed by opsonophago-cytosis	SLY	- complement consumption: activates complement, thereby reducing the amount available for opsonization of *S. suis*	(CHABOT-ROY et al. 2006, LECOURS et al. 2011)

cell type	host immune mechanism	*S. suis* immune evasion factor	proposed mechanism of *S. suis* immune evasion factor	reference
blood phagocytes (continued)	C3b deposition followed by opsonophago-cytosis (continued)	D-alanylation of LTA	- reduced C3 deposition, no mechanism discussed	(LECOURS et al. 2011)
		CPS	- sialic acid interferes with activation of alternative complement cascade by increasing the affinity constant of C3b to complement inhibitor factor H	(SEITZ et al. 2014)
		Fhb/Fhbp	- factor H is bound to *S. suis* and inhibits the alternative complement pathway	(PIAN et al. 2012, VAILLANCOURT et al. 2013, LI et al. 2016, ROY et al. 2016)
		Ide_{Ssuis}	- cleavage of porcine IgM → complement evasion, reduced opsonization	(SEELE et al. 2015, RUNGELRATH et al. 2018)
		SntA	- reduces C3 deposition, interacts with C1q, but also directly activates classical and lectin pathways (→ complement consumption)	(DENG et al. 2018)
	oxidative burst	SodA	- reduction of superoxide anions to hydrogen peroxide and oxygen	(TANG et al. 2012)
		STK/STP	- increased survival in blood or macrophages might be related to resistance to ROS	(ZHU et al. 2014, FANG et al. 2017)
		VirD4 (T4SS)	- upregulation upon oxidative stress → increased secretion of multiple protective *S. suis* proteins	(JIANG et al. 2016)
		NADH oxidase	- reduction of diatomic oxygen to water through the oxidation of NADH to NAD^+	(ZHENG et al. 2017)

cell type	host immune mechanism	*S. suis* immune evasion factor	proposed mechanism of *S. suis* immune evasion factor	reference
neutrophils	NETosis	SsnA, EndAsuis	- destruction of DNA backbone	(DE BUHR et al. 2014, DE BUHR et al. 2015)
		CPS	- protects *S. suis* from entrapment, but is not necessary to prevent killing by NETs	(DE BUHR et al. 2014, ZHAO et al. 2015)
		biofilm	- inhibits NET formation	(MA et al. 2017)
	degranulation	SLY	- Ca^{+} influx induces degranulation	(CHEN et al. 2016)
	AMPs and antimicrobial enzymes	D-Alanylation of LTAs	- alteration of surface structure - increase in positive charges	(FITTIPALDI et al. 2008a)
		N-deacetylation of PG	- alteration of surface structure	(FITTIPALDI et al. 2008b)
		ApdS	- cleaves the AMP LL-37	(XIE et al. 2019)
	chemotaxis	serine protease	- degrades IL-8 (neutrophil chemoattractant)	(VANIER et al. 2009)
	multiple neutrophil functions	N-deacetylation of PG	- prevents recognition by host receptors	(FITTIPALDI et al. 2008b)
		SLY	- cytotoxicity	(HE et al. 2014)
		Ssads	- activates A2aR receptor, which inhibits neutrophil functions	(LIU et al. 2014)
		soluble MRP	- binds human fibrinogen, which leads to an aggregation and exhaustion of neutrophils in an αxβ2 integrin-dependent manner	(PIAN et al. 2016)
monocytes	cytokine production	SLY	- triggers IL-6 release - released hemoglobin due to cytotoxicity increases IL-1β, TNF-α, IL-6 and IL-8 - inflammasome activation → IL-1β	(LUN et al. 2003) (TANABE et al. 2008) (LIN et al. 2019, SONG et al. 2020, XU et al. 2021)

cell type	host immune mechanism	*S. suis* immune evasion factor	proposed mechanism of *S. suis* immune evasion factor	reference
monocytes (continued)	cytokine production (continued)	PrsA	- modulates secretion of *S. suis* virulence-associated factors that trigger cytokine release	(JIANG et al. 2016, LIU et al. 2019)
	Chemotaxis	SLY	- binds complement receptors C3aR/C5aR and inhibits monocyte chemotaxis	(DENG et al. 2020)
lymphocytes	Release of T cell derived cytokines	CPS	- inhibits functions of antigen-presenting cells (like DCs) → dampens Th1 response	(LECOURS et al. 2016)
	Secretion of opsonizing antibodies	Ide_{Ssuis}	- proteolytic cleavage of porcine IgM	(SEELE et al. 2013)
		IgdE	- proteolytic cleavage of porcine IgG	(SPOERRY et al. 2016)
		IgA1 protease*	- proteolytic cleavage of IgA1	(ZHANG et al. 2010)

* based on experimental data the described function of IgA proteolysis is questionable (DUMESNIL et al. 2018)

Short list of abbrevations of evasion factors in table 2

ApdS	Aminopeptidase	LTAs	Lipoteichoic acids	SodA	Superoxide dismutase A
CPS	Capsular polysaccharide	MRP	muramidase-released protein	Ssads	Adenosine synthase
EndAsuis	Endonuclease A	NADH	Nicotinamide adenine dinucleotide + hydrogen	SsnA	*S. suis* secreted nuclease A
Fhb/Fhbp	Factor H binding proteins			STK/STP	Serine/threonine kinase/phosphatase
HP	Hypothetical protein	PG	Peptidoglycan		
Ide_{Ssuis}	IgM-degrading enzyme of *S. suis*	PrsA	Parvulin-like peptidyl-prolyl isomerases	T4SS	Type 4 secretion system
				VirD4	Protein component of T4SS
Ig	Immunoglobulin	SLY	Suilysin		
IgdE	IgG protease of *S. suis*	SntA	*S. suis* surface protein		

2.3.2 Complement evasion and *S. suis* cell wall modifications

Viewed from the hosts perspective, a major goal for immune cell-bacteria-interaction is killing the respective bacteria. As already described, phagocytosis and ROS play an important role in this. ROS production needs receptor-mediated signaling. Integrin receptor stimulation can lead to potent superoxide production (NGUYEN et al. 2017). The β2 integrin receptor family contains also complement receptor (CR) 3 and CR4, making complement factors an important trigger of ROS production. *S. suis* has developed mechanisms to evade complement recognition and opsonization. Sialic acid, present in the CPS of several serotypes, was found to increase the affinity constant of complement component C3b to complement inhibitor factor H and thereby reduces opsonization of *S. suis* with C3b (SEITZ 2014). However, CPS with or without sialic acid was shown to take part in recruitment of factor H (ROY et al. 2016). Additionally, factor H binding proteins Fhb and Fhbp cause an accumulation of factor H on the bacterial surface, but a *S. suis* mutant deficient in Fhb, Fhbp and CPS still had some factor H binding capacity (ROY et al. 2016). This suggests the presence of more surface components in *S. suis*, that are able to bind this complement inhibitor factor. The histidine triad protein identified in 2011 could be a candidate, as it was show to be involved in preventing C3 deposition (SHAO et al. 2011), by a so far unknown mechanism. In adaptive immune conditions, IgM plays a very important role in activating the classical complement pathway. The degradation of IgM via Ide_{Ssuis} significantly reduces complement opsonization of *S. suis* (SEELE et al. 2015, RUNGELRATH et al. 2018). Another possible mechanism is the consumption of complement components before they can become dangerous for the bacteria. As it was shown for a SLY-related toxin, pneumolysin, of *S. pneumoniae* (ALCANTARA et al. 2001), it was also suggested for SLY to be able to activate complement and reduce its availability for opsonization of *S. suis* (LECOURS et al. 2011). The role of complement is not restricted to opsonization but certain components like C3a and C5a also trigger chemotaxis. SLY was found to bind C3a and C5a, thereby preventing chemotaxis of monocytes (DENG et al. 2020). In 2018 a recently characterized cell wall anchored protein of *S. suis*, SntA, was found to reduce C3 deposition on *S. suis* and to be able to bind complement factor C1q, thereby competitively reducing C1q-IgG binding. It also directly activated the classical and the lectin complement pathway (DENG et al. 2018).

Other cell wall components of *S. suis* also influence the interaction of the bacteria with complement. Compensation of the negative surface charge by D-Alanylation of LTA was suggested to reduce C1q-binding and an increased C3 deposition on the surface of a *S. suis* Δ*dltA* mutant was observed by confocal microscopy (LECOURS et al. 2011). In the same study C3 deposition seemed also increased for the Δ*pgdA* mutant, which does not perform N-deacetylation of its peptidoglycan. This indicates, that the bacterial cell wall plays a very important role in interaction with the host complement system.

3 Publications

3.1 Survival of *Streptococcus suis* in Porcine Blood Is Limited by the Antibody- and Complement-Dependent Oxidative Burst Response of Granulocytes

Viktoria Rungelrath[#], Sophie Öhlmann[#], Gottfried Alber, Wieland Schrödl, Maren von Köckritz-Blickwede, Nicole de Buhr, Alexander Martens, Christoph Georg Baums, Nicole Schütze

# contributed equally, Author order was determined on the basis of the chronological sequence

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Author contributions

Victoria Rungelrath and I conducted oxidative burst experiments, bactericidal assays and flow cytometry measurements together. Viktoria Rungelrath performed growth analysis under addition of H_2O_2 as well as experiments to investigate the influence of moderate and hyperimmune antibody levels and of different *S. suis* serotypes. Nicole Schütze and I developed and conducted combined oxidative burst - phagocytosis assays in reconstituted whole blood and opsonophagocytosis assay with porcine granulocytes. I supported the animal experiment, which was designed and conducted by Maren von Köckritz-Blickwede, Nicole de Buhr and Alexander Martens. ELISA measurements and Western Blot analysis were conducted by myself. Viktoria Rungelrath, Nicole Schütze and I designed experiments, analyzed data and drafted the manuscript. Viktoria Rungelrath, Nicole Schütze and Christoph Georg Baums conceived the study. All authors read and approved the final manuscript.

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HOST RESPONSE AND INFLAMMATION

Survival of *Streptococcus suis* in Porcine Blood Is Limited by the Antibody- and Complement-Dependent Oxidative Burst Response of Granulocytes

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Viktoria Rungelrath and Sophie Öhlmann contributed equally. Author order was determined on the basis of the chronological sequence of their work.

ABSTRACT Bacteremia is a hallmark of invasive *Streptococcus suis* infections of pigs, often leading to septicemia, meningitis, or arthritis. An important defense mechanism of neutrophils is the generation of reactive oxygen species (ROS). In this study, we report high levels of ROS production by blood granulocytes after intravenous infection of a pig with high levels of *S. suis*-specific antibodies and comparatively low levels of bacteremia. This prompted us to investigate the working hypothesis that the immunoglobulin-mediated oxidative burst contributes to the killing of *S. suis* in porcine blood. Several *S. suis* strains representing serotypes 2, 7, and 9 proved to be highly susceptible to the oxidative burst intermediate hydrogen peroxide, already at concentrations of 0.001%. The induction of ROS in granulocytes in *ex vivo*-infected reconstituted blood showed an association with pathogen-specific antibody levels. Importantly, inhibition of ROS production by the NADPH oxidase inhibitor apocynin led to significantly increased bacterial survival in the presence of high specific antibody levels. The oxidative burst rate of granulocytes partially depended on complement activation, as shown by specific inhibition. Furthermore, treatment of IgG-depleted serum with a specific IgM protease or heat to inactivate complement resulted in >3-fold decreased oxidative burst activity and increased bacterial survival in reconstituted porcine blood in accordance with an IgM-complement-oxidative burst axis. In conclusion, this study highlights an important control mechanism of *S. suis* bacteremia in the natural host: the induction of ROS in blood granulocytes via specific immunoglobulins such as IgM.

KEYWORDS *Streptococcus suis*, oxidative burst, NADPH oxidase, neutrophil, reactive oxygen species, complement, IgM, respiratory burst

Streptococcus suis colonizes the mucosal surfaces of healthy pigs but can also become an invasive pathogen causing meningitis, septicemia, arthritis, endocarditis, and pneumonia in growing piglets (1). The species *S. suis* is very heterogeneous, comprising 29 serotypes and more than 700 sequence types (2). The prevalence of serotypes depends on the geographical region. In Europe, most invasive *S. suis* isolates belong to serotypes 9, 2, and 7 (3). Despite pigs being the main hosts, *S. suis* can also cause disease in other mammals and is a zoonotic pathogen, having so far been responsible for more than 1,600 human cases worldwide (4, 5). During infection, *S. suis* encounters neutrophils as the first line of defense not only in the bloodstream but also in infected tissue. Whereas numerous virulence factors, such as the polysaccharide

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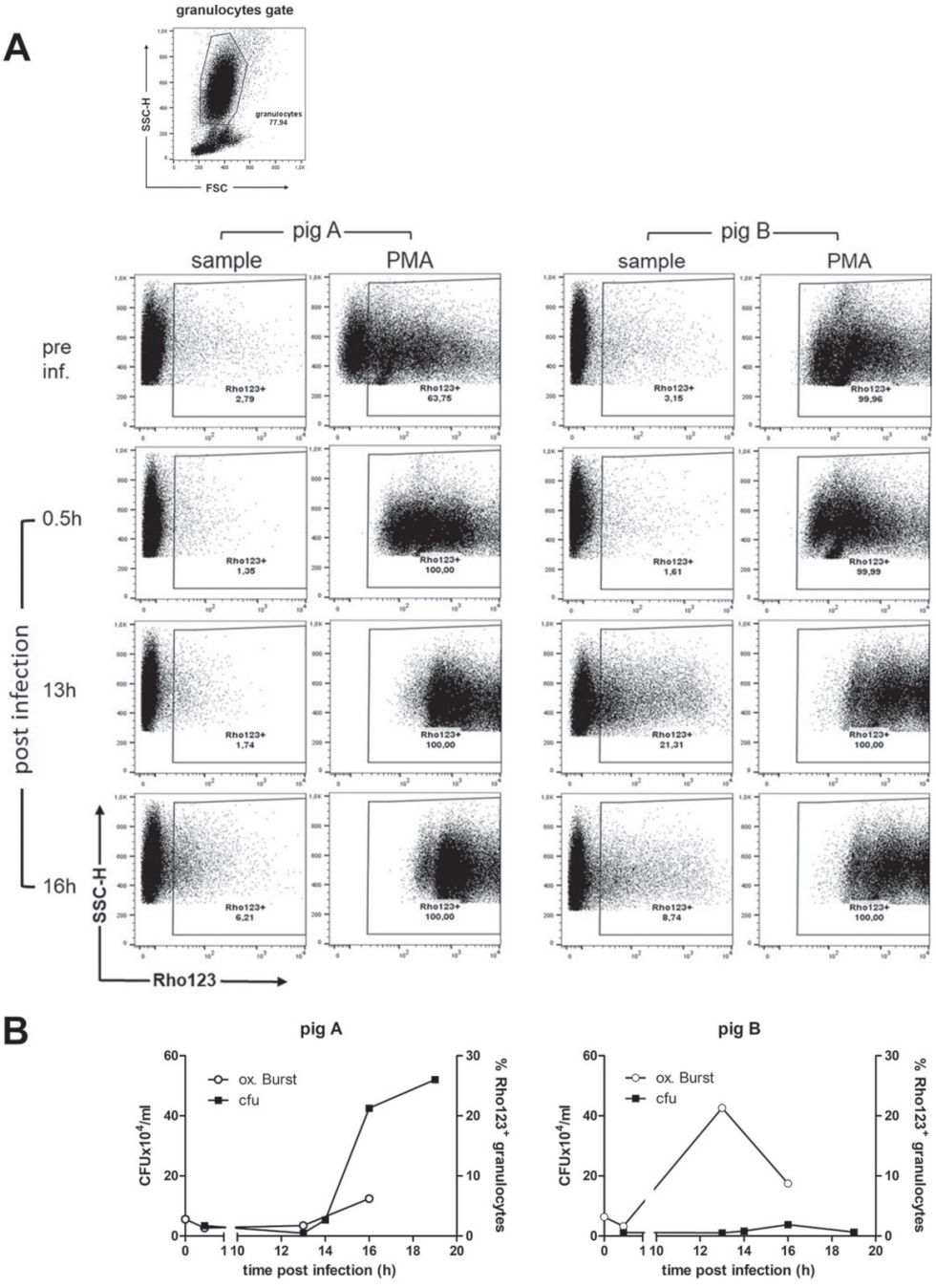
capsule or the D-alanylation of lipoteichoic acid, have been investigated with regard to their potential to prevent phagocytosis by neutrophils (6–8), few studies have been conducted to elucidate the mechanisms by which these important immune cells actually kill *S. suis*. Specific antibodies and complement have been shown to promote the killing of *S. suis* in whole blood or by isolated neutrophils (6, 9). Neutrophils are known to produce reactive oxygen species (ROS) in a process called the oxidative or respiratory burst (10). The oxidative burst is regarded as a rapid and extremely effective antimicrobial defense mechanism of the innate immune system against invading pathogens (11, 12). Catalase-negative streptococci are generally sensitive to ROS but have developed evasion mechanisms to circumvent the oxidative burst attack, as described previously for *Streptococcus pneumoniae* and *S. pyogenes* (13–15). The first enzyme in the oxidative burst cascade is the NADPH oxidase (NOX), which is a multicomponent enzyme complex with a catalytic core (gp91phox and p22phox), located in the cell membrane or the membrane of phagosomes, vesicles, and specific granules, and with regulatory subunits (Rac2, p67phox, p47phox, and p40phox) located in the cytosol of resting neutrophils. Once the neutrophil is stimulated, the cytosolic NADPH oxidase subunits translocate to the membrane-located core units to activate the NADPH oxidase. The active enzyme complex converts NADPH to $NADP^+$ and $2O_2^-$. These oxygen radicals are immediately transformed to hydrogen peroxide by the superoxide dismutase (SOD). The final bactericidal products of the oxidative burst, the hypohalites OCl^- and SCN^-, are generated by the activity of the myeloperoxidase and kill bacteria by oxidizing their proteins, DNA, and lipids (10). ROS exert their bactericidal activity both within the phagolysosome and also extracellularly when they are released by bursting neutrophils (16). Whereas the binding of immune complexes (ICs) or of antibody-labeled antigen to Fc receptors and integrin receptors can stimulate the oxidative burst directly, cytokine receptor, Toll-like receptor, or G-protein-coupled receptor binding only primes neutrophils and requires additional signaling for robust burst induction (16). ROS production has more far-reaching consequences than the direct killing of bacteria by oxygen radicals since it can also induce so-called neutrophil extracellular trap (NET) formation, the release of granules, and the production of proinflammatory cytokines (16). This combination leads to the increased killing of invading pathogens. Knowledge of the oxidative burst response to *S. suis* is very limited. Previous studies focused on the role of superoxide dismutase A (*sodA*) and NADH oxidase (NOX) of *S. suis* serotype 2 in susceptibility to oxidative stress and survival within murine macrophages and reported susceptibility to ROS intermediates only for the respective deletion mutants (17, 18).

In this study, we investigated the oxidative burst response of porcine granulocytes to different *S. suis* strains representing the three most common European serotypes to elucidate whether the oxidative burst is involved in the killing of *S. suis*. The role of IgM and complement in oxidative burst induction and the killing of *S. suis* was analyzed using a defined hyperimmune serum.

RESULTS

ROS production by blood granulocytes of an experimentally infected piglet with high *S. suis*-specific antibody levels and limited bacteremia. Since the oxidative burst in neutrophils is an important strategy of the immune system to attack invading pathogens, we wanted to investigate whether the oxidative burst is induced *in vivo* in blood granulocytes following intravenous (i.v.) infection of two pigs with *S. suis cps2* strain 10. Blood samples were drawn at different time points to assess the time course of ROS production and bacterial loads. Oxidative burst analysis in granulocytes was performed directly in blood samples by staining with ROS-sensitive dihydrorhodamine 123 (DHR123). Oxidative burst measurements in the blood of pig A revealed no clear increase in the frequency of rhodamine 123-positive ($Rho123^+$) granulocytes at 0.5 h or 13 h postinfection. Only a slight increase from 2.8 to 6.2% $Rho123^+$ cells was detectable at 16 h postinfection (Fig. 1A). At the same time, pig A developed progressive bacteremia, starting with 3.4×10^4 CFU/ml blood (30 min postinfection) and



C

anti- *S. suis* (strain 10; cps2)	pig A	pig B
IgM (ELISA units/ml)	46.7	55.4
IgG (ELISA units/ml)	46.5	276.4

FIG 1 The oxidative burst of porcine granulocytes in response to *S. suis* infection *in vivo* corresponds to lower bacterial burdens in blood at later time points of infection. Two piglets were intravenously infected with 3×10^8 CFU of *S. suis* strain 10. Blood samples

(Continued on next page)

reaching 5.2×10^5 CFU/ml blood at 19 h postinfection (Fig. 1B). The pig developed a fever of 40.7°C at 10 h postinfection. In contrast, pig B did not show clinical signs of *S. suis* disease but had a robust oxidative burst response in blood at 13 h postinfection (21.0% $Rho123^+$ granulocytes), representing an almost 7-fold increase in comparison to the sample taken prior to infection (preinfection, 3.1% $Rho123^+$ cells) (Fig. 1A). At 16 h postinfection, the oxidative burst response decreased to 8.7% $Rho123^+$ granulocytes. Moreover, *S. suis* could be isolated from the blood of this piglet at all time points, but the CFU count in the blood of this piglet was remarkably lower than for the first piglet, with a maximum count of 3.8×10^4 CFU/ml at 16 h postinfection (Fig. 1B).

S. suis-specific IgM and IgG levels in the sera of the two piglets before infection were measured by an enzyme-linked immunosorbent assay (ELISA). Pig A had *S. suis*-specific IgM and IgG levels (46.7 and 46.5 ELISA units [EU]/ml, respectively) that were substantially lower than the *S. suis*-specific IgM and IgG levels of pig B (55.4 and 276.4 EU/ml, respectively) (Fig. 1C).

In summary, induction of ROS was shown *in vivo* in blood granulocytes of a pig experimentally infected with *S. suis*. Based on the high levels of *S. suis*-specific antibodies and the controlled bacteremia in this pig, we postulated a crucial role for the antibody-induced oxidative burst of blood granulocytes in the control of *S. suis* bacteremia.

Growth of *S. suis* is impaired in the presence of hydrogen peroxide. Based on the *in vivo* data, we hypothesized that *S. suis* is susceptible to hydrogen peroxide, an important intermediate of the oxidative burst of phagocytic cells. We therefore investigated the susceptibility of four *S. suis* strains representing three different serotypes and an unencapsulated mutant to hydrogen peroxide. The growth of all five investigated *S. suis* strains was severely impaired in the presence of hydrogen peroxide concentrations of ≥0.01% (Fig. 2). A hydrogen peroxide concentration of 0.001% led to delayed growth of all investigated *S. suis* strains. The unencapsulated *S. suis* mutant 10cpsΔEF showed a susceptibility to hydrogen peroxide comparable to those of the four wild-type (wt) strains. Since all strains were susceptible to hydrogen peroxide, we tested if they possessed the *sodA* gene, which had previously been described to play a role in defense against oxidative stress (17). PCR analysis revealed that all five investigated *S. suis* strains were *sodA* positive (see Fig. S1A in the supplemental material). Furthermore, quantitative PCR (qPCR) of reverse-transcribed RNA confirmed that all four investigated wt strains of serotypes 2, 7, and 9 as well as the unencapsulated mutant 10cpsΔEF expressed *sodA* mRNA (Fig. S1B). We thus conclude that the ROS hydrogen peroxide impairs the growth of different *S. suis cps* types at concentrations as low as 0.001% despite *sodA* mRNA expression.

The oxidative burst of porcine granulocytes in response to *S. suis* strain 10 is induced in the presence of *S. suis*-specific antibodies. Granulocytes express both high- and low-affinity Fc receptors that are involved in the recognition and phagocytosis of antibody-opsonized pathogens but also in the induction of ROS production (19). To test our hypothesis that *S. suis*-specific antibodies are associated with the induction of the oxidative burst in blood granulocytes, we performed *in vitro* assays to define the factors involved in more detail. We therefore investigated the oxidative burst response of porcine granulocytes to *S. suis* in the presence of various antibody concentrations by using previously characterized porcine sera. As shown in Fig. 3A, the oxidative burst level in reconstituted blood infected with *S. suis* strain 10 was significantly higher in the presence of hyperimmune serum than in the presence of serum containing moderate anti-*S. suis* IgM and IgG antibody levels (22% ± 3.96% versus

FIG 1 Legend (Continued)
were drawn immediately before infection and at 0.5 h, 13 h, 14 h, 16 h, and 19 h postinfection. (A) The oxidative burst of porcine granulocytes was determined by DHR123 (Rho123) staining and flow cytometry. The Rho123 signal is depicted on the *x* axis. At each time point, PMA stimulation was used as a positive control. SSC, side scatter; FSC, forward scatter. (B) The CFU in blood were determined by plating of serial dilutions and plotted alongside the Rho123 signal. (C) Anti-*S. suis* strain 10 IgM and IgG antibody levels were determined in the serum of both piglets and are listed as relative ELISA units (EU) per milliliter.

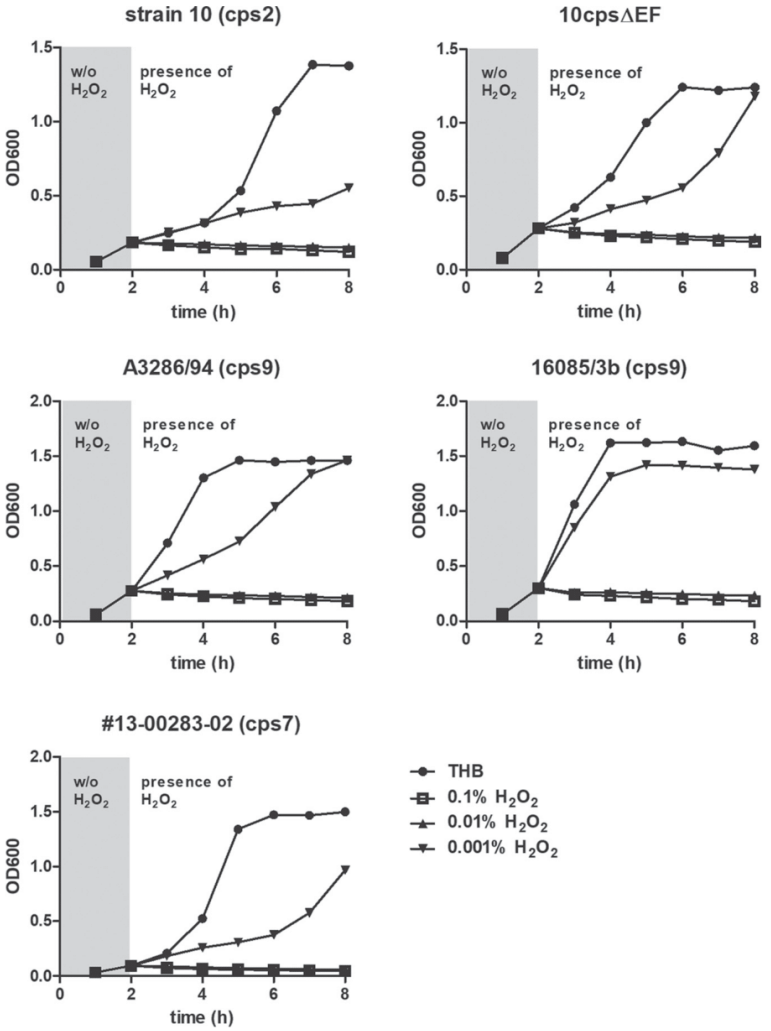
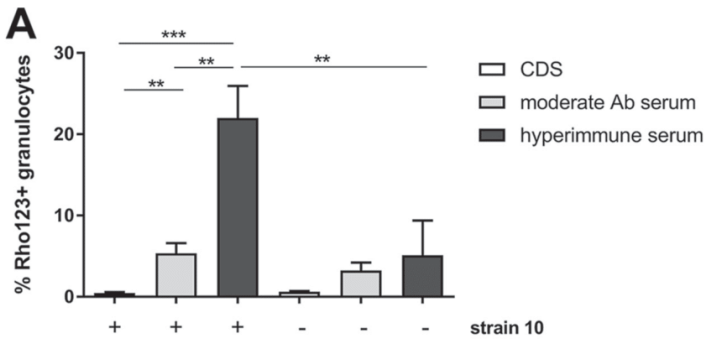


FIG 2 Growth of *S. suis* is impaired in the presence of hydrogen peroxide. *S. suis* strains 10, A3286/94, 16085/3b, and 13-00283-02, representing three different serotypes (*cps2*, *cps9*, and *cps7*), as well as a capsule-deficient *S. suis* mutant (10cpsΔEF) were grown in THB medium. After 2 h, hydrogen peroxide was added at the indicated concentrations (percent, weight per volume). The OD_{600} was measured every hour and is depicted on the *y* axis. Data from one representative experiment out of four are shown.

5.36% ± 1.25% Rho123$^+$ cells). The oxidative burst level was also significantly higher upon *S. suis* infection of reconstituted blood containing moderate antibody serum levels (5.36% ± 1.25% Rho123$^+$ cells) than in colostrum-deprived piglet serum (CDS) (0.45% ± 0.13% Rho123$^+$ cells). Noteworthy, the high oxidative burst response in blood reconstituted with hyperimmune serum depended on the presence of *S. suis* (22% ± 3.96% infected versus 5.11% ± 4.27% uninfected Rho123$^+$ cells). An oxidative burst was not detectable in blood reconstituted with CDS containing only natural antibodies, even in the presence of *S. suis* (0.45% ± 0.13% Rho123$^+$ cells). In fact, values were comparable to those for uninfected samples supplemented with CDS (0.61% ± 0.11% Rho123$^+$ cells) and significantly lower than the oxidative burst response to *S. suis* strain 10 in the presence of hyperimmune serum. Thus, ROS induction in blood granulocytes was not detectable in the absence of *S. suis*-specific antibodies.



B

	anti- *S. suis* **IgM** (ELISA units/ml)	anti- *S. suis* **IgG** (ELISA units/ml)
CDS	0	0
moderate Ab serum	20.4	35.8
hyperimmune serum	100	100

FIG 3 Antibody-mediated induction of the oxidative burst in porcine granulocytes in response to *S. suis*. (A) *S. suis* strain 10 was incubated with three sera, serum from colostrum-deprived piglets (CDS) (free of IgG), serum containing moderate levels of IgM and IgG antibodies against *S. suis* strain 10 (moderate Ab serum), and anti-*S. suis* strain 10 hyperimmune serum. Subsequently, the whole-blood cell pellet of healthy donor piglets was added, and ROS production in blood granulocytes was measured by flow cytometry ($n = 3$). Bars and error bars represent means and standard deviations, and significant differences are indicated (**, $P < 0.01$; ***, $P < 0.001$). (B) Anti-*S. suis* IgM and IgG antibody levels in the chosen experimental sera, listed as ELISA units. The prime-booster hyperimmune serum was defined to include 100 ELISA units of IgG and 100 ELISA units of IgM. Note that ELISA units for the different Ig classes are therefore not comparable.

We therefore conclude that specific antibodies are crucial for the induction of an oxidative burst in porcine blood granulocytes in response to *S. suis*.

Inhibition of the serum-induced oxidative burst in porcine granulocytes leads to increased survival of *S. suis*. We next wanted to find out if the oxidative burst response to *S. suis* in the presence of hyperimmune serum is also involved in the killing of *S. suis*. We therefore used the NADPH oxidase inhibitor apocynin (Apo) in oxidative burst and bactericidal assays. Control experiments confirmed that apocynin alone does not influence the growth of *S. suis* in Todd-Hewitt broth (THB) at the concentration used (Fig. S2A). The immune sera used for the assays described below were previously tested for their ability to mediate the killing of different *S. suis* strains. As shown in Fig. 4A, the oxidative burst responses to *S. suis* strain 10, 10cpsΔEF, A3286/94, 16085/3b, and 13-00283-02 in the presence of hyperimmune sera were significantly reduced by the addition of apocynin. The inhibition of the oxidative burst by apocynin led to significantly increased survival factors (SFs [calculation of the values is described in the legend for Fig. 4B]) for *S. suis* strains 10 (*cps2*), 16085/3b (*cps9*), and 13-00283-02 (*cps7*), as demonstrated in Fig. 4B. The bacterial survival factors were approximately 3-fold higher in these strains in the presence of apocynin, indicating an important bactericidal role of ROS generated in blood granulocytes by NADPH oxidase activity. The addition of apocynin also resulted in increased bacterial survival of the unencapsulated strain 10cpsΔEF and the *cps9* wt strain A3286/94, although the differences were smaller and not significant. Noteworthy, the mean survival factor for wt strain 10 was lower than the respective value for its unencapsulated mutant 10cpsΔEF in the absence of apocynin (Fig. 4B), and the inhibitory effect of apocynin on ROS generation was greater in the case of the *cps2* wt strain 10 (Fig. 4A). These results suggest that an important portion of the antibodies in this hyperimmune serum, raised against *cps2* strain 10 inducing ROS in response to strain 10, was directed against capsular polysaccharides. As capsular polysaccharides are T lymphocyte-independent antigens inducing mainly IgM antibodies, we specifically focused on IgM in the course of this study.

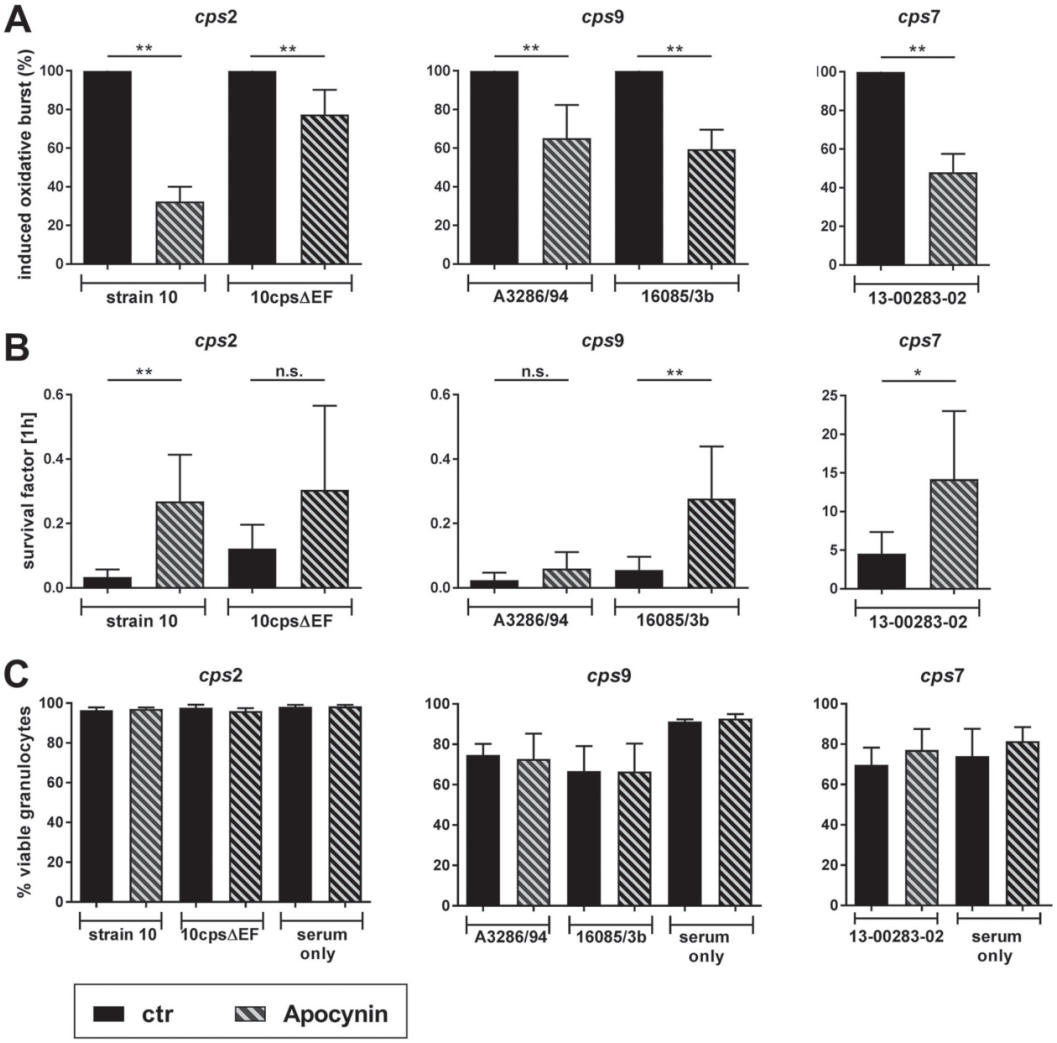


FIG 4 Inhibition of NADPH oxidase by apocynin leads to a decreased oxidative burst in porcine granulocytes and increased survival of *S. suis* in blood reconstituted with hyperimmune sera, without affecting granulocyte viability. (A) Oxidative burst of granulocytes in porcine blood in response to *in vitro* infection with different *S. suis* serotypes with or without the NADPH oxidase inhibitor apocynin. The oxidative burst induced by *S. suis* and hyperimmune sera was set at 100% (control [ctr]). The reduction in the oxidative burst by the addition of apocynin ($n = 5$ to 6) is depicted as a percentage of the oxidative burst without the inhibitor. (B) Survival factors of *S. suis* serotypes 2, 9, and 7 in the presence of hyperimmune sera with or without the addition of the NADPH oxidase inhibitor apocynin as the quotients of CFU per milliliter after 1 h and CFU per milliliter directly after *in vitro* infection with *S. suis* ($n = 5$ to 6). (C) The viability of granulocytes after the bactericidal assay (B) was measured by flow cytometry by staining with the fixable viability dye eF506 ($n = 3$). Bars and error bars represent means and standard deviations. Statistical analyses between controls and the respective apocynin-treated samples were done by an unpaired *t* test ($n = 5$ to 6/group) (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant).

To ensure that the increased survival of *S. suis* by the addition of apocynin was not due to a toxic effect of the inhibitor on phagocytic granulocytes, we investigated the viability of these cells subsequent to the bactericidal assay. The viability of porcine granulocytes was not reduced by the addition of the NADPH oxidase inhibitor, neither in samples with *S. suis* nor in samples with serum only (Fig. 4C). Viability dye staining results were validated using additional blood samples that were stressed by heat treatment (Fig. S2B). To demonstrate a direct impact of apocynin on granulocytes, we additionally conducted an assay with isolated granulocytes in 20% hyperimmune

serum to analyze the oxidative burst and bacterial survival. We observed a reduction of the oxidative burst and an increase in bacterial survival (Fig. S2C) by apocynin treatment, as shown in Fig. 4A and B for the reconstituted blood assays. This shows that oxidative burst induction in granulocytes does not require additional cellular interactions and that apocynin has a direct impact on granulocytes.

Taken together, these experiments demonstrate that the immune serum-induced oxidative burst in blood granulocytes is an important part of the immune response leading to the killing of *S. suis*.

Complement is involved in oxidative burst induction of porcine granulocytes in response to *S. suis*. To investigate the involvement of complement in the induction of the oxidative burst and killing of streptococci, we used vaccinia virus complement control protein (VCP), which was previously described to be an effective complement inhibitor in porcine blood (9, 20). Bactericidal and oxidative burst assays with *S. suis* strain 10 were conducted in the presence of VCP, apocynin, or the combination of both inhibitors. In these assays, porcine blood cells were resuspended in hyperimmune serum raised against *S. suis cps2* strain 10. As shown in Fig. 3, this hyperimmune serum induced high frequencies of $Rho123^+$ granulocytes (oxidative burst rate) in the presence of *S. suis* strain 10 (23.81% $\pm$ 4.72% [$n = 6$]) in comparison to the control without *S. suis* (5.065% $\pm$ 2.26% [$n = 6$]) (Fig. 5A). In infected samples, VCP treatment significantly reduced the frequencies of $Rho123^+$ granulocytes (11.17% $\pm$ 3.60% [$n = 6$]). As expected, apocynin also reduced the oxidative burst (7.58% $\pm$ 2.50% [$n = 3$]). The combination of VCP and apocynin led to highly significantly lower frequencies of $Rho123^+$ granulocytes (3.33% $\pm$ 2.31% [$n = 6$]). However, the bacterial survival factors increased significantly only in the presence of apocynin (4.03 $\pm$ 1.73 for Apo [$n = 3$] versus 0.02 $\pm$ 0.01 for the control [$n = 6$]) or by the combination of VPC and apocynin (4.84 $\pm$ 2.63 [$n = 6$]). Survival factors were only marginally increased by VCP alone (0.15 $\pm$ 0.11 [$n = 6$]), as demonstrated in Fig. 5B. From these results, we conclude that complement is involved in ROS induction. However, in the presence of complete hyperimmune serum (containing *S. suis*-specific IgG and IgM), the killing of streptococci also involves complement-independent mechanisms.

We observed a stronger inhibitory effect of apocynin in the *cps2* wt- than in the 10cpsΔEF-infected sample (Fig. 4A). Since capsule-specific antibodies are primarily of the IgM isotype, this observation encouraged us to investigate the role of IgM in the induction of ROS. To eliminate the effects of IgG, hyperimmune serum against *S. suis* strain 10 was depleted of total IgG. The removal of IgG from serum was verified by affinity chromatography (Fig. S3). As the IgG depletion process goes along with a high dilution and loss of serum components, the IgG-depleted hyperimmune serum was subsequently concentrated and supplemented with 10% CDS. As shown in Fig. 6A, we observed an *S. suis*-induced oxidative burst in the absence of IgG (8.52% $\pm$ 2.63% for the control with *S. suis* strain 10 [$n = 6$] versus 3.16% $\pm$ 0.85% for the control with no *S. suis* [$n = 3$]) but at a level lower than that with complete hyperimmune serum. In the absence of IgG, the impact of VCP on the oxidative burst and on bacterial survival was stronger than that in complete hyperimmune serum. VCP treatment reduced the oxidative burst rate >4.5-fold (1.88% $\pm$ 0.59% [$n = 6$]) (Fig. 6A). Apocynin treatment alone again reduced the oxidative burst significantly (0.99% $\pm$ 0.25% [$n = 6$]), as did the combination of both inhibitors (1.06% $\pm$ 0.31% [$n = 6$]). Interestingly, the inhibition of complement in IgG-depleted hyperimmune serum had a significant effect on bacterial survival: whereas the untreated control still mediated bacterial killing (survival factor, 0.09 $\pm$ 0.10 [$n = 6$]), VCP treatment resulted in a significantly increased survival factor (3.99 $\pm$ 2.38 [$n = 6$]) (Fig. 6B). Similarly, treatment with apocynin also led to a significant increase of the survival factor (4.29 $\pm$ 1.14 [$n = 6$]). In contrast to complete serum, the combination of apocynin and VCP increased bacterial survival immensely in IgG-depleted serum (17.38 $\pm$ 4.56 [$n = 6$]). This additive effect of both inhibitors in the absence of IgG suggests that besides the complement-induced oxidative burst axis, killing activities of complement and the oxidative burst also work independently of each other (Fig. 6B).

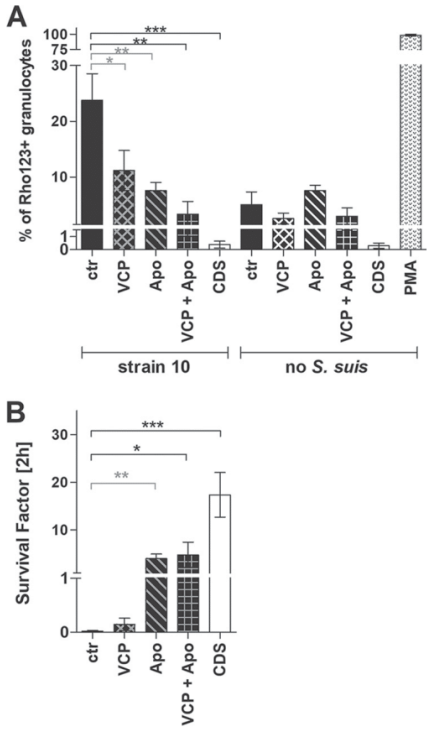


FIG 5 Complement partially mediates oxidative burst induction but is not crucial for the killing of *S. suis* strain 10 in blood reconstituted with hyperimmune serum raised against *cps2* strain 10. Hyperimmune serum raised against *S. suis* strain 10 was used to reconstitute porcine blood subsequently infected with *S. suis* strain 10 to analyze the oxidative burst (shown as percentages of $Rho123^+$ granulocytes) (A) and bacterial survival (shown as survival factors) (B) in the untreated control sample (ctr) or in the presence of the complement inhibitor VCP (100 μg/ml), the NADPH oxidase inhibitor apocynin (Apo) (1.5 mM), or a combination of both. Survival factors were determined after 2 h at 37°C after *in vitro* infection with 2×10^6 CFU/ml *S. suis* strain 10. To investigate the *S. suis*-induced oxidative burst, the same sample treatments were conducted in the absence of *S. suis* ($n = 3$). PMA (0.1 μg/ml) was used as a positive control in oxidative burst experiments. Bars and error bars represent means and standard deviations. For statistical analysis, the Kruskal-Wallis test with Dunn's multiple-comparison test was used ($n = 6$). All *S. suis in vitro*-infected groups (black brackets) or only the subgroups of controls versus VCP and VCP plus Apo (gray brackets) were included in statistical analyses (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

In summary, complement is strongly involved in the IgG-independent induction of the oxidative burst in blood granulocytes of an *S. suis*-immunized pig. Our results suggest that the role of complement for bacterial killing is especially important in the absence of or at low levels of antigen-specific IgG.

Cleavage of IgM by rIde$_{Ssuis}$_h reduces the oxidative burst and increases the survival of the *S. suis cps2* strain in porcine blood in a partially complement-independent manner. To further characterize the role of IgM and the possible IgM-complement-oxidative burst axis that might mediate the killing of *S. suis*, we used IgG-depleted hyperimmune serum but now in combination with Ide$_{Ssuis}$, a protease highly specific for porcine IgM, derived from *S. suis* itself (21). Noteworthy, previous bactericidal assays showed that the expression of Ide$_{Ssuis}$ by *S. suis* is not sufficient to cleave most of the IgM in porcine blood (9). Consequently, we conducted bactericidal and oxidative burst experiments with IgG-depleted hyperimmune serum after cleaving IgM with a recombinant Ide$_{Ssuis}$ homologue (rIde$_{Ssuis}$_h) (21), a truncated variant of the enzyme containing the active center. To reveal accidental side effects caused by potential contamination with endotoxin due to the expression of recombinant Ide$_{Ssuis}$_h in *Escherichia coli*, we used rIde$_{Ssuis}$_h that was heat inactivated at 95°C

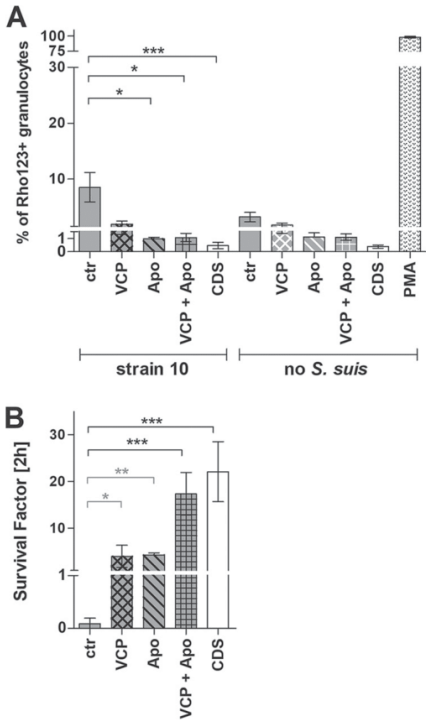


FIG 6 Influence of complement on the oxidative burst (A) and the survival of *cps2 S. suis* strain 10 (B) in porcine blood reconstituted with IgG-depleted hyperimmune serum. IgG-depleted hyperimmune serum raised against *cps2* strain 10, supplemented with 10% CDS, was used to reconstitute porcine blood samples subsequently infected with *S. suis*. The oxidative burst (shown as percentages of $Rho123^+$ granulocytes) (A) and bacterial survival (shown as survival factors) (B) were analyzed in the presence of the complement inhibitor VCP (100 μg/ml), the NADPH oxidase inhibitor apocynin (Apo) (1.5 mM), or a combination of both as well as in an untreated sample (ctr). Bacterial survival factors were determined after 2 h at 37°C after *in vitro* infection with 2×10^6 CFU/ml *S. suis* strain 10. To determine *S. suis*-specific mechanisms of oxidative burst induction, the same sample treatments were conducted in the absence of *S. suis* ($n = 3$). PMA (0.1 μg/ml) was used as a positive control in oxidative burst experiments. Bars and error bars represent means and standard deviations. For statistical analysis, the Kruskal-Wallis test with Dunn's multiple-comparison test was used ($n = 6$). All *S. suis in vitro*-infected groups (black brackets) or only the subgroups of controls versus VCP and VCP plus Apo (gray brackets) were included in statistical analyses (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(rIde$_{Ssuis}$_h_95°C) as a negative control as well as the loss-of-function point-mutated variant rIde$_{Ssuis}$_h_C195S (9). Complete cleavage of IgM in IgG-depleted hyperimmune serum through the addition of rIde$_{Ssuis}$_h was proven by Western blotting, as demonstrated in Fig. S4. The killing of *S. suis* strain 10 in the bactericidal assay with blood reconstituted with the IgG-depleted hyperimmune serum was primarily mediated via IgM (Fig. 7A, left), as shown by the addition of rIde$_{Ssuis}$_h. Survival factors of *S. suis* strain 10 in IgG-depleted serum (control, 0.03 ± 0.02 [$n = 3$]) were considerably increased by the addition of rIde$_{Ssuis}$_h (26.08 ± 5.69 [$n = 3$]) but not by the addition of the nonfunctional rIde$_{Ssuis}$ variants rIde$_{Ssuis}$_h_95°C (0.01 ± 0.002 [$n = 3$]) and rIde$_{Ssuis}$_h_C195S (0.01 ± 0.01) (Fig. 7A, left). Simultaneously, the oxidative burst response to *S. suis* strain 10 (control, $7.55\% \pm 2.16\%$ [$n = 3$]) was significantly reduced by the addition of the IgM protease rIde$_{Ssuis}$_h ($2.05\% \pm 0.25\%$ [$n = 3$]), in contrast to the addition of rIde$_{Ssuis}$_h_95°C ($6.81\% \pm 1.30\%$ [$n = 3$]) and rIde$_{Ssuis}$_h_C195S ($8.62\% \pm 1.50\%$ [$n = 3$]) (Fig. 7B, left). Additionally, complement inactivation (heat inactivation for 30 min at 56°C) decreased the oxidative burst regardless of the integrity of IgM (Fig. 7B, middle). These results demonstrate the relevance of the IgM-complement-oxidative burst axis as a mechanism for the killing of *S. suis* in the blood of a bacterin-immunized piglet,

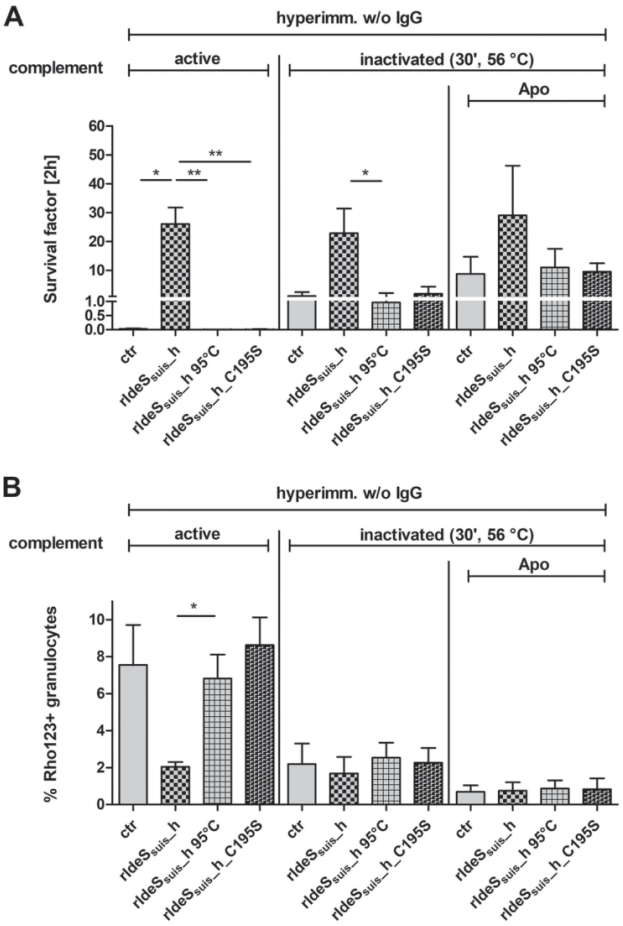


FIG 7 Impact of IgM on killing of *S. suis* (A) and induction of the oxidative burst in granulocytes (B) in porcine blood reconstituted with IgG-depleted hyperimmune serum. Shown are bacterial survival factors (A) and the oxidative burst responses of granulocytes (B) in *S. suis* strain 10-infected porcine blood reconstituted with IgG-depleted hyperimmune serum (raised against *cps2* strain 10) pretreated with PBS as a control (ctr) or with the IgM protease Ide$_{ssuis}$_h or its nonfunctional control rIde$_{ssuis}$_h_95°C (heat inactivated) or rIde$_{ssuis}$_h_C195S. Particular serum treatments were used without further treatment (left), were heat inactivated (56°C for 30 min) (middle and left) for complement inactivation, or were additionally treated with the NADPH oxidase inhibitor apocynin (1.5 mM) (right). The oxidative burst was measured by flow cytometry and is depicted as a percentage of Rho123$^+$ cells of all granulocytes. Bars and error bars represent means and standard deviations, and significant differences are indicated. Statistical significances were calculated using the Kruskal-Wallis test with Dunn's multiple-comparison test ($n = 3$ to 6) (*, $P < 0.05$; **, $P < 0.01$).

independent of IgG. Interestingly, in bactericidal assays using heat-inactivated IgG-free serum, treatment with rIde$_{ssuis}$_h also resulted in a substantial increase of the bacterial survival factor. This indicates that the killing activity of IgM does not depend solely on complement (Fig. 7A, middle).

To investigate whether the complement-independent bactericidal activity of IgM is still mediated via the oxidative burst, samples with the NADPH oxidase inhibitor apocynin were included. Inhibition of the oxidative burst led to a substantial increase of the bacterial survival factor of strain 10 in blood reconstituted with IgG-depleted and heat-inactivated serum, as shown in Fig. 6B. However, treatment of reconstituted IgG-depleted, complement-inactivated, apocynin-treated blood with rIde$_{ssuis}$_h again led to a higher survival factor for *S. suis* strain 10 than for the respective samples

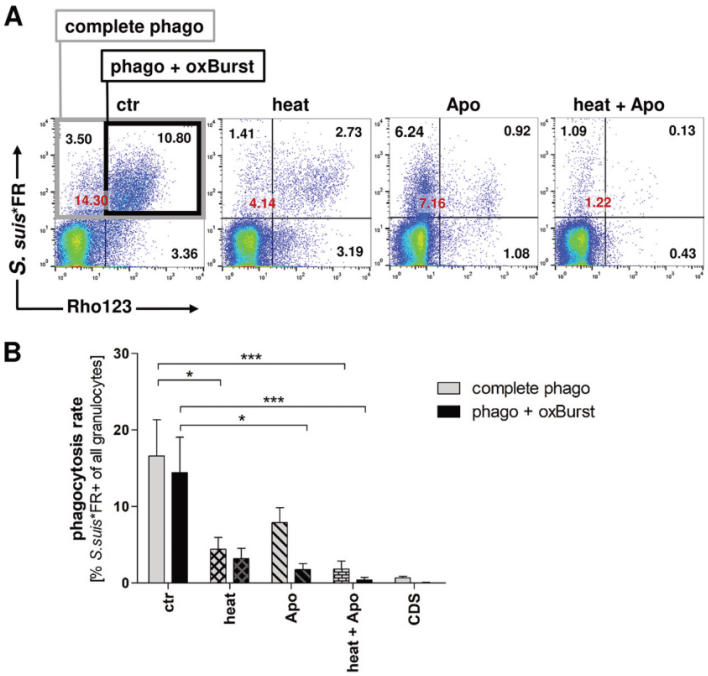


FIG 8 Influence of complement and NADPH oxidase inhibition on the rate of phagocytosis of porcine granulocytes in blood reconstituted with hyperimmune serum. (A) Blood reconstituted with hyperimmune serum raised against *cps2* strain 10 was infected with CellTrace far-red (FR)-labeled *S. suis* strain 10, and samples were analyzed by flow cytometry to estimate the oxidative burst response (Rho123 signal) within phagocytic granulocytes. (B) Complete phagocytosis rate depicted as a percentage of *S. suis* FR-positive granulocytes. Serum was untreated, heat inactivated (56°C for 30 min) to inhibit complement, and/or treated with the NADPH oxidase inhibitor apocynin (1.5 mM). Bars and error bars represent means and standard deviations, and significant differences are indicated. Statistical significances were calculated using the Kruskal-Wallis test with Dunn's multiple-comparison test ($n = 7$) (*, $P < 0.05$; ***, $P < 0.001$).

preincubated with nonfunctional rIde$_{Ssuis}$ constructs, although these differences were not significant (Fig. 7A, right). This suggests that the killing or at least inhibition of proliferation of *S. suis* by IgM is not mediated only via the complement system or the oxidative burst.

In summary, for blood reconstituted with IgG-depleted serum of a bacterin-vaccinated piglet, we showed IgM-mediated killing of *S. suis* in association with a pronounced induction of ROS. Complement inactivation and/or IgM degradation in this serum led to an overall reduction in ROS production corresponding to increased survival factors for *S. suis*. These results are in accordance with an IgM-complement-oxidative burst axis mediating the killing of *S. suis*, although IgM-mediated but complement-independent mechanisms were also observed (see Fig. 10).

Phagocytosis of *S. suis* in hyperimmune serum is primarily linked to ROS production and reduced by complement inactivation. In order to investigate the phagocytosis of *S. suis* in the context of ROS production in granulocytes, we used *S. suis* labeled with CellTrace far-red (FR) fluorescent dye in combination with DHR123 in assays with reconstituted blood to quantify complete phagocytosis or the subset showing both phagocytosis and ROS production (Fig. 8A, left).

Blood reconstituted with hyperimmune serum and infected with FR-labeled streptococci showed that 16.61% ± 4.73% of granulocytes were positive for engulfed fluorescent streptococci. The majority (89%) of these granulocytes (equal to 14.48% ± 4.58% of the total granulocytes) showed simultaneous ROS production (Fig. 8A and B). These data confirm ROS induction in association with the phagocytosis of

S. suis in porcine blood granulocytes. However, subsets of granulocytes showed either exclusively phagocytosis (control, 2.13% ± 0.65%) or exclusively ROS production (control, 8.64% ± 5.90%). Consequently, the phagocytosis assay allows a more precise analysis of *S. suis*-specific ROS induction. Heat treatment of hyperimmune serum reduced the percentages of both granulocytes with engulfed streptococci and granulocytes with detectable phagocytosis and ROS induction, to similar extents (Fig. 8B). This indicates that complement-mediated phagocytosis is associated with ROS induction. Apocynin treatment reduced the percentage of granulocytes showing ROS induction in association with phagocytosis significantly (1.75% ± 0.76% versus 14.48% ± 4.58%). However, the complete phagocytosis rate was also reduced in apocynin-treated samples albeit to a lesser extent (7.9% ± 1.9% versus 16.61% ± 4.73%), as shown in Fig. 8A and B. To prove the specific reduction of ROS production by apocynin in porcine granulocytes, we additionally treated phorbol myristate acetate (PMA)-stimulated blood samples with apocynin. We detected a clear reduction of the Rho123 signal by apocynin treatment even when PMA, as a strong stimulus for ROS induction, was used (Fig. S2D). In samples reconstituted with complement-inactivated hyperimmune serum, the addition of apocynin led to a statistically significant decrease of complete phagocytosis (1.85% ± 1.04%) and particularly to a strong reduction in ROS-producing phagocytic cells (0.41% ± 0.35%) compared to the untreated control.

Our results indicate that the majority of apocynin-treated blood granulocytes having phagocytosed *S. suis* do not produce ROS (Fig. 8B). This supports the conclusion that apocynin is an effective inhibitor of the oxidative burst in granulocytes with demonstrated phagocytic activity. However, since apocynin treatment leads not only to reduced ROS production but also to reduced phagocytosis, the increase of bacterial survival by this inhibitor might be a result of a combination of reduced ROS production and phagocytosis. Recently, it was shown for porcine alveolar macrophages that NADPH oxidase-dependent ROS generation has a positive effect on phagocytosis via mitogen-activated protein kinase (MAPK) activation and that apocynin treatment efficiently reduces this ROS-mediated stimulation of phagocytosis (22). Whether this also applies to porcine granulocytes is not clear, but inhibition of phagocytosis through apocynin treatment has also been described for human granulocytes (23).

The detection of *S. suis*-specific phagocytosis in combination with ROS confirmed our above-described findings of complement-mediated induction of the oxidative burst.

IgM-mediated phagocytosis of fluorescently labeled *S. suis* cells is associated with ROS production and decreased bacterial survival. We further investigated IgM-mediated effects on phagocytosis, ROS induction, and bacterial survival using FR-labeled *S. suis* and a new preparation of IgG-depleted hyperimmune serum. Noteworthy, fluorescently labeled streptococci showed significantly reduced survival factors in blood reconstituted with CDS (Fig. S5), indicating diminished viability, which makes it difficult to compare bacterial survival data with the results of the experiments with unlabeled bacteria described above. Using IgG-depleted hyperimmune serum for bactericidal assays with reconstituted blood, we observed reductions of phagocytosis and the oxidative burst in comparison to blood reconstituted with complete hyperimmune serum (Fig. 9). Cleavage of IgM with Ide_{Ssuis} or treatment with VCP significantly reduced the phagocytosis rate from 4.6% ± 2.8% to 0.2% ± 0.2% and 0.4% ± 0.2%, respectively. This indicates that phagocytosis in the absence of specific IgG is mainly driven by IgM and complement. Since the phagocytosis rates were not significantly different between control samples with cleaved IgM, VCP-treated samples containing intact IgM, and VCP-treated samples with cleaved IgM, we conclude that IgM-mediated phagocytosis is mainly driven by complement activation. The increased phagocytosis in the presence of intact IgM and active complement was associated with a percentage of ROS-positive granulocytes that was at least 30-fold higher than that in the samples with cleaved IgM or complement inhibition (2.6% ± 1.9% versus 0.02% ± 0.02% and 0.08% ± 0.06%, respectively) (Fig. 9A). Determination of bacterial survival confirmed the IgM-mediated killing of streptococci under these conditions, as Ide_{Ssuis} treatment

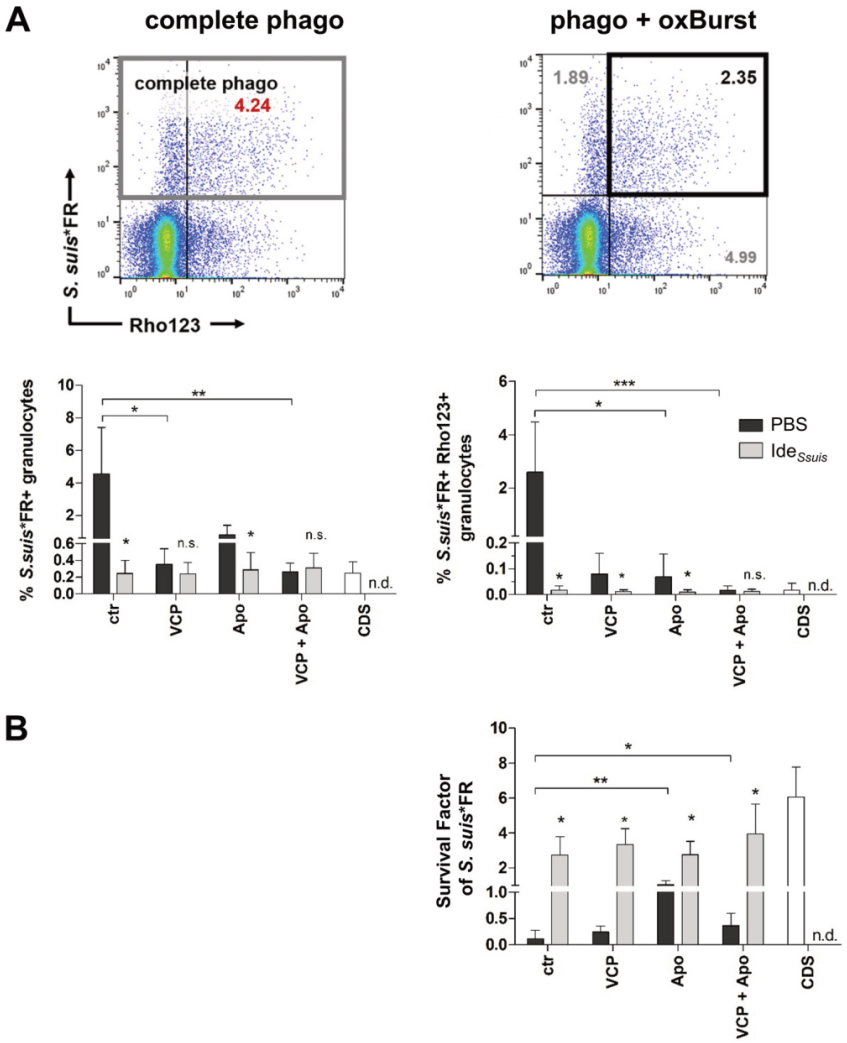


FIG 9 Impact of IgM and complement on phagocytosis and ROS production of porcine granulocytes in blood reconstituted with IgG-depleted hyperimmune serum. Fluorescently labeled *S. suis* strain 10 bacteria were used to infect porcine blood reconstituted with IgG-depleted hyperimmune serum (raised against *cps2* strain 10) pretreated with PBS as a control (PBS) or with the IgM protease Ide_{Ssuis}–h to analyze the phagocytosis rate and oxidative burst within phagocytic granulocytes (A) and bacterial survival (B). Serum was left without inhibitor (ctr), treated with VCP (100 μg/ml) for complement inactivation, and/or treated with the NADPH oxidase inhibitor apocynin (1.5 mM). The phagocytosis rate and oxidative burst response of granulocytes were measured by flow cytometry and are depicted as percentages of *S. suis* FR-positive and $Rho123^+$ cells of all granulocytes, respectively. Bars and error bars represent means and standard deviations, and significant differences are indicated. Statistical significances were calculated using the Kruskal-Wallis test with Dunn's multiple-comparison test ($n = 6$) (marked with brackets) and the Wilcoxon test for each group of samples versus the Ide_{Ssuis}-treated equivalent (without brackets) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). n.d., not determined.

led to an increase of the survival factor from 0.1 ± 0.2 (control) to 2.7 ± 1.1 (Ide_{Ssuis}), indicating efficient killing and bacterial proliferation, respectively (Fig. 9B). Inhibition of complement by VCP treatment resulted in only a moderate increase of the bacterial survival factor to 0.2 ± 0.1, which is significantly lower than the bacterial survival factor in blood with cleaved IgM.

Apocynin treatment reduced the frequency of ROS-positive granulocytes containing *S. suis* 37-fold, from 2.6% ± 1.9% to 0.07% ± 0.09%, whereas the phagocytosis rate was

reduced only 5.8-fold, from 4.6% ± 2.9% to 0.8% ± 0.6%. A significant increase in the bacterial survival factor to 1.0 ± 0.2 was observed in apocynin-treated samples, whereas the combination of VCP and apocynin treatment resulted in a survival factor of 0.4 ± 0.2. However, as the killing of *S. suis* under these conditions was IgM dependent and apocynin-treated samples had significantly higher bacterial survival factors, we conclude that IgM-dependent ROS induction is important for the killing of *S. suis*. This might include a putative direct effect of ROS generation on the phagocytosis rate.

DISCUSSION

The killing of invading bacteria via the oxidative burst of phagocytic cells is an important defense mechanism of innate immunity (10, 24). However, knowledge of the susceptibility of *S. suis* to ROS and the contribution of the oxidative burst to the killing of this important pathogen remains limited. As shown in this study, *S. suis* is highly susceptible to oxidative stress. We demonstrate the antibody-mediated induction of the oxidative burst in blood granulocytes with a partial contribution from complement. The involvement of the oxidative burst in the killing of *S. suis* was demonstrated by using an NADPH oxidase inhibitor.

Although streptococci are catalase negative, alternative antioxidant systems have been described for *S. pneumoniae* and *S. pyogenes* (13, 14). Whether *S. suis* is sensitive to H_2O_2, a potent bactericidal intermediate of the oxidative burst, was investigated in a previous study. Using H_2O_2 concentrations of up to 0.04%, Y. Tang et al. concluded that *S. suis* wt serotype 2 strains are not impaired in growth by the addition of hydrogen peroxide to brain heart infusion broth (17). Our results, on the other hand, demonstrate a clear impact of as little as 0.001% hydrogen peroxide on the growth of *S. suis* in THB, added after a 2-h growth phase without hydrogen peroxide. Using virulent *S. suis* strains of three different serotypes, we show that susceptibility to hydrogen peroxide is not limited to a certain strain or serotype, whereas previous studies were conducted only with strains of serotype 2 (17, 18).

In order to test if the polysaccharide capsule of *S. suis* protects against oxidative burst intermediates, we included a nonencapsulated strain in our study. We observed that the unencapsulated *S. suis* mutant 10cpsΔEF was not more susceptible to hydrogen peroxide than the wt strain 10. Whereas the role of the polysaccharide capsule in the susceptibility of *S. suis* to ROS had not been investigated to date, the influence of the capsule on burst induction was studied for *S. pneumoniae*. Barbuti et al. showed that the presence of a capsule in pneumococci does not alter the extent of ROS production by human neutrophils (25). We generated similar results by comparing oxidative burst levels induced by an *S. suis* serotype 2 wt strain and an isogenic capsule-deficient mutant (raw data not shown).

Previous studies investigating the oxidative burst response to *S. suis* were conducted using murine macrophages or field-infected animals whose cells were stimulated with fMLP (*N*-formyl-Met-Leu-Phe) (17, 26). Here, we investigated for the first time the induction of the oxidative burst in porcine blood granulocytes in response to *S. suis* in the context of defined humoral factors. The experimental conditions of our study are of substantial biological relevance, as septicemia and meningitis in pigs following bacteremia are predominant pathologies of *S. suis* infections in the field. Consecutive measurements of ROS and associated specific bacterial loads revealed for the first time the induction of the oxidative burst *in vivo* in blood granulocytes of a pig following an experimental challenge. Although conclusive evidence from the animal experiment is limited, as it included only two animals, the results are in accordance with the concept that *S. suis*-specific antibodies lead to the induction of ROS in blood granulocytes, which in turn contributes to the killing of streptococci during bacteremia. In any case, the results demonstrate that two pigs of the same age and herd may exhibit substantial differences in ROS production during bacteremia. This was reason enough for us to focus on the biological role of ROS in the control of bacteremia in pigs with specific antibodies.

The use of the NADPH oxidase inhibitor apocynin revealed that inhibition of the

oxidative burst leads to increased survival of *S. suis* strains belonging to different serotypes. Apocynin acts on the NADPH oxidase subunit p47phox that is present only in the NOX2 isoform, and as it needs preactivation by myeloperoxidase, it inhibits the oxidative burst in phagocytic leukocytes only (27, 28). Lu et al. (22) showed that NOX2-based NADPH oxidase-dependent ROS generation is responsible for increased phagocytosis of porcine alveolar macrophages. Additionally, Almeida et al. (23) described a link between ROS production and phagocytosis in granulocytes. Accordingly, our data also suggest a reduction in the percentage of phagocytic granulocytes after inhibition of NADPH oxidase activity with apocynin. Thus, increased survival of *S. suis* under these conditions might be a combined result of decreased phagocytosis and increased bacterial survival within phagolysosomes of granulocytes incapable of producing ROS under the influence of apocynin. From these results, we conclude that the oxidative burst of porcine granulocytes is indeed an important defense mechanism contributing to the killing of *S. suis*. In addition, we demonstrate that there is no derogatory effect of apocynin on the viability of isolated porcine granulocytes (see Fig. S2B in the supplemental material).

Human granulocytes express both high- and low-affinity Fc receptors that are involved in the recognition and phagocytosis of antibody-opsonized pathogens (19). It is known that the activation of Fc receptors on neutrophils directly induces high levels of ROS (16), and some studies suggest that only Fc receptor-mediated phagocytosis can induce ROS production (29). Our data show a distinct impact of antibody concentrations in serum on oxidative burst levels. However, the depletion of total IgG from hyperimmune serum does not result in a complete abrogation of the oxidative burst. As the concentration of serum and the addition of CDS (free of IgG) were conducted after IgG depletion, differences between original and IgG-depleted hyperimmune sera cannot be attributed solely to the absence or presence of IgG. Nevertheless, the higher level of ROS induction mediated by the original hyperimmune serum (compare Fig. 5A and Fig. 6A) is in accordance with IgG-FcγR-mediated oxidative burst induction in granulocytes (Fig. 10, pathway 1).

The results obtained with IgG-depleted hyperimmune serum clearly show that other mechanisms besides IgG might induce an oxidative burst and killing of streptococci in porcine blood of a bacterin-immunized piglet. Similar results with regard to the role of IgG-independent oxidative burst induction were obtained by Nilsson et al., who showed that blocking of Fc receptors on human neutrophils prevented neither the oxidative burst nor the killing of *S. pyogenes* (30). Besides IgG, complement can also induce the oxidative burst in neutrophils, which possess five distinct complement receptors (31). For group A *Streptococcus*, it was demonstrated that the activation of human complement receptor 3 (CR3 or CD11b/CD18) via iC3b is required for ROS production and killing of *S. pyogenes* by human neutrophils (30). Similarly, we show in this study that inhibition of complement in blood significantly reduced the oxidative burst. Since porcine neutrophils express the CR3 orthologue wCD11R3, it is likely that the process of complement-mediated burst induction in porcine granulocytes (Fig. 10, pathway 2) resembles the process in human cells (32). However, inhibition of complement in blood reconstituted with complete hyperimmune serum including high IgG levels showed marginal effects on the killing of *S. suis*.

The killing of *S. suis* could still be observed in blood reconstituted with IgG-depleted serum, but in this case, it was complement dependent, as shown by complement inhibition via VCP. However, this effect was less pronounced in experiments using fluorescently labeled streptococci. As IgM induces the classical complement pathway (33), we investigated whether IgM-induced complement activation (Fig. 10, pathway 3) leads to the subsequent induction of the oxidative burst (Fig. 10, pathway 4), here designated the IgM-complement-oxidative burst axis. The existence and relevance of this pathway were confirmed by the reconstitution of blood with IgG-depleted and IgM protease-treated hyperimmune serum. These treatments result in a reduction of the oxidative burst and an increase in bacterial survival. However, our results suggest that IgM also possesses antibacterial activity against *S. suis* independently of complement

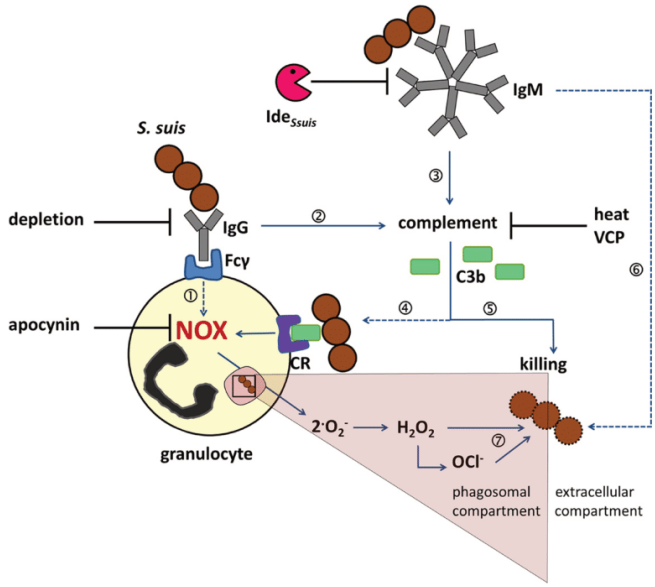


FIG 10 Schematic overview of mechanisms inducing the oxidative burst and mediating killing of *S. suis* by blood granulocytes. Mechanisms inducing the oxidative burst in porcine granulocytes and the killing of *S. suis* as well as inhibitors of the different pathways are depicted. Inhibitors used in this study to block certain players in the ROS induction cascade are shown as black blind-ended arrows, whereas pathways observed in this study are indicated by blue arrows or as dotted blue arrows in case of supposed pathways. Binding of IgG to *S. suis* can induce the oxidative burst via direct IgG-Fcγ receptor binding (pathway 1) or via complement (2). IgM induces the oxidative burst via complement (3). The complement-induced oxidative burst is potentially mediated via complement receptor (CR) signaling (4). Complement-mediated killing, independent of ROS production, could also be observed (5) and presumably occurs extracellularly. However, we observed IgM-mediated killing of *S. suis* independent of a complement-induced oxidative burst (6). Note that FcμR is absent from phagocytic cells (34). The activation of NADPH oxidase (NOX) ultimately leads to the production of bactericidal hydrogen peroxide (H_2O_2) and hypohalites such as hypochlorite (OCl^-) that mediate the killing of *S. suis* (7) in the suggested phagosomal compartment (depicted as a pink-shaded trapeze-shaped extension).

and the oxidative burst (Fig. 7A and Fig. 9B). It is conceivable that the aggregation of *S. suis* by IgM pentamers is involved in the oxidative burst- and complement-independent antimicrobial activity of IgM since aggregation might prevent bacterial proliferation (Fig. 10, pathway 6). This might even be the reason why the survival factors of fluorescently labeled streptococci were lower in samples with combined apocynin and VCP treatment than in samples treated with apocynin alone (Fig. 9B). However, we do not know why this is contrary to the findings obtained for unlabeled bacteria shown in Fig. 6B, and we can only speculate that this might be related to the different viability of fluorescently labeled streptococci in porcine blood (Fig. S5). In any case, the complement-independent killing mechanism of IgM is unlikely to be due to direct IgM-Fc receptor-mediated signaling since phagocytic cells do not possess an Fcμ receptor (34). Instead, IgM might mediate antibacterial activity and complement-independent burst induction via immune complexes (ICs). Lucisano et al. and Furriel et al. showed that IgM-ICs can bind to rabbit polymorphonuclear cells and induce the oxidative burst and lysosomal enzyme release (35, 36). This mode of action does not require the phagocytosis of IgM-ICs, and it is conceivable that *S. suis*-IgM-ICs also contribute to oxidative burst induction and killing of *S. suis*. Accordingly, the percentages of Rho123$^+$ granulocytes following complement inhibition were lower in IgM-cleaved samples, but the differences were rather small (Fig. 6B and Fig. 9B). For pneumococci, it has been shown that capsular polysaccharide-specific human monoclonal IgM has the ability to reduce the number of CFU even in the absence of phagocytes or complement and that this correlates with IgM aggregates (37).

In summary, this study was designed to investigate the oxidative burst responses of porcine granulocytes to different *S. suis* serotypes and elucidate in particular the oxidative burst-inducing factors as well as the role of the oxidative burst in the killing of *S. suis*. To conclude, in this study, we show for the first time the induction of the oxidative burst in porcine granulocytes in response to *S. suis* in porcine blood *in vitro* and *in vivo* and clearly demonstrate the involvement of the oxidative burst in the killing of *S. suis* in blood reconstituted with serum of a bacterin-vaccinated pig. The induction of the oxidative burst by *S. suis* was shown to be dependent on IgM antibodies as well as on complement, and our data suggest a likely role for IgG as well.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. suis* was grown in Todd-Hewitt broth (THB) (product no. 249240; Becton, Dickinson), and glycerol stocks were prepared at the late exponential growth phase. For experimental infection of pigs, *S. suis* was grown in tryptic soy broth (TSB) without dextrose (product no. 286220; Becton, Dickinson) at 37°C with 5% CO_2. The infection inoculum was prepared at the late exponential growth phase. *S. suis* strain 10 is a virulent muramidase-released protein-positive (MRP^+), extracellular factor-positive (EF^+), Ide_{Ssuis}-positive (Ide_{Ssuis}^+) suilysin-positive *cps2* strain that has been used for pathogenesis studies by different groups (21, 38, 39). *S. suis* 10cpsΔEF is an isogenic mutant of *S. suis* strain 10 deficient in capsule expression (38). *S. suis* A3286/94 is a virulent MRP^+ suilysin-positive *cps9* strain isolated from a pig with meningitis and has also been used in pathogenesis studies previously (40, 41). *S. suis* strain 16085/3b is a virulent MRP^+ suilysin-positive *cps9* strain (42). *S. suis* 13-00283-02 is a virulent MRP^+ suilysin-negative *cps7* strain (41). *Escherichia coli* strains harboring plasmids $pET45bide_{Ssuis_}$homologue and $pET45bide_{Ssuis_}$homologue_C195S were grown in Luria-Bertani medium (product no. X968.2; Carl Roth) at 37°C under constant shaking and with the addition of 100 μg/ml ampicillin (product no. K029.4; Carl Roth).

Experimental infection of piglets to measure the oxidative burst in *in vivo* blood samples. Two 8-week-old German Landrace piglets from a herd infected with numerous *S. suis* pathotypes were intravenously infected with 3×10^8 CFU of *S. suis* strain 10 under ketamine and azaperone anesthesia. At 12 h postinfection, the piglets were anesthetized again through the application of ketamine and azaperone, and anesthesia was maintained via inhalation of isoflurane. Blood samples were taken from the *vena jugularis* or *arteria femoralis* at defined time points (preinfection and 0.5 h, 13 h, 14 h, 16 h, and 19 h postinfection). CFU were determined in blood samples by plating of serial dilutions to monitor the status of bacteremia. Measurements of the oxidative burst *in vivo* were conducted using 0.1-ml heparinized whole-blood samples that were immediately stained with dihydrorhodamine 123 (DHR123) upon blood withdrawal. At each time point, an additional PMA-stimulated 0.1-ml blood sample (0.1 μg/ml) served as the positive control. Erythrocyte lysis, fixation, and sample analysis via flow cytometry were performed as described above for *in vitro* oxidative burst experiments. The animal experiment was approved by the Committee on Animal Experiments of the Lower Saxonian State Office for Consumer Protection and Food Safety under permit no. 33.8-42502-04-18/2879. Handling and treatment of animals were done in strict accordance with the principles of the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (43) as well as German animal protection law. Both piglets were euthanized 19 h after intravenous application of *S. suis* during inhalation anesthesia.

Growth of *S. suis* in the presence of hydrogen peroxide. Sixty milliliters of prewarmed THB medium was inoculated to an optical density at 600 nm (OD_{600}) of 0.02 with cultures of *S. suis* strains 10, 10cpsΔEF, A3286/94, 16085/3b, and 13-00283-02 grown overnight for 10 h. After 2 h of incubation at 37°C, the cultures were divided into four tubes to continue growth in either THB alone or THB supplemented with hydrogen peroxide to render the following final concentrations of H_2O_2: 0.1%, 0.01%, and 0.001% (wt/vol). The OD_{600} was measured every hour for 8 h.

Expression and purification of recombinant His-tagged proteins. Expression of recombinant His-tagged Ide_{Ssuis} homologue (r$Ide_{Ssuis_}$h) and r$Ide_{Ssuis_}$h_C195S was performed as described previously (9).

Oxidative burst experiments. Oxidative burst detection in blood granulocytes of i.v. infected pigs was performed directly in blood samples without further addition of *S. suis*. In addition, assays of reconstituted whole blood were conducted to assess the role of specific serum components in ROS induction and the killing of bacteria. Therefore, plasma-derived blood donor cells were resuspended with defined sera from other pigs. In detail, heparinized whole blood from healthy donor piglets from a commercial pig farm in Saxony, Germany, was washed two times with 10 ml 0.9% sodium chloride. The withdrawal of blood was approved under permit no. N19/14 by the responsible authorities of the state of Saxony, Germany (Landesdirektion Sachsen). Washed blood cells were resuspended in 0.9% sodium chloride and diluted in selected hyperimmune sera at a ratio of 1:1. *S. suis* was added from frozen glycerol stocks at a concentration of 2×10^6 CFU/ml. Positive controls were incubated with 0.1 μg/ml PMA (product no. 79346-1MG; Sigma-Aldrich). Samples were incubated at 37°C for 15 min in a water bath after the addition of *S. suis* or PMA. Subsequently, DHR123 (5 μg/ml) (product no. D1054-2MG; Sigma-Aldrich) was added, and incubation at 37°C in a water bath was continued for another 10 min. ROS interaction leads to the oxidation of DHR123 to cationic rhodamine 123 (Rho123). Finally, erythrocytes were lysed two times using erythrocyte lysis buffer (0.155 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM disodium EDTA [pH 7.2]), washed two times with phosphate-buffered saline (PBS), and fixed with

2% paraformaldehyde. Samples were measured immediately by flow cytometry (BD FACSCalibur) and analyzed by gating on granulocytes (FlowJo).

For experiments with the NADPH oxidase inhibitor apocynin (1.5 mM) (product no. 7884.1; Carl Roth), the inhibitor was added to reconstituted blood prior to the addition of *S. suis* and preincubated for 5 min at 37°C in a water bath. Complement inactivation of serum was achieved by treatment with VCP (vaccinia virus complement control protein) (100 μg/ml) (product no. GB-VCP250; Gene Balance Inc.) or by heat inactivation at 56°C for 30 min. For assays investigating the impact of IgM, serum was treated with 20 μg/ml of rIde$_{Ssuis}$_h (21), the point-mutated variant rIde$_{Ssuis}$_h_C195S (9), or heat-inactivated (10 min at 95°C) rIde$_{Ssuis}$_h (rIde$_{Ssuis}$_h_95°C), prior to reconstitution of blood by incubation at 37°C for 2.5 h on a rotator. To verify complete IgM cleavage, an anti-IgM Western blot assay under reducing conditions was conducted (see Fig. S4 in the supplemental material), as previously described (9).

Bactericidal assays. Bactericidal assays were always conducted in parallel with oxidative burst experiments, meaning that 0.2-ml samples containing reconstituted blood and *S. suis* at a concentration of 2×10^6 CFU/ml were divided in two immediately after the addition of bacteria. Whereas one half of the sample was used for oxidative burst experiments, the other half was used for bactericidal assays. CFU were determined by serial dilution at time zero and after incubation of samples at 37°C on a rotator for 1 or 2 h (indicated in the figures). The survival factor (SF) was calculated by dividing the CFU after the 1- or 2-h incubation period by the CFU at time zero.

Opsonophagocytosis assays. Opsonophagocytosis assays were conducted as previously described (44), with minor modifications. Briefly, 5×10^6 isolated porcine neutrophils resuspended in 500 μl RPMI supplemented with 20% (vol/vol) hyperimmune serum raised against *cps2* strain 10 were infected with 2.5×10^6 CFU *S. suis* strain 10 to obtain a multiplicity of infection (MOI) of 0.05. The samples were incubated for 2 h at 37°C on a rotator in the presence or absence of 1.5 mM apocynin.

Generation of hyperimmune sera and depletion of IgG from serum. The generation of hyperimmune sera in piglets was approved by the Landesdirektion Sachsen under permit no. N01/16. The hyperimmune serum used for experiments with *S. suis* strains 10 and 10cpsΔEF was obtained from a pig that had been prime-booster vaccinated with an *S. suis* strain 10 bacterin. The depletion of total IgG from hyperimmune serum against *S. suis* strain 10 was conducted by protein G affinity chromatography as described in detail previously (9). The moderate-antibody serum was derived from a piglet that had been experimentally infected with strain 10 and had to be euthanized due to meningitis at 5 days postinfection (the corresponding animal experiment was approved under permit no. TVV11/16 by the ethics committee of the Landesdirektion Sachsen). Hyperimmune serum against *S. suis* serotype 9 was drawn from a piglet that was prime-booster immunized with a bacterin based on strain A3286/94. The serum was drawn 18 days after the booster vaccination. The convalescent-phase serum used in oxidative burst and bactericidal assays with *S. suis cps7* strain 13-00283-02 was originally drawn from a piglet 14 days after experimental infection with *S. suis cps9* strain 16085/3b and is known to mediate the killing of different *cps* types, including *cps7* (the corresponding animal experiment was approved under permit no. TVV 28/16 by the ethics committee of the Landesdirektion Sachsen).

Anti-*S. suis* IgM and IgG ELISAs. IgM and IgG antibody levels against *S. suis* strain 10 were determined by ELISAs using plates coated with inactivated bacteria, as described previously (45). Moderate antibody levels were defined as lying within the range of 10 to 70 ELISA units. ELISA units were determined relative to reference serum that was defined as containing 100 ELISA units of both IgG and IgM.

Viability staining of granulocytes. The viability of granulocytes after bactericidal assays and subsequent erythrocyte lysis was proven by staining with a 1:500 dilution of eBioscience eF506 fixable viability dye (product no. 65-0866-14; Thermo Fisher Scientific) in PBS for 25 min. Afterwards, cells were washed, fixed, and measured via flow cytometry using an LSR Fortessa instrument (BD). As a positive control, an additional blood sample was stressed for 5 min at 95°C directly before staining with eF506 viability dye.

PCR for detection of the *sodA* gene of *S. suis*. For the detection of the *sodA* gene of *S. suis*, a colony PCR approach was used. Two colonies of each investigated *S. suis* strain (strains 10, 10cpsΔEF, A3286/94, 16085/3b, and 13-00283-02) were microwaved in 0.1 ml DNase- and RNase-free water (product no. T143.3; Carl Roth) at 100 W for 8 min, and 5 μl of this preparation served as the template for PCR with a total reaction mixture volume of 25 μl. Primers for the detection of the *sodA* gene were designed based on the *sodA* sequence of strain EA1832.92 (GenBank accession no. AB724057.1). The primer pair sodA_for (GCACCATGCAACTTATGTGGCAAATGC) and sodA_rev (CCTTCGCTGTTAACAACCAAFGAAAGCC), binding in a conserved region of the *sodA* gene, was used for the amplification of a 324-bp product. The PCR program using One*Taq* DNA polymerase (product no. M0480S; NEB) was as follows: an initial denaturation step for 30 s at 94°C, followed by 30 cycles of 30 s at 94°C, 60 s at 60°C, and 30 s at 68°C and a final elongation step for 5 min at 68°C. Gel electrophoresis was performed with a 1.5% agarose gel.

Real-time PCR for detection of *sodA* transcripts in *S. suis*. Bacterial RNA was extracted from stationary-phase THB cultures of the different *S. suis* strains used in this study. In detail, a 10-ml culture was centrifuged (10 min at 2,600 $\times$ *g* at room temperature [RT]), and the supernatant was discarded. The pellet was stored at −80°C until RNA extraction. Isolation was conducted as previously described (46). After RNA extraction, the concentration and purity of the isolated RNA were determined using the Agilent 2100 bioanalyzer (RNA 6000 Pico kit; Agilent Technologies Inc., Santa Clara, CA, USA). Quantitative real-time PCR (qRT-PCR) of reverse-transcribed RNA was designed to analyze the expression of *sodA* and the housekeeping gene *gyrB*. The respective primers are listed in Fig. S1B. qRT-PCR was conducted with the AriaMX real-time PCR system (Agilent Technologies Inc., Santa Clara, CA, USA), as previously described (46). The following modified program was used: an initial denaturation step at 95°C for 20 min

and 40 cycles of denaturation at 95°C for 25 s, annealing at 60°C for 30 s, and amplification at 72°C for 20 s. As negative controls, we included (i) water, (ii) a no-template control, and (iii) no reverse transcriptase to exclude contamination with DNA. Products were verified by melting-curve analysis and 2.0% agarose gel electrophoresis.

Combined phagocytosis and oxidative burst assays. The combined measurement of the oxidative burst and phagocytosis in a single blood sample was conducted essentially as described above for oxidative burst experiments. In order to be able to read out the oxidative burst and phagocytosis, living *S. suis* strain 10 bacteria were labeled with CellTrace far-red fluorescent dye (Thermo Fisher Scientific) and added to the blood samples at a concentration of 2×10^6 CFU/ml. All measurements using far-red-labeled bacteria were performed without the use of a quenching dye. Quenching of the extracellular signal with trypan blue was found to be unnecessary since the phagocytosis of carboxyfluorescein succinimidyl ester (CFSE)-labeled *S. suis* strain 10 bacteria was identical with and without the addition of trypan blue (data not shown).

Statistical analysis. Data were analyzed for normal distribution by the Shapiro-Wilk test. In the case of a normal distribution, unpaired two-tailed Student's *t* test was used. In the case of not-normally distributed data, the nonparametric two-tailed Mann-Whitney or Kruskal-Wallis test with Dunn's multiple-comparison test was applied. Significant outliers were calculated using GraphPad QuickCalcs (https://www.graphpad.com/quickcalcs/grubbs1/) and excluded from the analysis. A confidence interval of 95% was chosen for all analyzes. All figures and data in parentheses in the text represent the means and standard deviations (SD). Probabilities were considered as indicated in the figure legends. Flow cytometric data were analyzed using FlowJo_V10.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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Supplemental Material

Fig. S1 Detection of the superoxide dismutase gene *sodA* by PCR and its mRNA expression by RT-qPCR in different *S. suis* strains.

Fig. S2 Influences of the NADPH oxidase inhibitor apocynin on bacterial and granulocyte parameters.

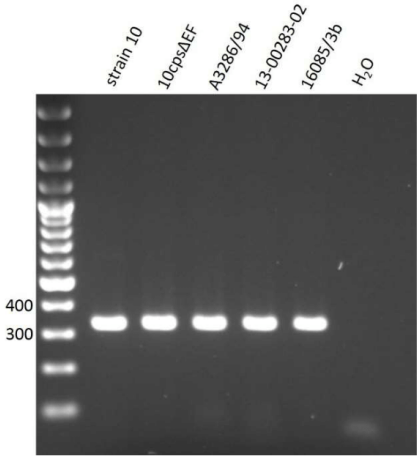
Fig. S3 Quality control of IgG-depletion from serum.

Fig. S4 Anti-porcine IgM Western blot of plasma samples incubated with functional or non-functional rIde$_{Ssuis}$ constructs.

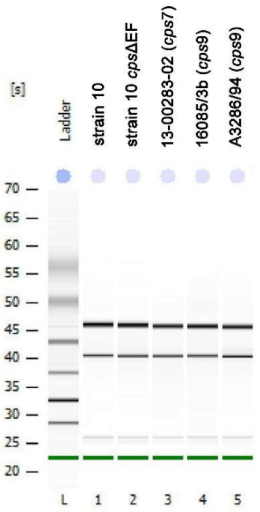
Fig. S5: Decreased survival factors of Far Red-labeled (FR) *S. suis* strain 10 compared to unlabeled *S. suis* strain 10

Fig. S1:

A



B



	gyrB	
	+ RT	- RT
S. suis strain 10 (*cps*2)	16.88 ± 1.08	23.41 ± 0.60
S. suis strain10 *cps*ΔEF	15.58 ± 0.96	21.25 ± 1.70
S. suis 13-00283-02 (*cps*7)	14.90 ± 1.00	24.34 ± 0.50
S. suis 16085/3b (*cps*9)	15.18 ± 1.23	17.33 ±1.94
S. suis A3286/94 (*cps*9)	17.08 ± 1.99	25.62 ± 0.42

	sodA	
	+ RT	- RT
S. suis strain 10 (cps2)	16.37 ± 0.86	23.02 ± 0.72
S. suis strain10 *cps*ΔEF	15.63 ± 0.73	20.89 ± 1.96
S. suis 13-00283-02 (*cps*7)	14.20 ± 1.05	23.81 ± 0.52
S. suis 16085/3b (*cps*9)	15.57 ± 0.84	17.05 ± 1.96
S. suis A3286/94 (*cps*9)	17.55 ± 2.05	24.79 ± 0.31

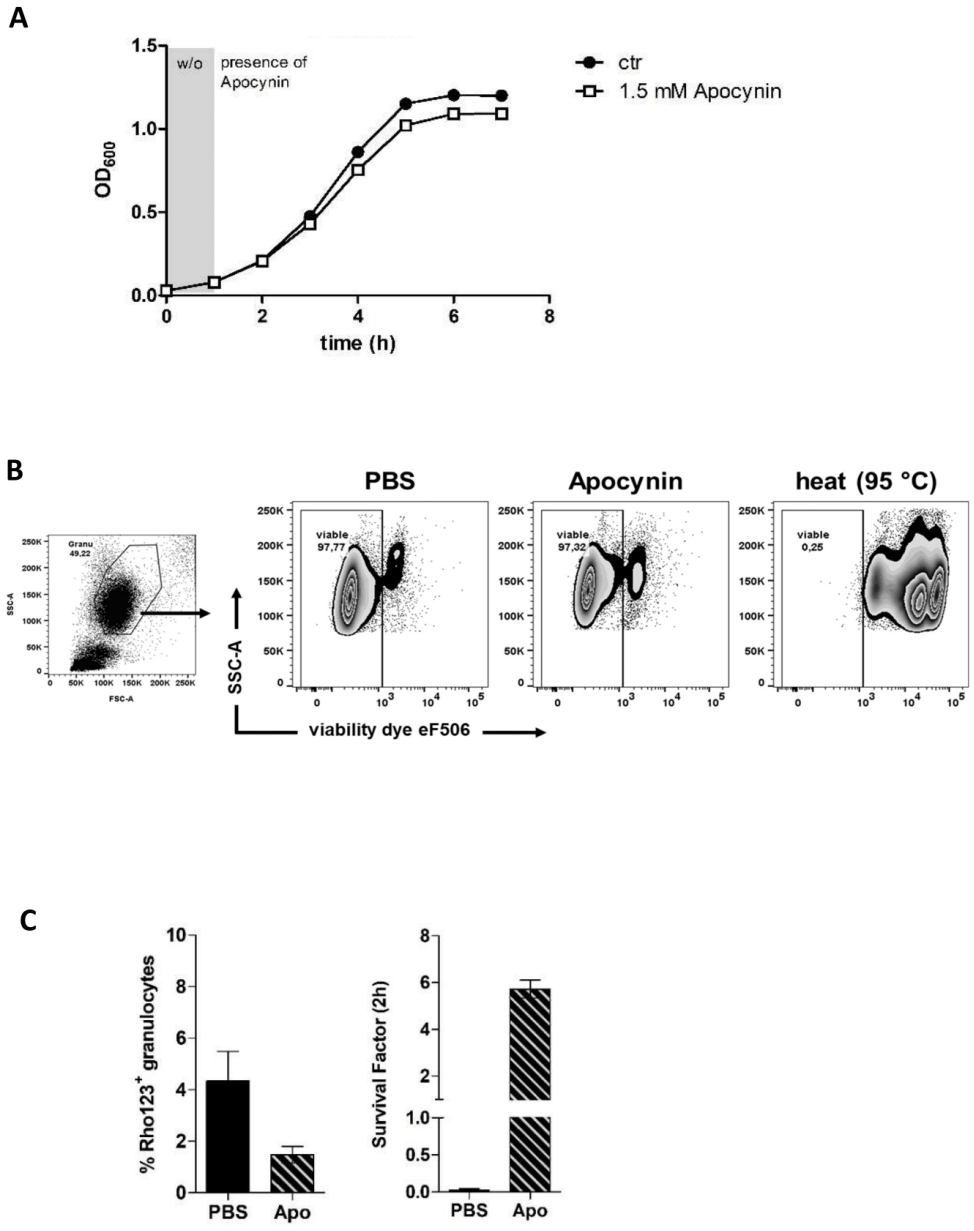
Oligonucleotide primers used in qRT-PCR

Gene	Primer sequence (5´-3´) forward	Primer sequence (5´-3´) reverse	Amplicon length (bp)
gyrB	GGACCTGGGTGCTTAACAGA	AGGTGGTACCCATGAGCAAG	158
sodA	AGACATCCGCCAAGCTTTGA	AAGCCCAACCTGAACCGAAA	197

Fig. S1 Detection of the superoxide dismutase gene *sodA* by PCR and its mRNA expression by qRT-PCR in different *S. suis* strains.

(A) A conserved fragment of the *sodA* gene was detected by PCR in the following *S. suis* strains: strain 10, 10cpsΔEF, A3286/94, 16085/3b and 13-00283-02. The amplification product has a theoretical size of 324 bp. Marker bands are shown in the first lane (sizes in bp). Primers were designed based on the *sodA* sequence of strain EA1832.92 (GenBank: AB724057.1). (**B**) After RNA isolation all samples were analyzed by Bioanalyzer (left). RNA integrity numbers (RIN) from *S. suis* strains are: strain 10 = 9.60; 10*cps* ΔEF = 9.50; 13-00283-02 = 9.10; 16085/3b = 9.20; A3286/94 = 9.30. The quantitative (q) reverse transcriptase (RT)-PCR was conducted with the isolated RNA shown on the left side. The complementary (c) DNA was synthetized in two independent technical runs. Expression of the housekeeping gene *gyrB* was used as a control. The oligonucleotide primers are listed in the table below. Data are presented as means ± SD (right) of Cq (cycle quantification) values of four independent technical qRT-PCR runs.

Fig. S2:


A
w/o
presence of Apocynin
ctr
1.5 mM Apocynin
OD600
time (h)
B
PBS
Apocynin
heat (95 °C)
Granu
49,22
viable
97,77
viable
97,32
viable
0,25
SSC-A
FSC-A
viability dye eF506
C
% Rho123+ granulocytes
Survival Factor (2h)
PBS
Apo
PBS
Apo

4

D

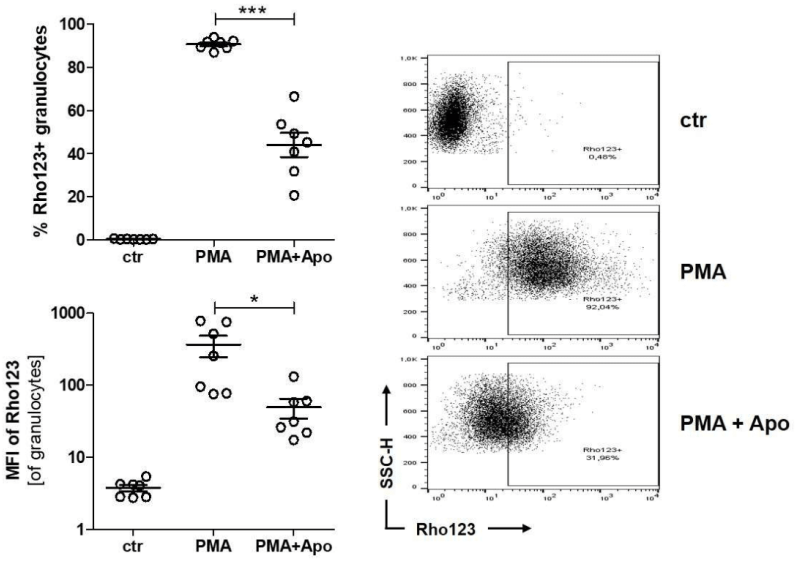


Fig. S2: Influences of the NADPH oxidase inhibitor apocynin on bacterial and granulocyte parameters.

The NADPH oxidase inhibitor apocynin (1.5 mM) was tested for potential effects on **A)** growth of *S. suis* strain 10 in THB **B)** viability of granulocytes after a 2 h incubation period in blood for PBS ctr- (left) or apocynin (1.5 mM)-treated samples. Heat-stressed granulocytes (5 min at 95 °C) were used as control for viability staining (right). **C)** Oxidative burst (left) and survival of *S. suis* strain 10 (right) in an opsonophagocytosis assay with purified porcine granulocytes (n=3). **D)** Inhibition of ROS production by apocynin treatment demonstrated by analysis of Rho123 signal of PMA stimulated (0.1 µ/ml, PMA) versus 5 min apocynin (1.5 mM) pretreated and PMA (PMA+Apo) stimulated blood samples, and non-stimulated control samples (ctr). Frequency of Rho123+ granulocytes (upper left panel) and mean fluorescence intensity (MFI, lower left panel) of Rho123 signal were statistically analyzed by unpaired t-test to compare PMA vs. PMA+Apo groups (n=7).

Fig. S3:

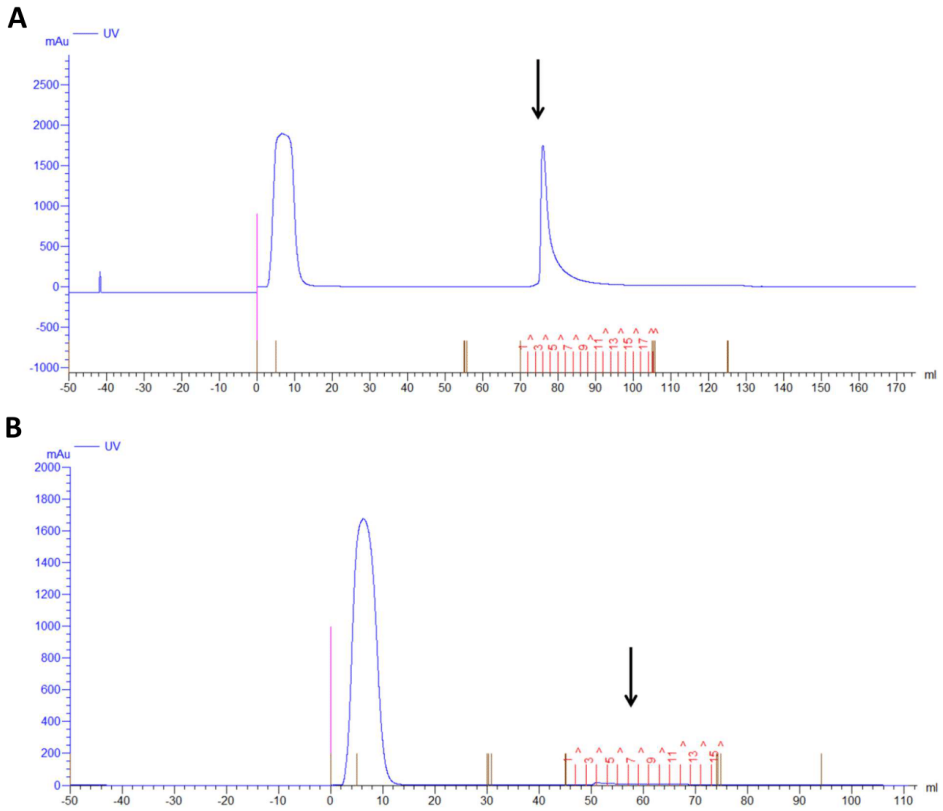


Fig. S3: Quality control of IgG-depletion from serum.

An anti-*S. suis* strain 10 hyperimmune serum was depleted from total IgG by affinity chromatography using protein G Sepharose (A). The first peak in both chromatograms indicates loading of the sepharose column with serum whereas the second peak in (A) represents eluted IgG (indicated by a downward pointing arrow). The flow through from the chromatographic step shown in (A) was loaded onto the protein G column again to verify complete depletion of IgG (downward pointing arrow in [B]).

Fig. S4:

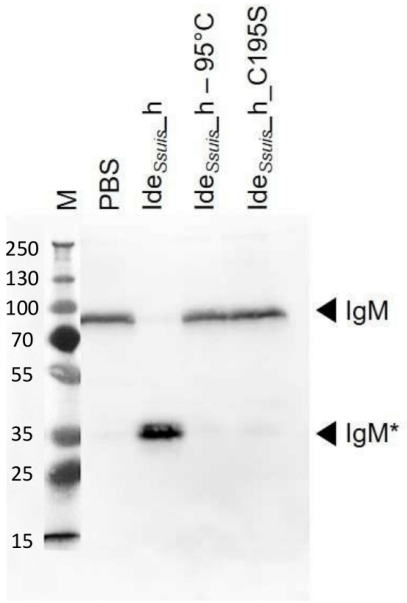


Fig. S4: Anti-porcine IgM Western blot of plasma samples incubated with functional or non-functional rIde*Ssuis* constructs.

IgG-depleted porcine hyperimmune serum was incubated with 20 µg/ml of the indicated rIde*Ssuis* constructs or PBS as a negative control prior to use in bactericidal and oxidative burst experiments. Samples were subjected to SDS-PAGE under reducing conditions using a 10% polyacrylamide gel. Anti-porcine IgM Western blot was conducted with a polyclonal goat anti-pig IgM antibody as first antibody and a peroxidase-conjugated goat anti-rabbit IgG secondary antibody. IgM cleavage products are marked with an asterisk (IgM*). Marker bands in kDa are shown in the first lane.

Fig. S5:

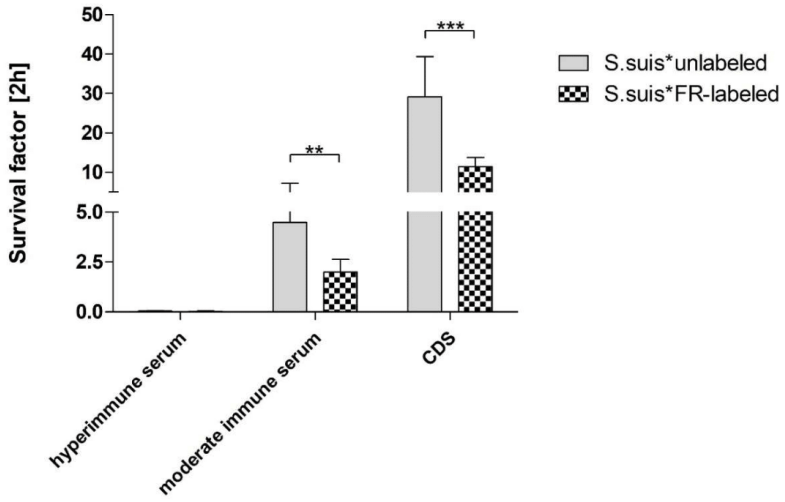


Fig. S5: Decreased survival factors of Far Red-labeled (FR) *S. suis* strain 10 compared to unlabeled *S. suis* strain 10

Porcine blood samples were reconstituted either with hyperimmune serum, serum containing moderate antibody levels against *S. suis* or with CDS. The unlabeled *S. suis* strain 10 was used for assays shown in Figure 2-7 and Far Red (FR)-labeled bacteria for assays shown in Figure 8 and 9. In general *S. suis* stain 10 were exponentially grown in THB. Unlabeled *S. suis* were shock frozen in liquid N_2 after addition of 15 % glycerin directly from THB culture. The Far Red-labeled bacteria were washed with PBS before and after labeling and afterwards resuspended in THB containing 15 % glycerin prior to shock freezing in liquid N_2. Data was statistically analyzed by unpaired t-test to compare the unlabeled versus FR-labeled groups.

3.2 D-Alanylation of Lipoteichoic Acids in *Streptococcus suis* Reduces Association with Leukocytes in Porcine Blood

Sophie Öhlmann, Ann-Kathrin Krieger, Nicolas Gisch, Marita Meurer, Nicole de Buhr, Maren von Köckritz-Blickwede, Nicole Schütze, Christoph Georg Baums

Front Microbiol. 2022 Apr 18;13:822369

Author contributions

I designed and conducted all experiments except LTA extraction, NMR spectroscopy and RNA analysis. Furthermore, I analyzed the data and drafted the manuscript. Ann-Kathrin Krieger supported blood taking and bactericidal assays. Nicolas Gisch supervised LTA extraction and NMR spectroscopy and analyzed NMR data. Nicole de Buhr and Marita Meurer supported experiments investigating interaction with antimicrobial peptides and performed RNA analysis. Nicole Schütze supervised flow cytometry analysis. Christoph Georg Baums, Maren von Köckritz-Blickwede and myself conceived the study and designed experiments. All authors have read and approved the final manuscript.

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ORIGINAL RESEARCH
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D-Alanylation of Lipoteichoic Acids in *Streptococcus suis* Reduces Association With Leukocytes in Porcine Blood

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Streptococcus suis (*S. suis*) is a common swine pathogen but also poses a threat to human health in causing meningitis and severe cases of streptococcal toxic shock-like syndrome (STSLS). Therefore, it is crucial to understand how *S. suis* interacts with the host immune system during bacteremia. As *S. suis* has the ability to introduce D-alanine into its lipoteichoic acids (LTAs), we investigated the working hypothesis that cell wall modification by LTA D-alanylation influences the interaction of *S. suis* with porcine blood immune cells. We created an isogenic mutant of *S. suis* strain 10 by in-frame deletion of the D-alanine D-alanyl carrier ligase (DltA). D-alanylation of LTAs was associated with reduced phagocytosis of *S. suis* by porcine granulocytes, reduced deposition of complement factor C3 on the bacterial surface, increased hydrophobicity of streptococci, and increased resistance to cationic antimicrobial peptides (CAMPs). At the same time, survival of *S. suis* was not significantly increased by LTA D-alanylation in whole blood of conventional piglets with specific IgG. However, we found a distinct cytokine pattern as IL-1β but not tumor necrosis factor (TNF)-α levels were significantly reduced in blood infected with the Δ*dltA* mutant. In contrast to TNF-α, activation and secretion of IL-1β are inflammasome-dependent, suggesting a possible influence of LTA D-alanylation on inflammasome regulation. Especially in the absence of specific antibodies, the association of *S. suis* with porcine monocytes was reduced by D-alanylation of its LTAs. This *dltA*-dependent phenotype was also observed with a non-encapsulated *dltA* double mutant indicating that it is independent of capsular polysaccharides. High antibody levels caused high levels of *S. suis*—monocyte—association followed by inflammatory cell death and strong production of both IL-1β and TNF-α, while the influence of LTA D-alanylation of the streptococci became less visible. In summary, the results of this study expand previous findings on D-alanylation of LTAs in *S. suis* and suggest that this pathogen specifically modulates association with blood leukocytes through this modification of its surface.

Keywords: *Streptococcus suis*, IL-1β, *dltA*, monocyte, oxidative burst, ROS, complement, lipoteichoic acids

INTRODUCTION

Streptococcus suis (*S. suis*) is a zoonotic pathogen, mainly associated with meningitis, arthritis, and septicemia in pigs (Gottschalk and Segura, 2019), but also a frequent colonizer of the upper respiratory tract and the tonsils of healthy pigs (Baele et al., 2001). To cause invasive infections, *S. suis* needs to enter the bloodstream, survive, spread, and proliferate in different tissues. Consequently, the immune response in blood represents an important line of defense and bacteremia plays a key role in the pathogenesis of invasive *S. suis* infections. Different virulence-associated factors of *S. suis* have been proposed to support bacterial survival in blood. Especially the polysaccharide capsule plays an important role, by protecting *S. suis* against phagocytosis (Smith et al., 1999; Fittipaldi et al., 2012). Its composition is very divers, resulting in 29 serotypes (Okura et al., 2016) of which serotype 2 is the most prevalent in invasive infections (Goyette-Desjardins et al., 2014).

As in other Gram-positive bacteria, lipoteichoic acids (LTA), which are linked to the cell membrane by a lipophilic, glycosylated diacyl-glycerol anchor (Percy and Gründling, 2014) are major cell wall components of *S. suis* (Gisch et al., 2018). One common modification of these LTAs is D-alanine residues that are non-stoichiometrically bound to glycerol or ribitol moieties within the polymeric LTA chains. The four essential enzymes required for this process are encoded by the dltABCD operon (Neuhaus and Baddiley, 2003; Brown et al., 2013; Wood et al., 2018). It has been established that DltA transfers D-alanine in the cytoplasm of the cell into the carrier protein DltC (Brown et al., 2013; Reichmann et al., 2013) and that inactivation of *dltA* leads to the abrogation of alanylation of LTA in *S. suis* (Fittipaldi et al., 2008). The ability to introduce D-alanine into the poly-glycerophosphate chains of LTAs has been recognized as a protective mechanism against cationic antimicrobial peptides (CAMPs) for numerous streptococcal species (Poyart et al., 2003; Kristian et al., 2005; Kovács et al., 2006; Chan et al., 2007; Fittipaldi et al., 2008). CAMPs commonly function by crossing the peptidoglycan barrier and interacting with the anionic phospholipid of the cytoplasmic membrane. The resistance induced by LTA D-alanylation might be due to the resulting alteration of the electric charge, the rigidity, and permeability of the bacterial cell wall (Saar-Dover et al., 2012), leading to an reduced interaction with CAMPs.

An increased susceptibility to neutrophils due to a lack of LTA D-alanylation was repeatedly described for different streptococci including *S. suis* (Poyart et al., 2003; Kristian et al., 2005; Fittipaldi et al., 2008). The exact mechanisms behind this remain unclear. Neutrophils can kill bacteria by different strategies like phagocytosis and oxidative burst, the formation of neutrophil extracellular traps (NETs), or degranulation. In these mechanisms, CAMPs released from neutrophil granules can be involved in bacterial killing (Wessely-Szponder et al., 2010).

Bacterial proliferation and exacerbated inflammation during bacteremia are important steps in the pathogenesis of meningitis and septicemia, often leading to sudden death in pigs and the streptococcal toxic shock-like syndrome (STSLS) described in human patients (Tang et al., 2006). While Interleukin (IL)-1 signaling was shown to be beneficial to control and clear streptococcal burden (Lavagna et al., 2019) an exacerbated inflammatory response due to inflammasome activation is able to induce STSLS (Lin et al., 2019).

D-alanylation of LTA in pneumococci might contribute to an increased production of IL-1β (IL-1F2; Sims et al., 2001) in the upper respiratory tract of infected infant mice (Zafar et al., 2019). Also for *S. suis*, a modification of the cytokine response in dependence on the level of LTA D-alanylation was described (Lecours et al., 2011). Nevertheless, the putative role of LTA as ligand of pattern recognition receptors (PRRs) is controversially discussed (Hashimoto et al., 2006; von Aulock et al., 2007; Zähringer et al., 2008; Gisch et al., 2013, 2018). A possible immunomodulatory effect of D-alanylation might not be attributable to direct interaction of LTA with PRRs but rather to changes in the bacterial cell wall and resulting differences in bacteria-host cell association. Accordingly, we investigated the working hypothesis in this study that D-alanylation of *S. suis* LTAs modulates the interaction with leukocytes, phagocytosis, and cytokine production in infected porcine blood.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Streptococcus suis serotype 2 strains 10 and 10cpsΔEF, a mutant deficient in capsule expression due to deletion of the genes *cps*E and *cps*F [*epf* encoding the extracellular factor (EF) is not deleted in this mutant], were kindly provided by Hilde Smith, DLO-Lelystad (Smith et al., 1999). The genome sequence of strain 10, which is part of clonal complex 1, has recently been published (Bunk et al., 2021). The strain was confirmed to be highly virulent in experimental infection of piglets leading to meningitis and other pathologies (Baums et al., 2006, 2009; Seele et al., 2013; de Buhr et al., 2014; Rungelrath et al., 2018). In this study, wild-type (wt) strain 10 and its non-encapsulated mutant 10cpsΔEF were used to generate the two isogenic knock out mutants 10Δ*dltA* and 10cpsΔEFΔ*dltA*, respectively.

Unless otherwise indicated, *S. suis* strains were cultured in Todd-Hewitt broth (THB, Becton Dickinson, 249240) or on Columbia agar plates with 5% sheep blood (Oxoid, PB5039A) at 37°C and 5% CO_2. *Escherichia coli* (*E. coli*) DH5α was grown in lysogeny broth (LB, Carl Roth, X968.1) at 37°C under constant shaking or on LB agar plates. When needed, chloramphenicol (Cm, Carl Roth, 3886.2) was added at a concentration of 3.5 μg/ml for *S. suis* and 8 μg/ml for *E. coli*.

DNA Techniques and Primers

Restriction enzymes (BamHI R0136, SalI R0138, PstI R0140, CIP M0525, and AccI R0161), ligase (ElectroLigase® M0369), and polymerases (One*Taq*® M0480, Phusion® M0530) were purchased from New England Biolabs and used according to manufacturer's recommendations. Standard DNA manipulations were performed as described (Green and Sambrook, 2012). Chromosomal DNA of *S. suis* strain 10 served as a template for all PCRs conducted for generation of inserts. Oligonucleotide primers were designed based on

the sequence of SSU0596 in the genome of *S. suis* P1/7.[1] This nucleotide sequence is also found without any difference in the recently published genome of strain 10 (SSU10_RS03070; Bunk et al., 2021) as well as in the 2020 published genome of S10 (van der Putten et al., 2020).

Targeted Mutagenesis of *dltA* in *Streptococcus suis* Strain 10 and 10cpsΔEF

The thermosensitive shuttle vector pSET5s (Takamatsu et al., 2001) was used as a backbone to generate the plasmid pSET5Δ*dltA* enabling allelic exchange of *dltA* as follows:

DNA fragments corresponding to regions upstream and downstream of the *dltA* gene were amplified using primers dltA_FrA_for_BamHI (TAAGGATCCTCACATTTTTTGCGAATG) and dltA_FrA_rev_SalI (ATGGTCGACCGCTCTAACATACTGCTAA) as well as dltA_FrB_for_SalI (TCCGTCGACTGCAAATGGGAAGATTG) and dltA_FrB_rev_PstI (GATCTGCAGGGCCACTCGAAATAGTTG). The fragments were digested with the restriction enzymes indicated in the names of the primers and inserted in the multiple cloning site of pSET5s.

Restriction analysis and sequencing were carried out to verify the sequence of the resulting plasmid prior to transformation into competent *S. suis* strain 10, whereby competence was induced using a synthetic peptide (H-GNWGTWVEE-OH, jpt) as described before (Zaccaria et al., 2014).

The isogenic mutant 10Δ*dltA* was verified by PCR, sequencing of the deletion site and non-radioactive Southern blot analysis as described before (Rungelrath et al., 2018). Chromosomal DNA was digested by AccI and primer pairs used for generation of the probes were dltA_Ssuis_for (5′-TATGTATTGGGCTCCGACGCTTG-3′) plus dltA_Ssuis_rev (5′-AAGTTGGCGAGTCTGGTTTGG-3′) to verify the absence of the *dltA* gene and pSET5sSondefor (5′-CGAAAAAAAGAGTTATGATTTCTCTG-3′) plus pSET5sSonderev (5′-GGTTTTTTATAGTGCTTTCCATTTTG-3′) to confirm complete excision of the plasmid from the bacterial chromosome.

To construct a 10cpsΔEFΔ*dltA* double mutant, we transformed the plasmid pSET5Δ*dltA* into the 10cpsΔEF mutant and followed the mutagenesis protocol described above.

Extraction and Isolation of LTA

Bacteria were grown in THB to an optical density at 600 nm (OD_{600}) of 1 and harvested by centrifugation at 5,000 × *g*, 4°C for 15 min. LTA isolation and purification were performed as described elsewhere (Heß et al., 2017). Yields of LTA preparations from 5 L of bacterial culture were as follows: strain 10, 8.7 mg; strain 10Δ*dltA*, 3.9 mg.

Nuclear Magnetic Resonance Spectroscopy

Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). Nuclear magnetic resonance (NMR) spectroscopic measurements were performed in deuterated 25 mM sodium phosphate buffer (pH 5.5; to suppress fast de-alanylation) at 300 K on a Bruker Avance^{III} 700 MHz (equipped with an inverse 5-mm quadruple-resonance Z-grad cryoprobe) as described (Gisch et al., 2018).

Extraction of mRNA and qRT-PCR

Bacterial RNA was extracted from exponential-phase THB cultures (OD_{600} of 0.5) of the different *S. suis* strains used in this study. In detail, the pellet of a 10-ml culture (centrifugation: 10 min, 2,600 × *g*, 4°C) was resuspended in 1 ml ice-cold Trizol (Sigma T9424) and stored at −80°C until RNA extraction. Isolation was conducted as previously described (Willenborg et al., 2011). Concentration and purity of the isolated RNA were determined using the Agilent 2100 bioanalyzer (RNA 6000 Pico kit; Agilent Technologies Inc., Santa Clara, CA, United States). Quantitative real-time PCR (qRT-PCR) of reverse-transcribed RNA was designed to analyze the expression of *dltA*, *dltC—dltD*, SSU10_RS03090 (low temperature requirement protein A, downstream of *dltD*), SSU10_RS03040 (glucosamin-6-phosphate deaminase, upstream of *dltA*), and the housekeeping gene *gyrB*. The respective primers are listed in **Supplementary Table S1**. qRT-PCR was conducted with the AriaMX real-time PCR system (Agilent Technologies Inc., Santa Clara, CA, United States), as previously described (Willenborg et al., 2011). The following modified program was used as: an initial denaturation step at 95°C for 15 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and amplification at 72°C for 30 s. As negative controls, we included (i) water, (ii) a no-template control, and (iii) no reverse transcriptase control to exclude false positive results due to contamination with DNA. Products were verified by melting-curve analysis and 1.5% agarose gel electrophoresis.

Cytochrome C Binding Assay

The assay was essentially performed as previously described (Kristian et al., 2005), except that bacteria were adjusted to an OD_{600} of 1 in morpholinepropanesulfonic acid (MOPS) buffer (20 mM, pH 7) before adding 0.5 mg/ml cytochrome C for 10 min at room temperature. After centrifugation (14,000 × *g*, 3 min, room temperature), the cytochrome C content of the supernatant was quantified photometrically at 530 nm (OD_B). MOPS buffer containing 0.5 mg/ml cytochrome C was incubated under the same conditions without bacteria as a control (OD_A). The percentage of bound cytochrome C was calculated as follows:

$$\text{Bound cytochrome C}\,(\%) = \left[\left(OD_A - OD_B\right) / OD_A\right] \times 100$$

Microbial Adhesion to Hydrocarbons Assay

Hydrophobicity of *S. suis* was evaluated by measuring bacterial adhesion to hexadecane (Sigma, H6703) following a previously described protocol (Srikham et al., 2021) with slight modifications.

[1]http://www.sanger.ac.uk

Briefly, *S. suis* strains were cultured overnight and harvested by centrifugation (3,900 × *g*, 10 min, 4°C). Pellets were resuspended in PBS and washed twice before adjusting the suspensions to an OD_{600} of 1 (OD_A). Then, 2 ml of bacterial suspension was mixed with 400 µl of hexadecane and tubes were vortexed for 30 s. The mixture was allowed to separate into two phases for 30 min at room temperature. The aqueous phase was collected and OD_{600} (OD_B) was measured. Cell surface hydrophobicity was calculated as follows:

$$\text{Hydrophobicity}(\%) = \left[1 - \left(OD_B / OD_A\right)\right] \times 100$$

Antimicrobial Susceptibility

The cathelicidin PR-39 (RRRPRPPYLPRPRPPPFFPPRLPPRIP PGFPPRFPPRFP) was kindly provided by Ralf Hoffmann from the Institute of Bioanalytical Chemistry of the University of Leipzig. Bacitracin was obtained from Carl Roth (5655.1), colistin, and polymyxin B were obtained from Sigma (C5561 and P4932). Minimal bactericidal concentrations (MBC) were defined as the lowest concentration of the antimicrobial agent where no bacterial growth occurred. To determine the MBC, we followed a previously described protocol for evaluation of minimal inhibitory concentrations (MIC; Fittipaldi et al., 2008) and additionally plated aliquots of the suspensions on blood agar plates after the incubation period of 24 h at 37°C. While *S. suis* was cultured in 100 µl THB with serial dilutions of bacitracin, colistin, and polymyxin B as described in the above mentioned protocol, evaluation of PR-39 MBC was performed in 50 µl Rosewell Park Memorial Institute (RPMI) 1640 (Gibco, 11835063) containing 5% cation-adjusted Mueller Hinton broth (CA-MHB, Oxoid CM0405, $MgCl_2.6H_2O$ Sigma M2670, and $CaCl_2.2H_2O$ Merck 2382) based on the finding that MICs measured in this medium resembled MICs determined in cerebrospinal fluid of pigs (Meurer et al., 2019). Each assay was performed in triplicates and repeated at least three times.

Bactericidal Assays in Whole Blood

Comparative analysis of survival of *S. suis* 10 and its mutants in heparinized porcine blood was conducted multiple times in samples drawn from 8-week-old piglets originating from different commercial pig farms. Collection of blood was approved by the state Saxony, Germany, under the permit numbers TVV 57-18 and A09/19. The assay was conducted as described before (Seele et al., 2013) with an increased infection dose of 1×10^7 CFU/ml. Survival factors (SF) were determined by dividing the CFUs after the indicated incubation time by the CFU value at time zero.

Far Red Labeling of *Streptococcus suis*

Stocks of *S. suis*, labeled with CellTrace Far Red fluorescent dye (Thermo Fisher Scientific, C34564; *S. suis**FR), were generated using exponential phase THB cultures (OD_{600} 0.5). Bacteria were harvested from 8 ml of these cultures (2,500 × *g*, 10 min, 4°C) and washed twice with PBS before resuspending the pellet in 1 ml PBS and adding 1 µl of FR stock solution (1 mM in DMSO). After an incubation for 20 min at 37°C under rotation in the dark, bacteria where washed again with PBS and finally resuspended in 1 ml THB containing 15% glycerol. Aliquots were frozen in liquid nitrogen. Unlabeled stocks were treated the same way without addition of FR.

Combined Oxidative Burst and Granulocyte Association Assay

Measurement of oxidative burst and the association of *S. suis* with porcine granulocytes was essentially conducted as described before (Rungelrath et al., 2020), except that whole blood samples were used. Briefly, *S. suis**FR stocks were added to 100 µl whole blood of 8-week-old piglets at a concentration of 10^7 CFU/ml. After 15 min of incubation at 37°C, dihydrorhodamine123 (DHR123, Sigma, D1054) was added to stain reactive oxygen species (ROS) within the granulocytes. While reacting with ROS, DHR123 is oxidized to fluorescent rhodamine123 (Rho123). Samples were measured by flow cytometry (BD FACSCalibur) and analyzed with FlowJo™_V10 software.

Flow Cytometry Analysis of *Streptococcus suis* Association With Porcine Monocytes and Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by a density gradient separation as described previously (Hohnstein et al., 2020). PBMCs (10^7 cells/ml) were infected with *S. suis**FR at an MOI of 1 for 30 min at 37°C, whereby *S. suis* had been pre-incubated in porcine serum of colostrum-deprived piglets (CDS) or in hyperimmune serum #4515 generated through prime-boost vaccination of a weaning piglet with a strain 10 bacterin (both obtained within previous studies; Weiße et al., 2021). Monocytes were stained using the myeloid marker CD172a-FITCs (BD Pharmingen™, 561498, 0.5 mg/ml). Lymphocytes were defined as CD172a-negative PBMCs. Samples were measured by flow cytometry (BD FACSCalibur) and analyzed with FlowJo™_V10 software. Following incubation with bacteria for 30 min or 2 h, in a subset of samples, PBMCs were stained with viability dye eFluor™ 506 (Thermo Fisher Scientific, 65-0866-14) according to manufacturer's recommendations. These samples were measured using BD FACS Fortessa.

Cytospin and Staining for Optical Microscopy

Directly after incubation of the PBMC samples with *S. suis*, an aliquot containing approximately 6×10^4 PBMCs was added to 200 µl PBS and centrifuged onto slides for 5 min at 70 × *g* in a Shandon Cytospin 4. Slides were air dried and stained with a Diff-Quik staining kit (Medion Diagnostics, 726443) according to manufacturer's recommendations. Microscopic analysis was performed using Olympus AX70 Provis microscope.

Cytokine Quantification

DuoSet ELISA kits for porcine tumor necrosis factor (TNF)-α and IL-1β were purchased from R&D Systems (DY690B and DY681) and performed essentially according to manufacturer's recommendations. The analysis was conducted with supernatants or plasma obtained before and after infection with *S. suis* in

PBMC samples or bactericidal assays in whole blood as described above. The streptavidin-horseradish peroxidase used to couple the detection antibodies was detected with a 3,3′,5,5′-Tetramethylbenzidin (TMB) solution (SeraCare, Milford, MA, United States, formerly KPL) and the reaction was stopped after 20 min with 1 M H_3PO_4 (Roth, 6366.1). OD values were measured with a microplate reader SpectraMax 340PC384 (Molecular Devices, LLC San Jose, CA, United States) at 450 and 630 nm as a background reference and analyzed with SoftMax® Pro v5.0 software (Molecular Devices, LLC; Hohnstein et al., 2020).

C3 Deposition on the Surface of *Streptococcus suis*

Deposition of complement on the streptococcal surface was measured essentially as described before (Rungelrath et al., 2018). Briefly, *S. suis* strains were grown to an OD_{600} of 0.5 in THB. Then, 50 µl of the respective culture was incubated with 100 µl of CDS for 1 h at 37°C under rotation (8 rpm). As negative control, CDS was incubated for 30 min at 56°C to inactivate all complement factors. Staining of C3 labeled bacteria was conducted with 200 µl of a 1:150 diluted FITC-labeled cross-reactive rabbit anti-human C3c antibody (Dako, F020102-2, 3 g/L) for 1 h at 4°C. Samples were measured using BD FACS Fortessa and analyzed using FlowJo™_V10 software.

Anti-*Streptococcus suis* IgG and IgM ELISA

ELISAs measuring serum IgG or IgM binding to the surface of formaldehyde inactivated and immobilized *S. suis* strain 10 were conducted as described previously (Seele et al., 2015), using horseradish peroxidase (HRP) conjugated polyclonal goat anti-pig IgG (Bethyl, A100-105P) and goat anti-pig IgM (Bethyl, A100-117P) antisera. Hyperimmune serum #4515, which was also used in cell association assays, served as reference standard and was defined as containing 100 ELISA units of both IgG and IgM.

Statistical Analysis

Statistical analysis was performed using Prism software, version 9 (GraphPad, San Diego, CA, United States). Normality was tested by Shapiro–Wilk test. Differences between multiple groups or time points were determined using ANOVA followed by Holm-Sídáks or Tukeys multiple comparisons test, respectively. Differences between wt and *dltA* mutant were tested using two-tailed paired *t*-test or Wilcoxon test when appropriate. Treatments with the two different porcine sera were compared by two-tailed unpaired *t*-test or Mann–Whitney test. A CI of 95% was chosen for all analyzes. All figures and data in parentheses in the text represent the means and SD. Probabilities were considered as indicated in the figure legends.

RESULTS

Deletion of the *dltA* Gene Abolishes D-Alanylation of *Streptococcus suis* LTA

We created an isogenic mutant of the virulent serotype 2 strain 10 by in-frame deletion of the *dltA* gene, encoding for the D-alanine-D-alanyl carrier protein ligase in order to investigate the role of D-alanylation of LTAs in pathogen-host interaction. To verify the absence of alanine residues in the LTAs of *S. suis* 10Δ*dltA*, LTA was isolated from late exponential wt and mutant cultures and subjected to NMR spectroscopy. Overall, the ^{1}H NMR spectrum recorded for LTA isolated from the wt (**Supplementary Figure S1**, top panel) is virtually identical with those obtained earlier for LTA isolated from other *S. suis* serotype 2 strains (strains P1/7 and SC84; Gisch et al., 2018) and further NMR analysis confirmed the identical overall structure. The peaks indicative for D-alanine residues present at the position O-2 of glycerol within the poly-(glyco) glycerolphosphate chain [δ_H 1.65–1.60 (Ala-CH_3), 4.33–4.27 (Ala-CH), and 5.42–5.36 (glycerol-H2) ppm; Fittipaldi et al., 2008; Gisch et al., 2018] were absent in the spectra recorded from LTA of the Δ*dltA* strain (**Supplementary Figure S1**). This result confirms that the *dltA* gene is necessary for D-alanylation of LTA in *S. suis*, as already described previously (Fittipaldi et al., 2008).

mRNA Expression Analysis of the dlt Operon and Adjacent Genes

Quantitative real-time PCR verified the absence of mRNA expression of *dltA* in the mutants 10Δ*dltA* and 10cpsΔEFΔ*dltA* (**Supplementary Figure S2**). Transcript levels of the genes *dltC* and *dltD*, as well as those of the genes SSU10_RS03040 and SSU10_RS03090 up- and downstream of the dlt locus, were not different between *S. suis* 10 wt and 10Δ*dltA*, confirming that in-frame deletion did not result in polar effects. The non-encapsulated mutant 10cpsΔEF showed a slightly increased ΔΔCT of 1.8 (SD 0.36) for SSU10_RS03040 in comparison with *S. suis* strain 10 wt, whereas the double mutant 10cpsΔEFΔ*dltA* obtained a slightly decreased ΔΔCT of 0.5 (SD 0.22) for SSU10_RS03040. This open reading frame encodes putatively a glucosamine-6-phosphate deaminase, which is called NagB in *Staphylococcus aureus* and *Streptococcus mutants* (Komatsuzawa et al., 2004; Kawada-Matsuo et al., 2016). NagB is responsible for the synthesis of fructose from glucosamine. High concentrations of amino sugars like N-acetylglucosamine (GlcNAc) in the medium result in upregulation of the expression of NagB (Kawada-Matsuo et al., 2016). We speculate that the slightly modified expression of SSU10_RS03040 in the non-encapsulated mutants might be related to the deletion of the capsule biosynthesis genes, as GlcNAc is part the capsular polysaccharide of *S. suis* serotype 2 (van Calsteren et al., 2010).

Effects of LTA D-Alanylation on Surface Charge, Hydrophobicity, and Susceptibility to Cationic Antimicrobial Peptides

We hypothesized in agreement with results for Group A streptococci (Kristian et al., 2005) and pneumococci (Saar-Dover et al., 2012) that the net negative charge of the bacteria is increased in the two Δ*dltA* mutants. However, we found no significant difference in binding of the cationic peptide cytochrome C between *S. suis* wt and 10Δ*dltA* (**Figure 1A**; strain 10: mean 38.27%, SD 4.43%; 10Δ*dltA*: mean 34.99%,

SD 3.46%). In contrast, loss of LTA D-alanylation in the non-encapsulated mutant caused a highly significant increase in bound cytochrome C (10cpsΔEF: mean 25.57%, SD 2.32%; 10cpsΔEFΔ*dltA*: mean 33.25%, SD 2.76%). These results suggest that changes in local charges due to LTA D-alanylation in the wt bacteria are masked by the capsular polysaccharides. We further investigated hydrophobicity of the different streptococci by the microbial adhesion to hydrocarbons assay. While the capsule provided a hydrophilic character to the streptococcal surface and its absence caused a strongly significant increase of hydrophobicity (strain 10: mean 22.18%, SD 3.66%; 10cpsΔEF: mean 82.60%, SD 5.10%), 10Δ*dltA* and 10cpsΔEFΔ*dltA* showed a significantly reduced cell surface hydrophobicity compared to the wt and the capsular mutant 10cpsΔEF, respectively (10Δ*dltA*: mean 12.39%, SD 6.08%; 10cpsΔEFΔ*dltA*: mean 73.24%, SD 8.80%; **Figure 1B**). In conclusion, LTA D-alanylation increases cell surface hydrophobicity of encapsulated as well as non-encapsulated *S. suis*.

We also determined the MBC of the bacteria-derived peptides bacitracin, colistin, and polymyxin B and the porcine cathelicidin PR-39 (**Figures 1C–F**). Bacitracin, colistin, and polymyxin B showed a significantly lower MBC for 10Δ*dltA* than for the wt strain, confirming and expanding the results obtained for MIC of an independent laboratory (Fittipaldi et al., 2008). Thus, it has been shown by independent laboratories that D-alanylation in *S. suis* serotype 2 decreases the susceptibility to several CAMPs.

The Oxidative Burst Response of Porcine Granulocytes and the Association With *Streptococcus suis* Is Reduced by D-Alanylation of LTAs

The role of LTA D-alanylation in protection of streptococci against neutrophils is described in different studies (Poyart et al., 2003; Kristian et al., 2005; Fittipaldi et al., 2008). To specifically investigate whether D-alanylation of LTAs influences phagocytosis of *S. suis* by porcine granulocytes during bacteremia, whole blood of 8-week-old piglets was infected with Far Red (FR)-labeled bacteria (*S. suis**FR). The labeling of bacteria was verified by flow cytometry (**Supplementary Figure S3**). Flow cytometry analysis of the infected blood samples enabled us to read out the number of granulocytes associated with *S. suis**FR but did not allow us to distinguish extracellular binding or intracellular presence of *S. suis* within the granulocytes. Therefore, measurement of *S. suis*-granulocytes association was combined with the evaluation of ROS production to identify granulocytes performing phagocytosis. Gating of double positive granulocytes is visualized exemplarily for one blood donor in **Figure 2A**.

We found a higher number of granulocytes in association with strain 10Δ*dltA* (FR positive granulocytes) generating ROS (Rho123 positive) than observed for the wt (10Δ*dltA*: mean 7.83% and SD 5.03%; strain 10: mean 5.04% and SD 3.34%), indicating a higher level of phagocytosis of the Δ*dltA* mutant (**Figure 2B**). The observation that D-alanylation might protect *S. suis* from phagocytosis and oxidative burst by granulocytes goes in line with the described increased killing of the *S. suis dltA* mutant by neutrophils (Fittipaldi et al., 2008).

To answer the question, whether increased oxidative burst and association of granulocytes with the *dltA* mutant result in an increased killing of the mutant in whole blood, we investigated the oxidative burst and streptococci-cell association at different time points and plated the Far Red-labeled bacteria at the same time points (**Figure 2C**). Again, we saw a higher percentage of granulocytes in association with the 10Δ*dltA* mutant, while performing oxidative burst after 30 min of incubation (10Δ*dltA*: mean 14.78%, SD 7.32%; strain 10: mean 11.88% SD 6.81%), while after 1 and 2 h differences between wt and *dltA* mutant were not significant. During time, the mean percentage of granulocytes showing oxidative burst simultaneously to association with *S. suis* significantly increased for both strains and reached values around 33 and 36% after 2 h. In all six blood donors, the bacteria were efficiently killed with significant reduction of the mean CFU already after 30 min. These results suggest that D-alanylation of LTA reduces the early association with granulocytes and their activation as *S. suis* enters porcine blood but might not ensure efficient escape in the progress of infection, at least not in the blood of conventional 8-week-old piglets that generally carry specific IgG and IgM antibodies against *S. suis* but are nevertheless often affected by *S. suis* disease.

LTA D-Alanylation Reduces the Level of Association of *Streptococcus suis* With Porcine Monocytes

The interaction of *S. suis* with porcine brain microvascular endothelial cells can be inhibited through addition of LTA (Vanier et al., 2007). Furthermore, D-alanylation of LTA is described to influence the interaction of the streptococci with dendritic cells (Lecours et al., 2011). We hypothesized that LTA and its modification through D-alanylation might also modulate the interaction with porcine monocytes in blood.

Accordingly, we isolated PBMCs from freshly obtained porcine blood and applied *S. suis**FR strain 10 or 10Δ*dltA* at an MOI of 1. The myeloid marker CD172a was used to identify monocytes (**Supplementary Figure S4B**) and samples were analyzed for FR positive monocytes by flow cytometry (**Figure 3A**). To evaluate the impact of serum components on the bacteria-cell-association, we pre-incubated the streptococci with either serum of CDS, containing no specific antibodies against *S. suis*, or with hyperimmune serum raised against *S. suis* strain 10.

In the absence of specific antibodies (CDS-treated bacteria), LTA D-alanylation significantly reduced the level of association of *S. suis* with monocytes (**Figure 3B**, left graph, CDS; strain 10: mean 8.78%, SD 2.95%; 10Δ*dltA*: mean 12.63%, SD 3.6%), while the association was strongly enhanced for both strains when bacteria were opsonized with specific antibodies (**Figure 3B**, left graph, hyperimmune serum; strain 10: mean 36.41%, SD 6.43%; 10Δ*dltA*: mean 39.87%, SD 5.48%). In these hyperimmune conditions, the influence of LTA D-alanylation on the association of *S. suis* with monocytes remained significant but less pronounced. In microscopic analysis, the association

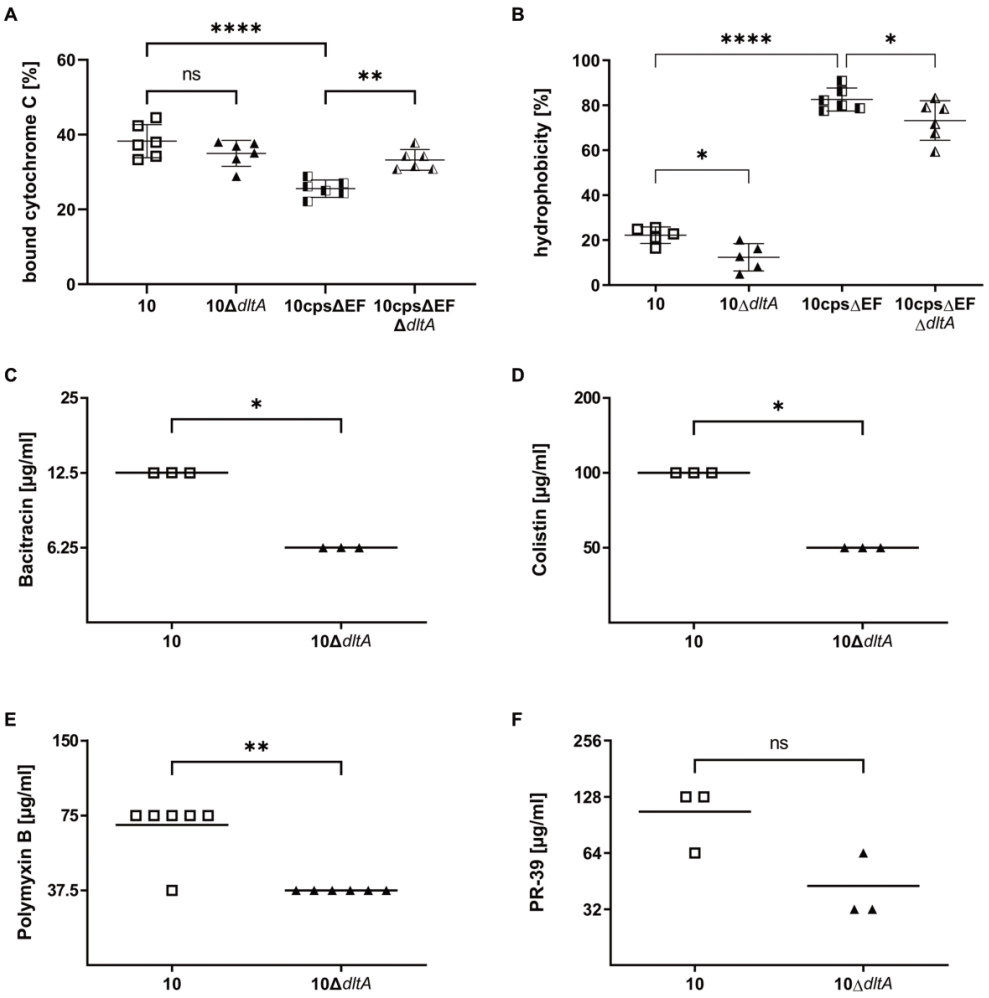

FIGURE 1 | D-alanylation of lipoteichoic acids (LTAs) increases the ability to bind cationic cytochrome C in the absence of the capsule in *Streptococcus suis*, reduces the hydrophobic character of the streptococcal surface, and provides protection against several cationic antimicrobial peptides (CAMPs). Binding of the cationic cytochrome C (Cyt C) to the bacterial surface **(A)** was evaluated indirectly by photometrical measurement of the bacterial supernatants after 10 min of incubation with 0.5 mg/ml Cyt C in morpholinepropanesulfonic acid (MOPS) buffer, compared to a control containing 0.5 mg/ml Cyt C without bacteria. **(B)** Shows the hydrophobicity of *S. suis* strains, as evaluated by rigorous mixing of bacterial PBS suspensions (adjusted to OD_{600} of 1) with the hydrophobic solvent hexadecane. The mixtures were left to settle for 30 min followed by measuring the OD_{600} of the aqueous (PBS) phase and calculation of the hydrophobicity as written in the materials and methods section. **(C–F)** Demonstrate minimal bactericidal concentrations (MBC) that were evaluated by incubating *S. suis* strain 10 or 10Δ*dltA* (1×10^4 CFU/ml) in a 2-fold serial dilution of the antimicrobial agent for 24 h at 37°C and subsequently plating on blood agar plates. MBC was defined as the lowest concentration of the antimicrobial agent where no bacterial growth occurred on agar plates. Each symbol represents a measurement in triplicates and each experiment was repeated at least three times with freshly cultured bacteria. Assays were performed in Todd-Hewitt broth (THB; **C,D,E**) or Rosewell Park Memorial Institute (RPMI) with 5% cation-adjusted Mueller Hinton broth (CA-MHB; **F**). One-way ANOVA was performed followed by Holm-Sídáks multiple comparisons test and results are indicated for the comparisons of each isogenic mutant to its parent strain **(A,B)**. One-tailed Mann–Whitney test was performed to compare wild-type (wt) and *dltA* mutant samples in **(C–F)**. Not significant (ns) $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

of monocytes with CDS-treated *S. suis* resembled a binding of the streptococci to the cell surface, whereas opsonization with hyperimmune serum partly resulted in streptococci visible within the membrane borders of monocytes, indicating a possible uptake by phagocytosis (**Figure 3C**).

As the capsule of *S. suis* strongly influences its interaction with mononuclear cells (Smith et al., 1999; Meijerink et al., 2012), we repeated the assays using the non-encapsulated mutant 10cpsΔEF and the double mutant 10cpsΔEFΔ*dltA* to exclude the influence of capsular polysaccharides on the *dltA* phenotype.

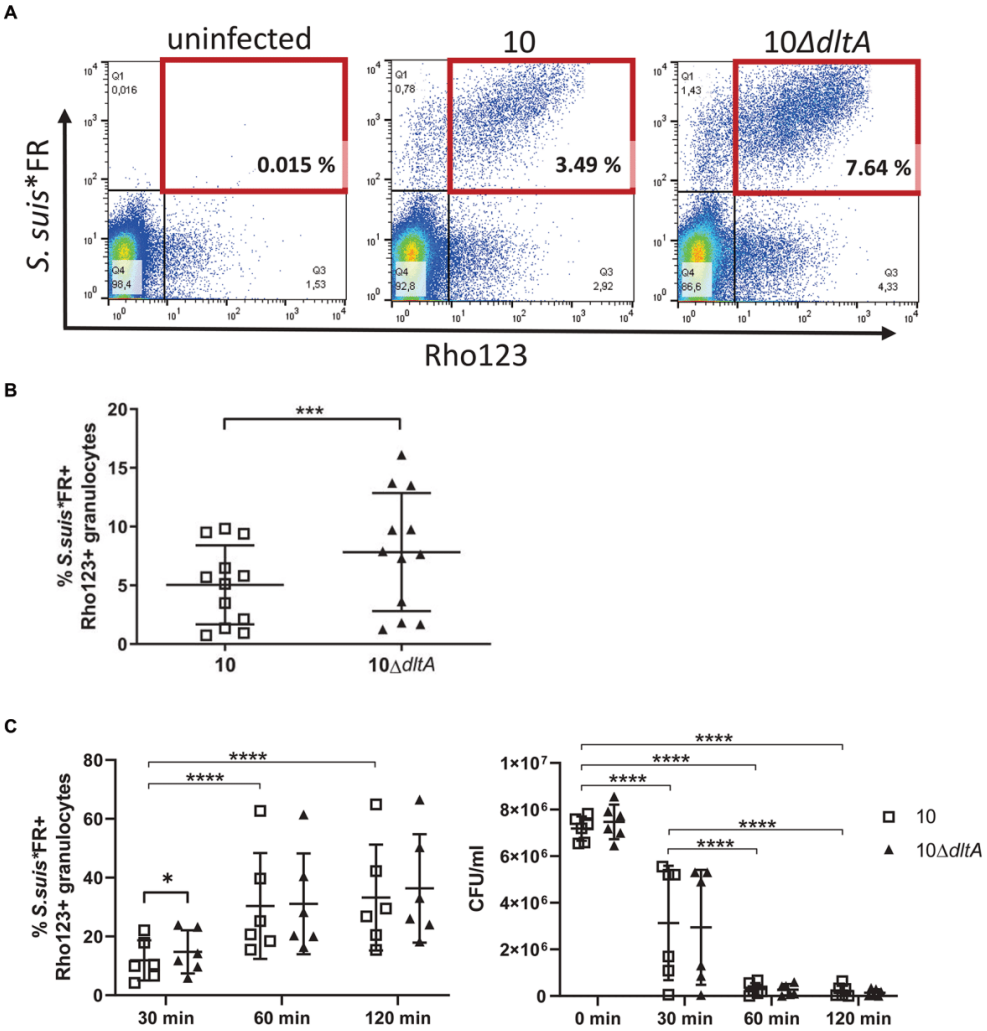

FIGURE 2 | D-alanylation of LTAs in *Streptococcus suis* reduces early association of *S. suis* with granulocytes and the proportion of granulocytes performing oxidative burst. Whole porcine blood was infected with Far Red (FR)-labeled *S. suis* at a concentration of 1×10^7 CFU/ml at 37°C for 15 min, followed by intracellular staining of reactive oxygen species (ROS) with dihydrorhodamine123 (Rho123) at 37°C for 10 min. After erythrocyte lysis, samples were analyzed by flow cytometry. While gating of granulocytes in whole blood is depicted in **Supplementary Figure S4A**, **(A)** demonstrates the gating strategy for double positive granulocytes (red square) in the blood of one piglet and **(B)** shows the % of granulocytes interacting with *S. suis**FR (FR+) and producing ROS at the same time (Rho123+; $n = 12$). **(C)** Demonstrates the course of the association of streptococci with granulocytes that simultaneously show oxidative burst response over time, as well as the CFU of *S. suis**FR in blood of six piglets. Error bars represent SDs. Paired *t*-test was performed for statistical analysis to compare wt and mutant samples in blood of the same animals **(B,C)** and repeated measures ANOVA followed by Tukeys multiple comparisons test was performed for each strain at different time points **(C)**. For clarity, significant differences between the time points are indicated for the wt samples only but are equally seen for the *dltA* mutant. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Even in the absence of specific antibodies, the non-encapsulated mutants showed a very high level of association with monocytes (**Figure 3B**, right graph, 10cpsΔEF: mean 68.59%, SD 4.99%) and the loss of LTA D-alanylation significantly increased the number of FR positive monocytes (10cpsΔEFΔ*dltA*: mean 71.76%, SD 5.10%), similar to what was observed for the encapsulated strains. These results indicate that D-alanylation of LTA modulates association of *S. suis* with monocytes independently of the capsular polysaccharides.

When analyzing the association of the streptococci with lymphocytes contained in the PBMC samples (**Figure 3D**), we found that the bacteria also adhered to lymphocytes in a

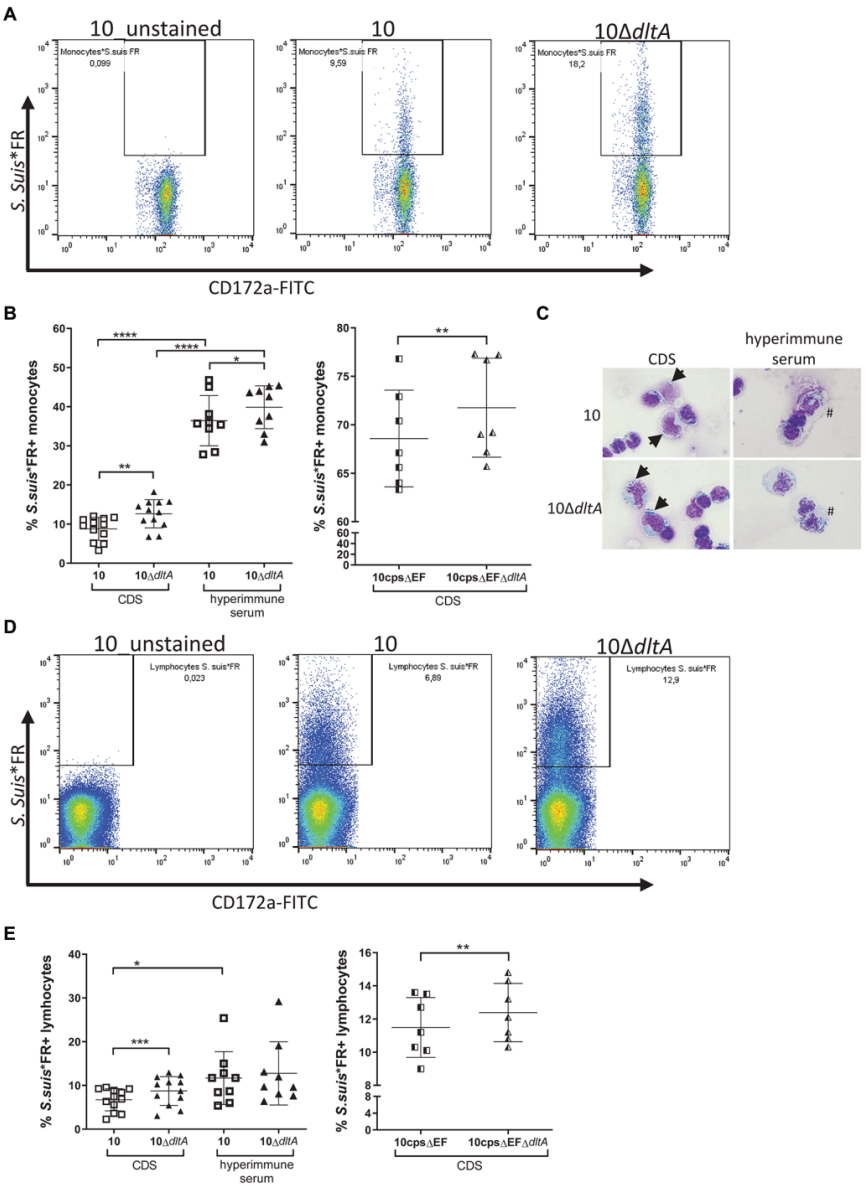

FIGURE 3 | Far Red-labeled *Streptococcus suis* Δ*dltA* mutants show a significantly higher level of association with porcine monocytes and lymphocytes. Peripheral blood mononuclear cell (PBMCs; freshly isolated from porcine blood) were incubated with Far Red-labeled *S. suis* strains (*S. suis**FR) at an MOI of 1 for 30 min at 37°C, whereby *S. suis**FR had been pre-incubated in serum of colostrum-deprived piglets (CDS) or in hyperimmune serum for 30 min, as indicated. Monocytes were stained using the myeloid marker CD172a-FITCs (as visualized in **Supplementary Figure S4**) and samples were measured by flow cytometry. The gating strategy for monocytes **(A)** and lymphocytes **(D)** in association with *S. suis* is visualized for PBMCs of one animal, while (**B**; monocytes) and (**E**; lymphocytes) show the entire results for the encapsulated strains on the left and the non-encapsulated strains on the right. The number of symbols represents the number of piglets PBMCs were obtained from. **(C)** Shows exemplary pictures of CDS-treated *S. suis* bound to the surface of monocytes (arrow) and uptake of antibody-opsonized *S. suis* (#) into monocytes as visualized by optical microscopy (x400 magnification). Horizontal lines and error bars represent mean values and SDs. For statistical analysis, paired *t*-test (wt or 10cpsΔEF vs. respective Δ*dltA* mutant) or unpaired *t*-test (CDS vs. hyperimmune samples) were performed. In case of not normally distributed samples, Wilcoxon test or Mann–Whitney test was used. Differences that are not indicated are not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

dltA-dependent manner when no specific antibodies where present, as CDS-treated 10Δ*dltA* showed a significantly increased association with lymphocytes (**Figure 3E**, left graph, CDS; strain 10: mean 6.73%, SD 2.53%; 10Δ*dltA*: mean 8.74%, SD 3.28%). The same was visible in the non-encapsulated strains, although the increase was small (**Figure 3E**, right graph; 10cpsΔEF: mean 11.48%, SD 1.81%; 10cpsΔEFΔ*dltA*: mean 12.39%, SD 1.76%). In contrast to monocytes, the increase of lymphocytes associated with *S. suis* due to opsonization with specific antibodies was far less pronounced (**Figure 3E**, left graph, hyperimmune serum; strain 10: mean 11.68%, SD 6.04%; 10Δ*dltA*: mean 12.76%, SD 7.24%) and an influence of LTA D-alanylation was no longer detected.

To verify our cell association data and exclude falsification of data due to cell death, staining with Viability Dye eFluor™ 506 (eF506) was performed with the PBMC samples and the percentage of cells containing eF506 was measured after 30 min (**Supplementary Figure S5**) and after 2 h of incubation with *S. suis* (**Figure 4A**). After 30 min, which corresponds to the time of evaluation of the bacterial association with monocytes, the number of cells displaying membrane damage (positive for eF506), in samples incubated with CDS-treated bacteria, was still very low and comparable to samples without bacteria (**Supplementary Figure S5**). In contrast, after 2 h of incubation, CDS-treated 10Δ*dltA* induced a higher number of eF506 positive monocytes than CDS-treated wt strain 10 (**Figure 4A** CDS; strain 10: mean 7.70%, SD 4.58%; 10Δ*dltA*: mean 15.85%, SD 7.02%). When bacteria were opsonized with specific antibodies contained in the hyperimmune serum, we found very high percentages of eF506 positive monocytes 2 h after infection with the wt (mean 45.30%, SD 4.21%) or the Δ*dltA* mutant (mean 53.87%, SD 5.25%; **Figure 4A** hyperimmune serum). The percentage of eF506 positive lymphocytes did not increase upon incubation with the opsonized *S. suis* (**Figure 4B** hyperimmune serum; 10 mean 1.73%, SD 0.50%; 10Δ*dltA* mean 2.03%, SD 0.36%) but was reduced compared to the samples incubated with CDS-treated bacteria (**Figure 4B** CDS; 10 mean 7.11%, SD 2.45%; 10Δ*dltA* mean 11.95%, SD 4.13%).

As we were interested in the immunomodulatory effect of the association of *S. suis* with monocytes, we measured the pro-inflammatory cytokines IL-1β and TNF-α in the supernatants of the infected PBMC samples after 2 and 6 h. Only in supernatants of PBMCs infected with bacteria pre-treated with hyperimmune serum, we found both cytokines already after 2 h of infection (**Figures 4C,D**). In samples with CDS-treated *S. suis*, the cytokines were undetectable at 2 h and still quite low at 6 h post-infection. There was no significant difference in the levels of the two cytokines between samples infected with *S. suis* wt or 10Δ*dltA*, but when *S. suis* was pre-opsonized in hyperimmune serum, it induced significantly higher levels of both pro-inflammatory cytokines, than without opsonization with specific antibodies (**Figures 4C,D**).

In conclusion, D-alanylation of LTA significantly reduces association of *S. suis* with porcine monocytes in isolated PBMCs infected *ex vivo*. Independent of LTA D-alanylation, opsonization with specific antibodies results in a strong increase of this association, followed by membrane damage in monocytes and induction of the pro-inflammatory cytokines IL-1β and TNF-α.

In the Absence of Specific Antibodies Complement Deposition on the Surface of *Streptococcus suis* Is Reduced Through D-Alanylation of LTAs

As suggested previously (Lecours et al., 2011), LTA D-alanylation might reduce complement deposition on the bacterial surface resulting in reduced interaction with myeloid cells such as dendritic cells. Since, we saw LTA D-alanylation-dependent differences in the association of *S. suis* with monocytes, we asked if the level of C3 deposition on the surface of CDS-treated *S. suis* depends on D-alanylation of LTAs. Flow cytometry was used to investigate opsonization of streptococci with C3 (**Figure 5A**). A significantly increased number of 10Δ*dltA* bacteria were stained positive for C3 on their surface compared to the wt (**Figure 5B**, strain 10 mean 2.21%, SD 0.48%; 10Δ*dltA* mean 4.03%, SD 0.38%). A similar tendency was visible for non-encapsulated *S. suis*, whereby the numbers of bacteria opsonized with C3 were higher than observed for the encapsulated strains (**Figure 5C**, 10cpsΔEF: mean 28.38%, SD 10.41%; 10cpsΔEFΔ*dltA*: mean 38.82%, SD 9.40%). For all strains, C3 deposition on bacteria incubated with heat-inactivated CDS was nearly absent, confirming that the measurement of C3 deposition depends on active complement. In conclusion, our results indicate that LTA D-alanylation is involved in complement evasion.

Mutation of the *dltA* Gene of *Streptococcus suis* Reduces the Amount of IL-1β but Not of TNF-α Induced by *Streptococcus suis* in Porcine Blood

To investigate the pro-inflammatory response in *S. suis* infected porcine blood in dependence of D-alanylation of LTAs, we focused on two differently regulated pro-inflammatory cytokines, namely, IL-1β and TNF-α. Again, the lack of LTA D-alanylation made no difference in bacterial survival after 2 h of infection observed in the blood of these animals originating from the same conventional herd as shown in **Figure 2** (**Figure 6A**, SF strain 10: mean 1.52, SD 2.80; 10Δ*dltA* mean 1.73, SD 3.38). However, while the number of CFU after 2 h in porcine blood was comparable between wt and the isogenic Δ*dltA* mutant, a distinct pattern of cytokine production was recorded. Whereas the amount of TNF-induced by the two strains was very similar, the wt induced with 0.88 ng/ml (SD 0.35) significantly higher amounts of IL-1ß than the *dltA* mutant with 0.48 ng/ml (SD 0.18; **Figure 6B**). This phenotypical difference, which we had not observed in isolated PBMCs, was reproducible in blood obtained from animals of an independent herd with a different history of *S. suis* diseases, mainly dominated by serotype 9 (**Supplementary Figure S6**).

Purified LTAs obtained from strain 10 and 10Δ*dltA* were applied to porcine blood (final LTA concentration 30 μg/ml), which was subsequently incubated for 2 and 6 h. The concentrations of TNF-α and IL-1β after 2 and 6 h varied between samples but did not seem to depend on the *dltA* genotype of the original strain. Of note, IL-1β levels were below the limit of detection of 0.032 ng/ml after 2 h but above

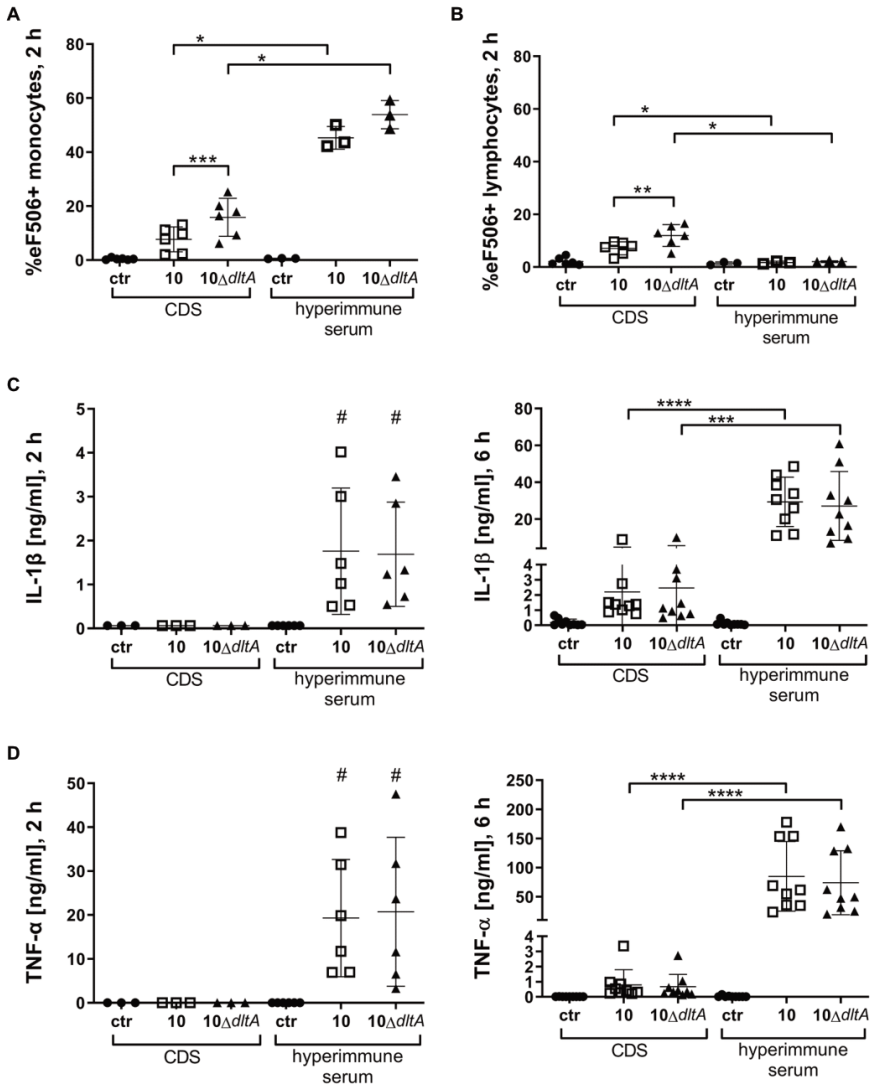

FIGURE 4 | Infection of PBMCs with *Streptococcus suis* opsonized in hyperimmune serum induces membrane damage in monocytes but not in lymphocytes and results in induction of IL-1β and tumor necrosis factor (TNF)-α. For **(A,B)**, a subset of PBMC samples visualized in **Figure 3** ($n = 6$ for CDS and $n = 3$ for hyperimmune serum) were stained with the viability dye eFlour™ 506 after 2 h of incubation with the respective *S. suis* strains. PBMC samples without bacteria served as negative control while heat-treated PBMCs served as a positive control (not shown: eF506+ monocytes 90.9%; eF506+ lymphocytes 89.6%). Samples were measured by flow cytometry. Results for monocytes are visualized in **(A)**, while lymphocytes are depicted in **(B)**. Pro-inflammatory cytokines IL-1β **(C)** and TNF-α **(D)** were measured by ELISA in the supernatant of PBMC samples visualized in **Figure 3** after 2 and 6 h of infection with respective *S. suis* strains. The limit of detection was 0.031 ng/ml for TNF-α and 0.063 ng/ml for IL-1β. Horizontal lines and error bars represent mean values and SDs. For statistical analysis, paired *t*-test (wt vs. mutant) or unpaired *t*-test (CDS vs. hyperimmune samples) was performed. In case of not normally distributed samples **(C,D)**, Wilcoxon test or Mann–Whitney test was used, respectively. One sample Wilcoxon was performed to evaluate how much IL-1β and TNF-α levels differ from the values 0.063 or 0.031 ng/ml, respectively, after 2 h of incubating PBMCs with *S. suis* opsonized in hyperimmune serum (all other values in this setting lay below the detection limit; #). $^{*/\#}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

5 ng/ml (mean 15.53 and 18.80 ng/ml for LTAs of wt and 10Δ*dltA*, respectively) after 6 h indicating a delayed induction of IL-1β secretion by the purified LTAs in comparison with whole living *S. suis* in this assay (**Figure 6C**).

In conclusion, infection of porcine blood *ex vivo* with *S. suis* wt and 10Δ*dltA* indicates that D-alanylation of LTAs results in increased secretion of IL-1β, while the level of alanylation of purified LTAs, used for *ex vivo* stimulation of

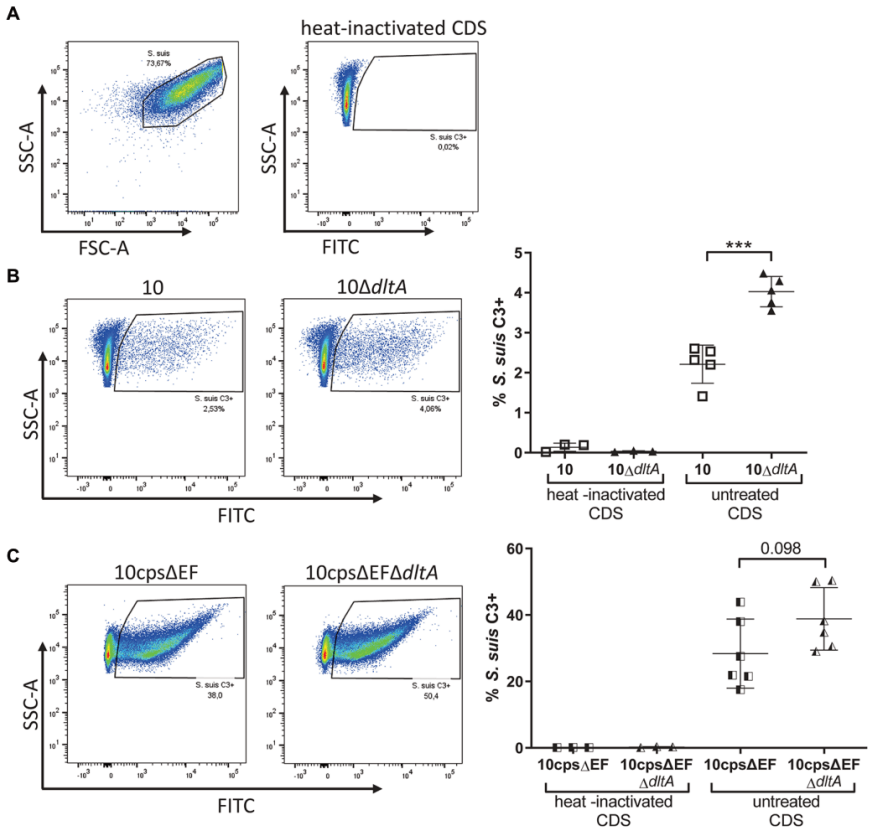

FIGURE 5 | Labeling with complement factor C3 is increased in *Streptococcus suis* 10Δ*dltA* after incubation in active CDS. *Streptococcus suis* strains were grown to early exponential phase (OD_{600} of 0.5) in THB and incubated with CDS for 1 h, followed by staining of C3 with a cross-reactive FITCs conjugated anti-human C3c antibody and measurement of C3 associated with the bacterial surface by flow cytometry. **(A)** Represents the gating strategy and negative control sample, while (**B**; encapsulated *S. suis*) and (**C**; unencapsulated *S. suis*) visualize representative examples on the left and the complete data sets on the right. Horizontal lines and error bars represent mean values and SDs. For statistical analysis, unpaired *t*-test was used to compare parent strain and respective Δ*dltA* mutant. ***$p < 0.001$.

porcine blood, did not influence the amount of secreted IL-1β. Therefore, the reduction of IL-1β levels, observed in blood infected with the Δ*dltA* mutant, is most likely not attributable to a directly reduced activation of PRRs in porcine immune cells by the LTAs of 10Δ*dltA* compared to the LTAs of the wt.

DISCUSSION

More than a decade ago, Fittipaldi et al. (2008) described D-alanylation of LTA in *S. suis* as a virulence mechanism. We revisited this phenotype and confirmed important findings of this previous study by independent loss-of-function experiments, namely, that *dltA* is necessary for D-alanylation of LTA and that an isogenic Δ*dltA* mutant is more susceptible to the antimicrobial agents colistin and polymyxin B.

When determining MBCs, we found an increased susceptibility of 10Δ*dltA* to several CAMPs, and as a tendency also for the cathelicidin PR-39, which is found in porcine NET structures (de Buhr et al., 2017). A common explanation for the protective effect of LTA D-alanylation is the introduction of positive charges into the bacterial cell wall, increasing the net positive charge of the bacterial surface and resulting in an increased electrostatic repulsion of CAMPs. In contrast to this theory, we did not find an increase of the binding of cationic cytochrome C to the encapsulated Δ*dltA* mutant, suggesting no difference to the wt in the net surface charge. A similar inconsistency was described by Poyart et al. (2003), when transmission electron microscopy of *Streptococcus agalactiae* showed stronger binding of positively charged metal ions to the wt rather than to the *dltA* mutant. It was discussed that the resulting local charge variation causes structural modifications that render the metal binding sites less accessible in the wall of the *dltA*

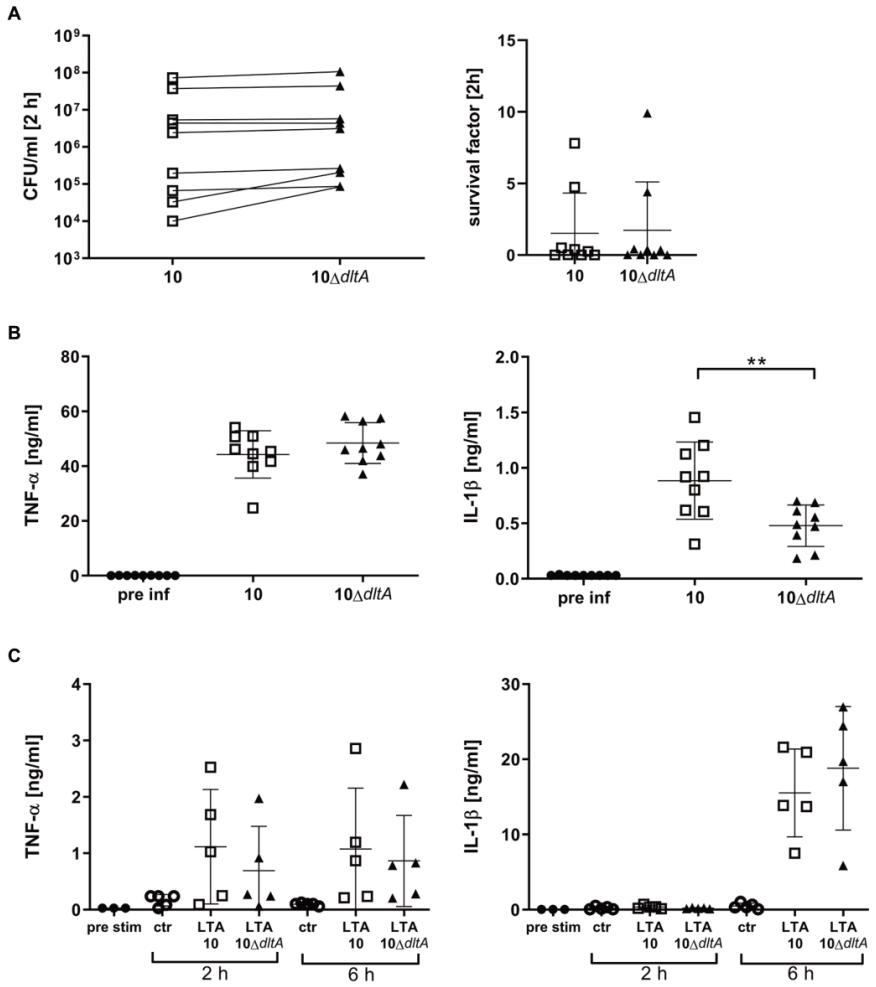

FIGURE 6 | *Streptococcus suis* strain 10 wt induces comparable levels of TNF-α, but higher levels of IL-1β in porcine blood than the mutant 10Δ*dltA*, while purified LTAs of wt and 10Δ*dltA* do not show differences in IL-1β induction upon addition to porcine blood. Whole porcine blood was infected with *S. suis* strain 10 and 10Δ*dltA* at an inital CFU of 1×10^7/ml. After 2 h infection, CFUs and survival factor **(A)** as well as TNF-α and IL-1β levels in plasma **(B)** were determined. For **(C)**, blood was treated with 30 μg/ml purified LTA obtained from strain 10 or 10Δ*dltA* as indicated, whereby citric acid was used as a solvent for LTA. The equivalent volume of citric acid was used in control samples (ctr). Plasma was obtained after incubation as well as prior to infection (pre-inf) or stimulation (pre-stim), respectively, to determine cytokine levels by ELISA. The limit of detection was 0.016 ng/ml for TNF-α and 0.032 ng/ml for IL-1β and all pre-inf and pre-stim samples lay below that limit. Lines in the left graph in **(A)** connect results of each individual blood donor. Horizontal lines and error bars represent mean values and SDs, respectively. For statistical analysis, paired *t*-test **(A,B)** or Wilcoxon matched-pairs signed rank test **(C)** was performed comparing wt and mutant samples. Differences that are not indicated are not significant. $^{**}p < 0.01$.

mutant. However, for the observed increase in resistance to CAMPs, it might not be crucial for *S. suis* to increase its net surface charge. Saar-Dover et al. (2012) suggest that the anionicity of the bacterial surface does not modulate the accumulation of CAMPs and the increased resistance resulting from D-alanylation of Group B *Streptococcus* LTA might rather be attributed to an altered conformation of the LTAs and resulting modulations in the density and surface properties of the bacterial cell wall. Therefore, even without increasing the global charge, LTA D-alanylation could contribute to CAMP resistance in *S. suis*. In the absence of the capsule, we observed an effect of LTA D-alanylation on the electric charge of the bacterial surface as determined by cytochrome C binding. The non-encapsulated Δ*dltA* mutant bound a significantly higher amount of cytochrome C than its non-encapsulated parent strain. This suggests that D-alanylation of LTA makes a difference for the electric charge of the bacterial surface if the capsule is downregulated and that capsular polysaccharides of *S. suis*

can mask this electric charge. Furthermore, we found an increased surface hydrophobicity due to the introduction of D-alanine into LTAs of *S. suis* as determined by the microbial adhesion to hydrocarbons assay. This might be caused by the hydrophobic character of the methyl group in alanine.

Many Gram-positive bacteria do not only possess LTA but also teichoic acids covalently attached to peptidoglycan, the so-called wall teichoic acids (WTAs; Brown et al., 2013). Deletion of the *dltA* gene has been shown to abolish D-alanylation in both TA polymers (Perego et al., 1995; Peschel et al., 1999). This might also be the case for *S. suis*. Although, the presence of WTAs in *S. suis* has not yet been proven on the molecular level, genes encoding members of the LCP family (LytR-CpsA-Psr), shown to be involved in cell wall anchoring of WTAs in *Staphylococcus aureus* (Chan et al., 2013; Schaefer et al., 2017) or *Bacillus subtilis* (Gale et al., 2017), namely, LytR and Psr, have also been identified in *S. suis* (Huang et al., 2021). Therefore, it seems likely that the phenotypes observed due to *dltA* deletion in *S. suis* are also partly attributable to a resulting loss of WTA D-alanylation.

Neutrophils represent a large part of the immune cells in blood and are major players in controlling bacteremia during *S. suis* infection. Comparing *S. suis* Δ*dltA* to its wt serotype 2 strain, Fittipaldi et al. (2008) describe increased killing of the mutant by porcine neutrophils, although the performed neutrophil killing assay was not able to distinguish between intra- and extracellular killing. Flow cytometry analysis enabled us to further clarify the role of LTA D-alanylation in the interaction of *S. suis* with neutrophils. After 30 min of infection, we observed a larger number of granulocytes in association with 10Δ*dltA* and simultaneously presenting an oxidative burst response in whole porcine blood, indicating an increased phagocytosis of the Δ*dltA* mutant compared to the wt. This is also in accordance with findings of Fittipaldi et al. (2008) showing increased killing of the Δ*dltA* mutant by porcine neutrophils after opsonization with complete porcine serum. Although antibody- and complement-dependent phagocytosis and oxidative burst of granulocytes are important to limit the survival of *S. suis* (Rungelrath et al., 2020), we did not record reduced killing of the Δ*dltA* mutant in whole porcine blood. In contrast, the specific bacterial content in the blood of a specific piglet was very similar between the wt and the isogenic mutant (**Figure 6A**), though there were substantial differences between piglets and blood of most piglets demonstrated killing of wt and mutant. Monitoring bacterial survival, *S. suis*-granulocyte association and oxidative burst over 2 h in whole blood of six piglets, proofed an efficient killing of both *S. suis* strains and, in parallel, a significant increase of association and oxidative burst. This is most likely due to opsonophagocytosis, with antibodies masking the initially observed effect of LTA D-alanylation on phagocytosis. We investigated bacterial survival in blood of 8-week-old conventional piglets which carried IgG and IgM antibodies binding to the surface of *S. suis* strain 10 as shown by ELISA (**Supplementary Figure S7**). Of note, this age class is severely affected by *S. suis* diseases in the field though IgG and IgM antibodies binding to streptococcal surface antigens are generally detectable in these piglets (Rieckmann et al., 2018; Mayer et al., 2021). We did not carry out bactericidal assays in the absence of specific antibodies, but based on results of previous investigations (unpublished results and Rungelrath et al., 2020), we speculate that wt and its isogenic *dltA* mutant will both exhibit high proliferation rates under such conditions.

The analysis of PBMC samples showed that D-alanylation of LTAs also reduces the association of *S. suis* with monocytes and lymphocytes. Macrophages/monocytes, polymorphonuclear leukocytes, B-lymphocytes, and subpopulations of T-lymphocytes have been shown to express the complement receptor 1 which binds to complement protein C3b on the bacterial surface (Vandendriessche et al., 2021). As already suggested by Lecours et al. (2011) when working with *S. suis* and dendritic cells of mice, a reduction in complement opsonization could be the underlying protective mechanism caused by the D-alanylation of LTA. Accordingly, the Δ*dltA* mutant showed an increased level of C3 deposition on its surface after incubation in serum that does not contain specific antibodies against *S. suis*. We assume that the influence of D-alanylation of LTAs on complement activation is mainly put down to the alternative pathway. The reasons for a reduced complement opsonization due to LTA D-alanylation might be found in electrostatic interactions, as stronger binding of C3 to negatively charged lipid membranes in comparison with neutral or positively charged lipid membranes has been described (Yorulmaz et al., 2016). In addition, surfaces with amino groups in combination with hydrophobic CH_3 groups (as it is found in alanine) might more strongly absorb serum proteins such as albumin, causing the formation of a protein layer that inhibits access of C3b (Toda and Iwata, 2010).

After opsonization of the streptococci with hyperimmune serum, we found high levels of association of *S. suis* wt and 10Δ*dltA* with monocytes but not with lymphocytes. In addition, the secretion of IL-1β and TNF-α strongly increased. This is likely due to a crosstalk between signaling of antibody-recognizing Fc receptors and PRR signaling in the monocytes associated with *S. suis*, as described for human dendritic cells exposed to opsonized *Staphylococcus aureus* (den Dunnen et al., 2012). IL-1β is produced by activated macrophages and monocytes, whereby the cleavage of pro-IL-1β to active IL-1β by caspase-1 is inflammasome-dependent (Martinon et al., 2002). Active inflammasomes also regulate gasdermin D activation resulting in pore formation and IL-1β release in the context of pyroptosis (Chen et al., 2020), which might account for the membrane damage we observed in monocytes after 2 h infection (**Figure 4A**). High local levels of the pore-forming toxin suilysin, produced by *S. suis*, might also be involved in this phenomenon as suilysin is known to induce IL-1β by inflammasome activation in murine macrophages (Song et al., 2020).

In difference to our finding of an augmented secretion of IL-1β and TNF-α in response to antibody-opsonized bacteria, Segura et al. (2006) described reduced mRNA expression of cytokines in a whole blood culture system with *S. suis* opsonized by specific antibodies, possibly due to suppressed bacterial growth. In our PBMC samples, bacteria proliferated even in the presence of high levels of specific antibodies (**Supplementary Figure S8**), most likely because there were no granulocytes present.

In contrast to the observed cytokine induction in isolated PBMCs, in whole porcine blood infected with *S. suis* 10Δ*dltA* for 2 h, we found IL-1β but not TNF-α levels to be reduced compared to blood infected with the wt (**Figure 6**; **Supplementary Figure S4**). This finding suggests a possible influence of LTA D-alanylation on inflammasome regulation, as TNF-α production and secretion are not inflammasome-dependent. Interestingly, direct recognition of cytosolic LTA of *Listeria monocytogenes* by the NLRP6 inflammasome was described for macrophages of mice (Hara et al., 2018), while the impact of D-alanylation was not investigated in this context. Since the reduction in IL-1β levels was only visible in whole blood, but not in isolated PBMCs, we speculate that a crosstalk between different immune cells in porcine blood might be important for this phenotype.

In summary, we found D-alanylation of LTAs in *S. suis* to be an important factor in CAMP resistance, defense against phagocytosis by granulocytes, reduction of complement deposition, and association with porcine monocytes and lymphocytes. Further studies are warranted to elucidate how *S. suis* modulates the host response through D-alanylation of its LTA and potentially also its WTA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the state Saxony (Landesdirektion Sachsen), Germany, under the permit numbers TVV 57-18 and A09/19 (collection of blood from piglets).

AUTHOR CONTRIBUTIONS

SÖ designed and conducted all experiments except RNA analysis, LTA extraction and NMR spectroscopy. Furthermore, SÖ analyzed the data and drafted the manuscript. A-KK supported bactericidal assays. NG supervised LTA extraction and NMR spectroscopy and analyzed NMR data. NB and MM supported experiments investigating interaction and antimicrobial peptides and performed RNA analysis. NS supervised flow cytometry analysis. SÖ, CB, and MK-B conceived the study and designed experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.822369/full#supplementary-material

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Supplementary Material

D-Alanylation of Lipoteichoic Acids in *Streptococcus suis* Reduces Association with Leukocytes in Porcine Blood

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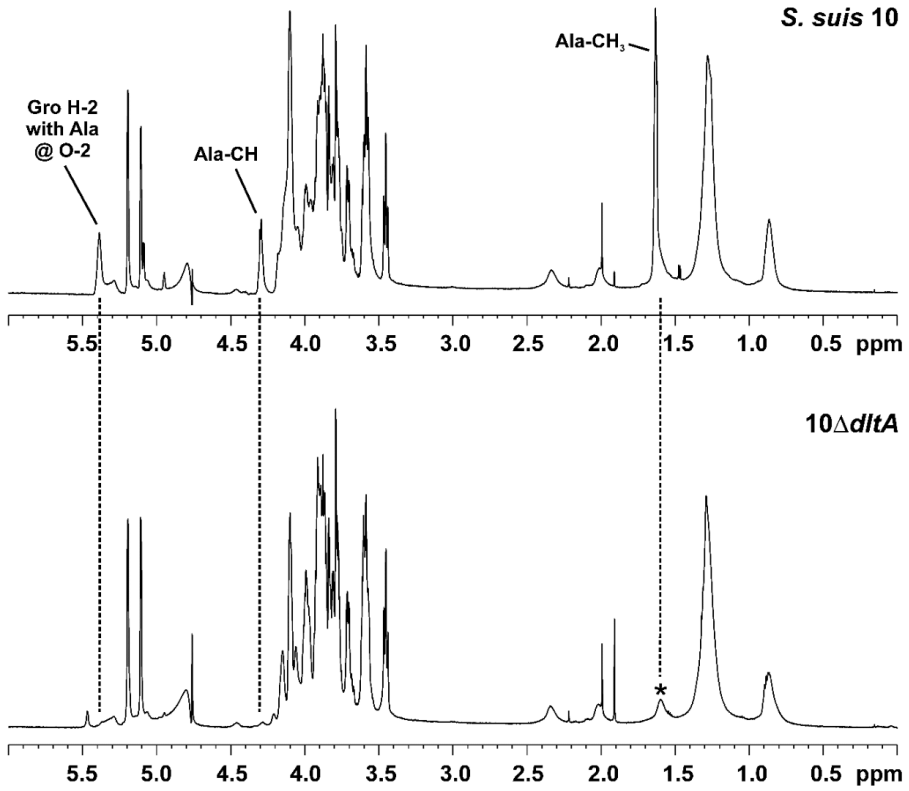

Figure S1: Lipoteichoic acids (LTA) of *S. suis* 10Δ*dltA* mutant does not possess alanine residues.

^{1}H Nuclear magnetic resonance spectroscopy of *S. suis* strain 10 (top panel) and 10Δ*dltA* mutant (bottom panel) LTA. The lines indicate the indicative peaks for D-alanine residues at the position O-2 of glycerol within poly-(glyco)glycerolphosphate chains, which are present in the spectrum recorded from LTA isolated from the wt but not in that of LTA isolated from the mutant. Residual signals at δ_H 1.65-1.60 ppm in spectra recorded from LTA of Δ*dltA* mutant (marked with *) represent the β-protons of fatty acids.

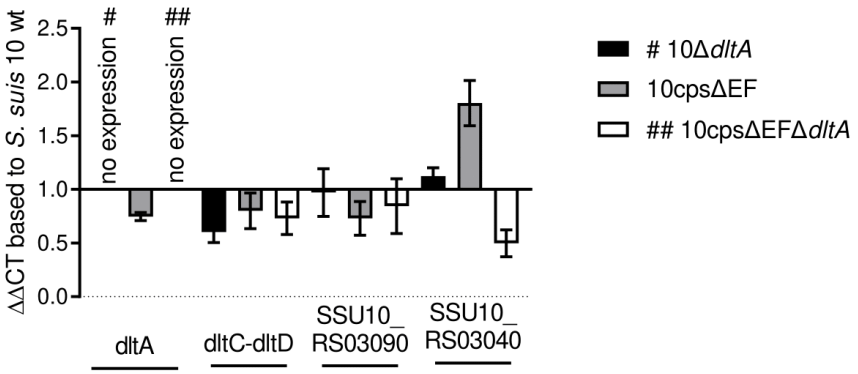

Figure S2: In frame deletion of *dltA* in 10Δ*dltA* does not affect expression of *dltC-dltD* nor of an ORF upstream (SSU10_RS03040, glucosamine-6-phosphate deaminase) or the ORF directly downstream of the *dlt* locus (SSU10_RS03090, low temperature requirement protein A).
RT-PCR was conducted with oligonucleotide primers listed in Table S1. Relative transcript levels of 10Δ*dltA* as well as the unencapsulated mutants 10cpsΔEF and 10cpsΔEFΔ*dltA* were compared to those of strain 10 wt by calculation of ΔΔCT using *gyrB* expression as reference. Additionally, we tested *dltA* RNA expression and did not measure any signal in the *dltA* mutants after 40 cycles. Data are presented as means ± SD of ΔΔCT of three independent experiments.

Table S1: Oligonucleotide primers used in qRT-PCR

Gene	Primer sequence (5´-3´) forward	Primer sequence (5´-3´) reverse	Amplicon length (bp)
gyrB	GGACCTGGGTGCTTAACAGA	AGGTGGTACCCATGAGCAAG	158
dltA	CCTGTCCTTGTTTTTGGCGG	CACCAAACTCGGCTCTGCTA	150
dltC-dltD	CATTTCTGGTGTTGTGGAGTTGA	ACCGAGAAGAACTCGGGAAAA	120
SSU10_ RS03090	ACGTTGCCAGCTGATTACCA	AGAGTCGCCATTGCGAAGAA	120
SSU10_ RS03040	TAGGTTTGGCAACAGGCTCC	TGGTCGCTCTCCTCACCTAA	127

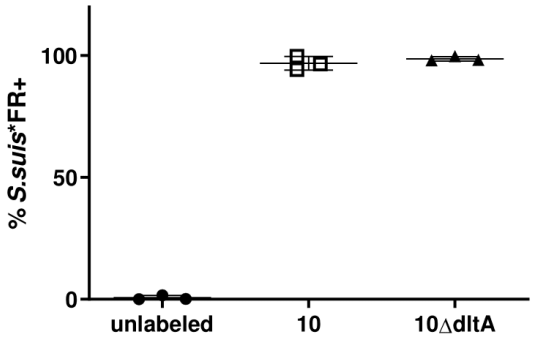

Figure S3: Comparison of Far Red labeling of *S. suis* 10 and 10Δ*dltA*.

Far Red labeled *S. suis* 10 wt and mutant strain were analyzed for the percentage of positively labeled bacteria by flow cytometry. Statistical analysis was performed by Mann Whitney test and no significant difference between wt and isogenic mutant was recorded.

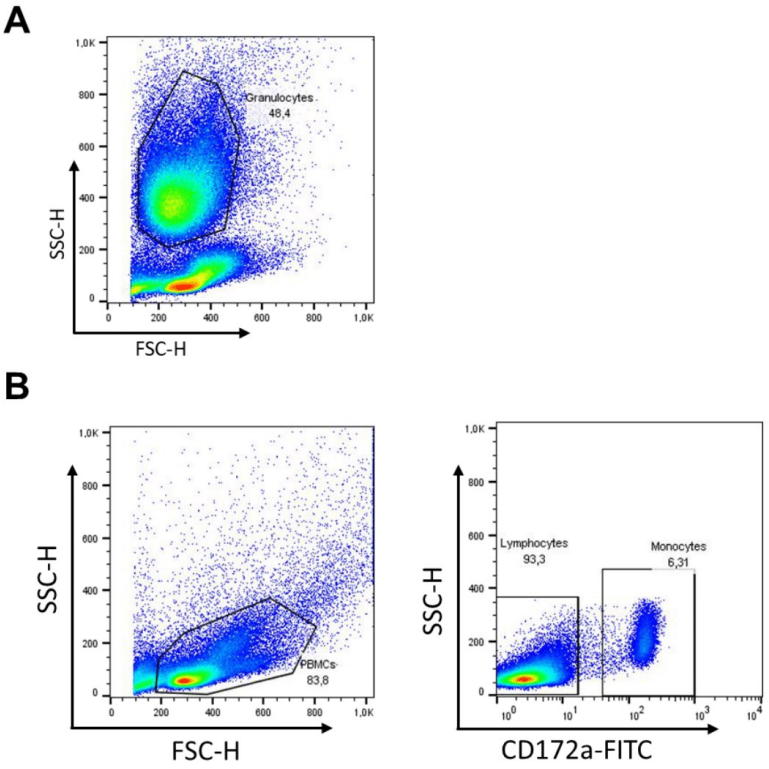

Figure S4: Gating strategy of granulocytes in whole blood (A) and monocytes in isolated PBMCs (B).

Flow cytometry measurement of granulocytes in whole blood was performed in assays depicted in Figure 2, while isolation of PBMCs and staining of monocytes with the FITC-labeled marker CD172a was conducted in experiments shown in Figure 3.

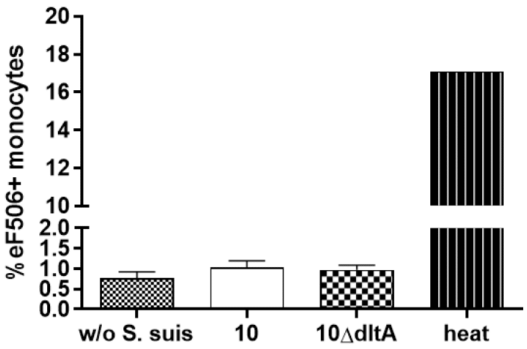

Figure S5: Viability staining of peripheral blood mononuclear cells (PBMCs) indicates no increased cytotoxicity towards monocytes of *S. suis* 10 or 10Δ*dltA* after 30 min of incubation.

PBMCs (1 x 10^7 /ml) were incubated with *S. suis* 10 wt or 10Δ*dltA*, that had been pre-incubated in serum of colostrum-deprived piglets (CDS). After 30 min viability dye eF506 was used to mark membrane damaged cells and monocytes were stained with FITC-coupled myeloid marker CD172a. Cells treated with PBS without bacteria (w/o *S. suis*) and heat treated (2 x 5 min at 56 °C) PBMCs were used as negative and positive control, respectively. Samples were analyzed by flow cytometry. Bars and error bars represent mean and SD of three biological replicates (heat treatment to verify eF506 staining was only performed with one sample). Statistical analysis was performed by Kruskal-Wallis and Dunn's multiple comparison test and no significant differences were detected between samples with or without *S. suis*.

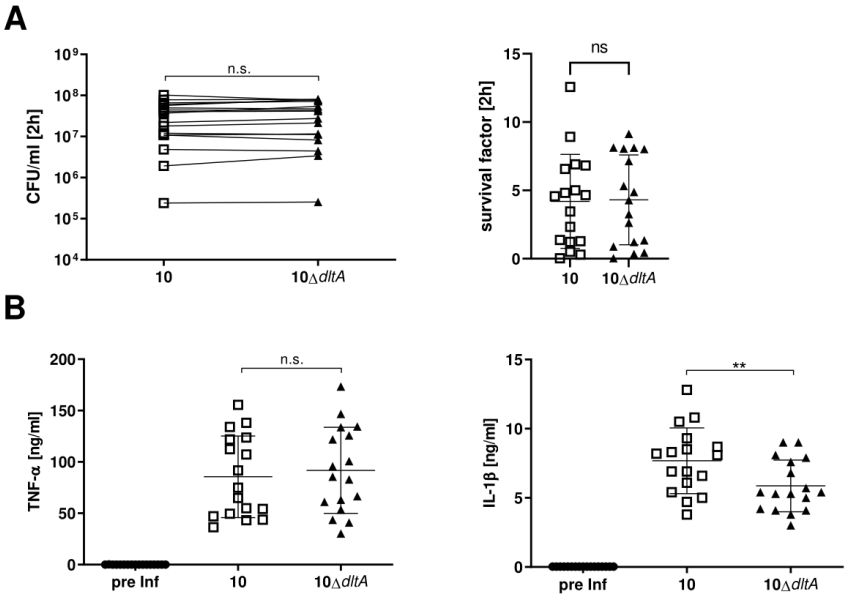

Figure S6: S. *suis* strain 10 induces significantly higher levels of IL-1β in porcine blood than the mutant 10Δ*dltA*.

Porcine plasma was obtained after incubation of blood with *S. suis* strain 10 or 10Δ*dltA* for 2h at 37 °C under constant rotation. CFU and survival data are shown in A. For B cytokine levels were determined by ELISA. Horizontal lines and error bars represent mean values and standard deviations, respectively. The limit of detection was 0.016 ng/ml for TNF-α and 0.031 ng/ml for IL-1β. Bars and error bars represent mean and SD. For statistical analysis Shapiro-Wilk normality test and paired t-tests between wt and mutant samples were performed. ** $p < 0.01$, not significant (n.s.) $p > 0.05$.

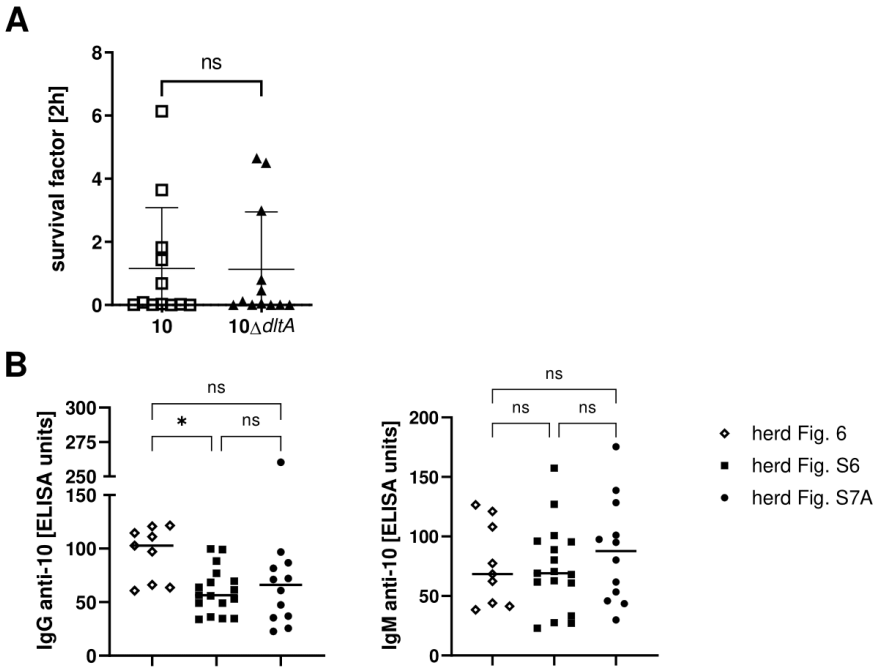

Figure S7: Comparison of survival of *S. suis* 10 wt and 10Δ*dltA* in whole blood obtained from piglets of a third conventional herd (A) and levels of plasma IgG and IgM antibodies binding to the surface of *S. suis* strain 10 present in the bactericidal assays shown in the indicated figures (blood samples collected in 3 different herds) (B).

A Whole porcine blood was infected with *S. suis* 10 and 10Δ*dltA* for 2 h at 37 °C under constant rotation. The survival factor was calculated by dividing the CFUs obtained after and before the 2h incubation period. **B** Levels of plasma IgG and IgM binding to the surface of inactivated and immobilized *S. suis* wt strain 10 as determined in ELISA. Plasma was obtained of all three indicated bactericidal assays (piglets from different herds for each assay) and stored at -80°C until measurement. A hyperimmune serum (#4515) obtained after prime-boost vaccination of a piglet with a *S. suis* strain 10 bacterin was used as a standard and defined to include 100 ELISA Units. Bars and error bars represent mean and SD. For statistical analysis Shapiro-Wilk normality test, Wilcoxon matched-pairs signed rank test between wt and mutant samples (**A**) as well as Kruskal-Wallis test followed by Dunn's multiple comparisons test (**B**) were performed. * $p < 0.05$, not significant (n.s.) $p > 0.05$.

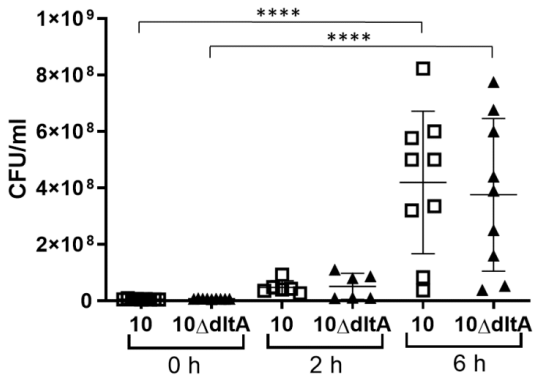

Figure S8: Colony forming units (CFU) in PBMC samples infected with *S. suis* 10 or 10Δ*dltA* for the indicated times after pre-opsonization in hyperimmune serum.

Serial dilution and plating on blood agar plates were conducted to determine CFU of *S. suis* 10 and 10Δ*dltA* in the PBMC samples used to evaluate cytokine production as shown in Fig. 5. Normality was tested using Shapiro-Wilk test. For statistical analysis Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed. Differences that are not indicated are not significant. **** $p < 0.0001$

4 Discussion

Bacteremia plays a key role in invasive *S. suis* infections. The interaction with blood leukocytes is a potentially dangerous obstacle for the bacteria in this process. At the same time, *S. suis* might also use the interaction with these leukocytes to its advantage. For example, WILLIAMS and BLAKEMORE (1990) suggested that *S. suis* is taken up into monocytes, without being killed, and then uses these cells as “trojan horse” to cross barriers into the CNS. The finding of the antiphagocytic properties of *S. suis* CPS and that unencapsulated mutants were phagocytoses and quickly died within monocytes and macrophages (CHARLAND et al. 1998, SMITH et al. 1999), led to the suggestion of a “modified trojan horse theory” with *S. suis* not being engulfed but rather adhering extracellularly on monocytes and macrophages (GOTTSCHALK and SEGURA 2000). Shortly afterwards high levels of adherence to murine macrophages, without ingestion of the bacteria, could be described by SEGURA and GOTTSCHALK (2002). This thesis illuminates the interaction of *S. suis* with porcine leukocytes during bacteremia, firstly focusing on neutrophil oxidative burst response and the influence of the porcine complement system on *S. suis* survival (RUNGELRATH et al 2020) and secondly on modification of *S. suis* cell wall by LTA D-alanylation as a defense mechanism and the resulting changes in interaction with blood neutrophils and monocytes (ÖHLMANN et al 2022) (Figure 1). The results of these studies are also discussed in light of the proposed “modified trojan horse theory” in comparison to “planktonic” *S. suis* traveling free in the bloodstream and highlight that the bacterial-cell-interaction is not restricted to the question of binding versus phagocytosis, but also involves the cytokine and chemokine response of blood immune cells.

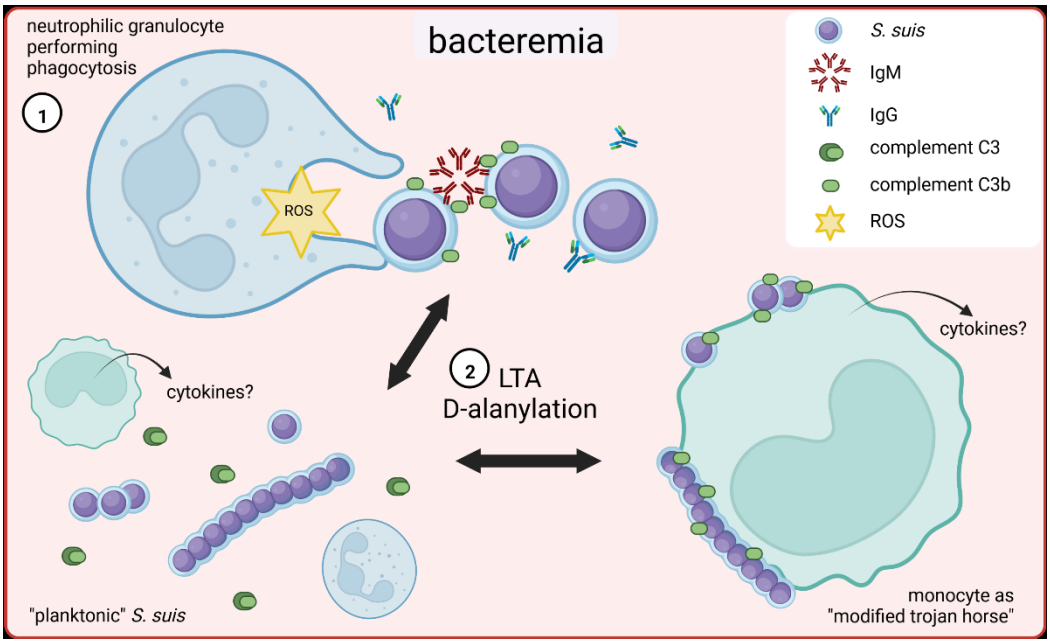

Figure 1 Interaction of *S. suis* with blood leukocytes. ① Factors involved in neutrophil oxidative burst and *S. suis* survival. ② Influence of *S. suis* LTA D-alanylation on phagocytosis by neutrophils and binding to monocytes. Created with BioRender.com

4.1 Phagocytosis and oxidative burst in porcine blood

In this thesis the role of the oxidative burst in response to *S. suis* infection was investigated in porcine blood granulocytes *in vitro* and *in vivo.* For this, the survival of *S. suis* in blood was related to the amount of induced oxidative burst intermediates in granulocytes. For the first time, the induction of ROS in blood granulocytes after experimental *S. suis* infection of a pig was demonstrated *in vivo* and the associated bacterial loads were measured. Although the results obtained from this animal experiment are not conclusive, as only two animals were included, they underline the hypothesis, that immunoglobulin-mediated oxidative burst contributes to the killing of *S. suis* in porcine blood.

Despite a number of described *S. suis* factors supposedly conferring resistance to oxidative stress (e.g. SodA, NADH oxidase) (TANG et al. 2012, ZHENG et al. 2017), we found a clear impact of hydrogen peroxide (H_2O_2) levels as low as 0.001% on the growth of serotype 2, serotype 7 and serotype 9 strains in Todd Hewitt broth. In contrast, TANG et al. (2012) used concentrations of up to 0.04% H_2O_2 without seeing impaired growth of a serotype 2 strain in brain heart infusion and related this ROS resistance to the presence of SodA. All strains investigated in our study were *sodA* positive and also expressed *sodA* mRNA. ZHENG et al. (2017) investigated another serotype 2 strain and found it to be tolerant to oxidative stress during growth in high oxygen conditions and also under addition of 0.5 mM H_2O_2. The serotype 2 strain, we worked with, is a well known virulent European strain, while the *S. suis* strains, used in the mentioned studies, derive from Asia, with at least one of them isolated during the Chinese outbreak 2005. Therefore, the genetical background is different, as these Asian strains probably belong to the highly virulent ST7. A pathogenicity island was identified exclusively in the epidemic Asian strains (CHEN et al. 2007) and a putative T4SS lies within this island (ZHAO et al. 2011), contributing to oxidative stress tolerance by influencing the secretion of several *S. suis* proteins under oxidative stress conditions (JIANG et al. 2016). The investigated Asian serotype 2 strains might possess a higher resistance to oxidative stress, than the virulent European serotype 2 strain used in this thesis. However, we could show the importance of the oxidative burst in porcine granulocytes for the killing of this strain by using an NADPH oxidase inhibitor in reconstituted porcine blood under different immunological conditions.

During our investigations specific antibodies in blood proofed crucial for the development of a robust oxidative burst response, as *S. suis* did not trigger ROS production in granulocytes in blood reconstituted with a serum containing only natural but no specific antibodies. It was suggested, that not complement receptor, but only Fc-receptor mediated IgG-dependent phagocytosis leads to ROS production (CARON and HALL 1998). Nevertheless, when using hyperimmune serum depleted of IgG, we still observed an oxidative burst response, that was accompanied by nearly complete killing of *S. suis*, indicating that other mechanisms beside Fc-receptor mediated phagocytosis can induce ROS formation and killing in porcine blood. This stands in agreement with an observation in human neutrophils, where blocking of Fc-receptors did not prevent oxidative burst response nor killing of *S. pyogenes*. Instead the human complement receptor CR3 was necessary in this context (NILSSON et al. 2005). The process of complement-mediated oxidative burst induction is probably similar in porcine

neutrophils as they express an orthologue to human CR3 (PIRIOU-GUZYLACK and SALMON 2008). However, inhibition of the complement cascade, either by vaccinia virus complement control protein (VCP) or by heat inactivation, showed only limited effect on survival of *S. suis* in the presence of hyperimmune serum, although ROS production was significantly decreased. This gives credit to the important roles of IgG and complement, respectively, in *S. suis* killing in porcine blood and points out the multifactorial character of this process.

Different tools were used to investigate the remaining strong killing capacity of reconstituted porcine blood in the absence of IgG. Under these conditions, complement-inhibition resulted in significantly increased survival of *S. suis* and cleavage of porcine IgM by the highly specific IgM protease Ide_{Ssuis} increased the survival factor even more. Both factors also proved important for ROS production in accordance with an IgM-complement-oxidative burst axis. However, IgM cleavage also increased the bacterial survival factor, when ROS production was already at a minimum due to the used inhibitors. This suggests that IgM-mediated killing or inhibition of *S. suis* proliferation is not only mediated via the complement system or the oxidative burst. The pentamer structure of IgM might play a role in this independent mechanism. It allows prominent agglutination activity (KLIMOVICH 2011). IgM specific for the capsular polysaccharide of *S. pneumoniae* caused aggregation of the pneumococci and reduced CFU counts even in the absence of phagocytes and complement (FABRIZIO et al. 2010). Since phagocytes do not possess an Fcμ receptor for IgM, Fc-receptor mediated signaling should not be involved in the complement-independent killing mechanism. Nevertheless, IgM immune complexes can induce oxidative burst and lysosomal enzyme release in rabbit polymorphonuclear cells, even without phagocytosis (FURRIEL et al. 1992, LUCISANO et al. 1998).

By using fluorescently labeled *S. suis,* we were able to simultaneously investigate the association of the streptococci with granulocytes and the oxidative burst response by flow cytometry. The majority of granulocytes associated with *S. suis* showed ROS production. Complement inactivation by VCP or heat significantly reduced the number of granulocytes associated with *S. suis,* despite the presence of specific antibodies. In an intranasal colonization model for *S. suis* serotype 2 with C3-deficient mice, an intact complement system proofed crucial to protect the mice against invasive disease and death (SEITZ et al. 2014). These different studies highlight the importance of the complement cascade as innate immune defense mechanism against *S. suis*. Hence there is a strong need for pathogens to avoid complement activation, or at least opsonization.

In the second manuscript I investigated the ability of *S. suis* to modify its surface by D-alanylation of LTA as a complement evasion mechanism. In *S. suis* serotype 2 wt, I found reduced opsonization with C3b compared to the Δ*dltA* mutant, which is incapable of LTA D-alanylation. This was also described by LECOURS et al. (2011) for a different serotype 2 strain. This might be explained by electrostatic changes in the cell wall, upon introduction of positive charges within D-alanine. Negatively charged lipid membranes have been shown to bind more C3b than neutral or positively charged lipid membranes (YORULMAZ et al. 2016). The formation of a protective protein layer that inhibits access of C3b to the bacterial surface, is also a possible explanation for the effect of LTA D-alanylation on complement deposition, since

a surface composed of amino groups with hydrophobic methyl groups (as it is found in alanine) might strongly absorb serum proteins (TODA and IWATA 2010). I found increased *S. suis*-cell-association and simultaneous oxidative burst response of granulocytes for the mutant, indicating increased phagocytosis of Δ*dltA* after 30 min of incubation in porcine blood. This might be a result of the increased C3b opsonization but also of the reduced surface hydrophobicity I recorded in the mutant (ABSOLOM 1988).

FITTIPALDI et al. (2008) described increased neutrophil-mediated killing of a *S. suis* serotype 2 Δ*dltA* mutant. Despite the initially observed increase in phagocytosis, we did not see a difference in survival between wt and mutant *in vitro* in blood of immunocompetent piglets after 2h of infection. Monitoring the phagocytosis rate and CFU counts at 30min, 1h and 2h in blood of piglets of the same herd, revealed that phagocytosis increased significantly over time for both strains indifferently, resulting in strong killing. As these piglets carried antibodies against *S. suis* serotype 2, I suspect antibody-mediated opsonization to mask the initially observed effect of LTA D-alanylation.

4.2 Interaction of *S. suis* with blood monocytes and lymphocytes

I found that D-alanylation of LTA reduced the association of *S. suis* with multiple blood leukocytes (granulocytes, monocytes and lymphocytes). This might be related to the observed reduction in C3b deposition on the surface of the wt. Monocytes, granulocytes, B cells and subpopulations of T cells have all been shown to express complement receptor CR1, lymphocytes additionally express CR2, and monocytes, neutrophils and activated lymphocytes also express CR3 and CR4, all of which bind to C3b or iC3b on bacterial surfaces (VANDENDRIESSCHE et al. 2021). LECOURS at al. (2011) observed a similar influence of LTA D-alanylation on the interaction of *S. suis* serotype 2 with murine DCs and also related this to the reduced complement deposition on the wt. Another effect of LTA D-alanylation could be the already mentioned capacity of a surface composed of amino groups with hydrophobic methyl groups (as in alanine) to absorb serum proteins (TODA and IWATA 2010), leading to a masking of PAMPs and to reduced interaction with PRRs of the blood immune cells. This has not been shown for *S. suis* so far.

The thought that *S. suis* actively reduces its association to monocytes in porcine blood by LTA D-alanylation stands somewhat in contrast to the suggestion that *S. suis* wants to use monocytes as a vehicle to reach tissues (“modified trojan horse theory”) (GOTTSCHALK and SEGURA 2000). The advantage of avoiding recognition by LTA D-alanylation might be more important than the advantage of traveling along with monocytes, bound to their surface. My observation, that the presence of the capsule strongly reduces the association of the *S. suis* with monocytes and the suggestion that CPS might be upregulated in blood (GOTTSCHALK and SEGURA 2000) also points rather to a free, “planktonic” way of dissemination of *S. suis* in the circulation. I created a Δ*dltA* mutant strain from a non-encapsulated *S. suis* parent strain and found that LTA D-alanylation still reduced the association with monocytes, independently of the capsule, whereby for both non-encapsulated strains a far higher number of monocytes were associated with *S. suis* than it was the case using the respective encapsulated strains.

Addition of specific antibodies increased the number of monocytes associated with *S. suis* very prominently, independently of the alanylation status of *S. suis* LTAs, while the number of lymphocytes associated with *S. suis* decreased under these conditions. Additionally, the *S. suis*-induced concentration of IL-1β and TNF-α increased in the presence of specific antibodies. Likely the crosstalk of Fc- and PRR-mediated signaling in monocytes was responsible for this observed strong activation, as this was also described for human DCs interacting with opsonized *Staphylococcus aureus* (DUNNEN et al. 2012). At the same time, membrane damage was observed in the monocytes – on the one hand possibly due to the cytotoxic effect of *S. suis*-secreted SLY (TENENBAUM et al. 2016) but likely also a result of Gasdermin D activation in the monocytes associated with *S. suis*. Gasdermin D is a pore forming molecule, which is activated in an inflammasome-dependent manner, like the cytokine IL-1β (MARTINON et al. 2002, CHEN et al. 2020). It is often proposed, that IL-1β is passively released during cell lysis and Gasdermin D deficiency abrogates IL-1β secretion upon canonical inflammasome activation (SHI et al. 2015, CHEN et al. 2020). Therefore, the observed high levels of IL-1β and the membrane damage can be seen in the context of a form of cell death called pyroptosis, which was induced in monocytes by association with antibody- and complement-opsonized *S. suis*. This leads to the suggestion, that not only neutrophils can give up themselves in a suicidal mission to defend the host against invading pathogens, but also blood monocytes are ready to die in order to increase the amount of pro-inflammatory molecules in a very efficient manner.

For the host this pro-inflammatory response is a double-edged sword as shown by the STSLS caused by *S. suis* in the two Chinese outbreaks in 1998 and 2005 (TANG et al. 2006, LIN et al. 2019). While IL-1 signaling was found to be beneficial in clearing the bacterial burden in blood, an exaggerated inflammation also causes severe damage to the host (LAVAGNA et al. 2019). This is aggravated in infections with the Chinese endemic ST7 strain, as it was found to produce high levels of SLY (HE et al. 2014), which is able to activate the NLRP3 inflammasome and augment the inflammatory response (LIN et al. 2019, SONG et al. 2020, XU et al. 2021). Although the European serotype 2 strain, I worked with, probably expresses lower levels of SLY, as it was shown for the genetically strongly related strain P1/7 (LIN et al. 2019), locally increased concentrations of this cytolysin might have also played a role in my experiments.

In my investigations of the interaction of *S. suis* with monocytes and lymphocytes, I used purified peripheral blood mononuclear cells (PBMCs). In contrast to my finding of strongly increased cytokine levels in PBMCs, SEGURA et al (2006) described reduced cytokine mRNA expression in whole blood infected with antibody-opsonized *S. suis*. While bacteria proliferated in my PBMC samples, in whole blood the opsonized *S. suis* were killed rapidly by neutrophil phagocytosis and oxidative burst response, as we observed in our first study. When investigating the cytokine response in whole blood of immunocompetent pigs I found substantial differences among the blood donors. Nevertheless, I made the observation, that the Δ*dltA* mutant induced lower levels of IL-1β than the wt, while TNF-α levels were similar, suggesting a possible influence of LTA D-alanylation on inflammasome activation. For pneumococci a similar observation is described, as IL-1β transcription was reduced in the

nasal fluids of infant mice infected with a *dltB*-negative mutant compared to the wt (ZAFAR et al. 2019).

AUGER et al. (2020) investigated the role of inflammatory and patrolling monocytes and neutrophils in a mouse model of *S. suis* infection. While patrolling monocytes played a minor role, inflammatory monocytes and neutrophils played a beneficial role for systemic bacterial control and elimination after intraperitoneal infection of the mice. Monocytes were also described to be important in reducing dissemination of Group A *Streptococcus*, whereby the mechanism remained unclear but might be related to TNF-α levels and macrophage recruitment to the site of infection (MISHALIAN et al. 2011). AUGER et al. (2020) propose an indirect antibacterial effect of monocytes by providing a certain level of inflammation, beneficial for bacterial clearance, as also described with regard to inflammation by type I interferon in *S. suis* disease in mice (AUGER et al. 2017). It is likely that monocytes play a similar important role in the porcine immune system in response to *S. suis* through modification of the inflammatory response.

4.3 Conclusion

For the first time, we could demonstrate the induction of ROS in granulocytes *in vivo* in two pigs infected with *S. suis* and found the number of bacteria to be lower in the pig with a robust oxidative burst response than in the pig with reduced ROS production. The usage of an *in vitro* blood model adjusted with sera containing different antibody levels, depletion and cleavage of IgG and IgM, respectively and the usage of different inhibitors allowed us to elucidate the factors leading to a robust oxidative burst response and killing of *S. suis*. Our results demonstrate that specific antibodies and the IgM-complement-oxidative burst axis play a crucial role in the control of survival of *S. suis* in porcine blood. Despite numerous described defense mechanisms of *S. suis* against oxidative stress, phagocytosis and ROS represent a very effective way of the porcine immune system to kill the invading streptococci and control bacteremia.

One defense mechanism of *S. suis* against the host immune system is the D-alanylation of LTAs. I verified and expanded described effects of LTA D-alanylation for the protection against AMPs and found a decreased deposition of complement on the bacterial surface and a certain protection from phagocytosis and ROS by granulocytes. Nevertheless, survival of the *dltA* mutant is not attenuated in porcine blood containing specific antibodies, where both wt and mutant were killed, in accordance with our findings of the first manuscript on the importance of these antibodies for killing in blood. This leads to the conclusion, that the effect of cell wall modification by LTA D-alanylation is to be found in defense against innate immunity, but it does not protect the streptococci form the adaptive immune system. However, the induction of IL-1β in porcine whole blood is influenced by the ability of *S. suis* to D-alanylate its LTAs and the association to monocytes is reduced in PBMC samples. Therefore, the role of this surface modification in blood might be found in modulating general mechanism of bacteremia, favoring a reduced association with blood leukocytes and a free dissemination in contrast to the proposed “modified trojan horse theory” and in influencing the cytokine response.

5 Zusammenfassung

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Interaktion von *Streptococcus suis* mit Blut-Immunzellen: Einfluss des Komplementsystems und der Modifikation von Lipoteichonsäuren

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Stichwörter: *Streptococcus suis*, neutrophils, monocytes, ROS, complement, LTA, *dltA*

Einleitung

Streptococcus (S.) suis ist weltweit in Schweinepopulationen verbreitet. *S. suis* verursacht hohe wirtschaftliche Verluste durch das Auftreten von Meningitis, Arthritis, Serositis und Endokarditis und stellt als Zoonoseerreger gleichzeitig ein Risiko für den Menschen dar. Im Zusammenhang mit Infektionen in europäischen Schweinebetrieben werden Serotyp 2, 7 und 9 am häufigsten nachgewiesen. Die Bakteriämie spielt eine Schlüsselrolle in der invasiven *S. suis* Erkrankung und die Interaktion der Streptokokken mit den Immunzellen des Wirts im Blut ist entscheidend für den Ausgang der Infektion.

Ziele der Untersuchung

Das Ziel der vorliegenden Arbeit, war die Untersuchung der Fähigkeit von porzinen Immunzellen *S. suis* durch Phagozytose und reaktive Sauerstoffspezies (ROS) zu töten. Dabei wurden vor allem die Bedeutung von IgM und des Komplementsystems beleuchtet. In diesem Sinne, wurde auch die Arbeitshypothese untersucht, dass eine Modifizierung der bakteriellen Zellwand durch D-Alanylierung von Lipoteichonsäuren (LTAs) *S. suis* hilft, angeborene Immunfunktionen zu umgehen, wie z. B. die Opsonisierung mit Komplementfaktoren, sowie dass diese Zellwandveränderung die Interaktion von *S. suis* mit Leukozyten beeinflusst.

Material und Methoden

Die Bestimmung von ROS und dem Überleben der Streptokokken wurde *in vivo* im Blut zweier mit *S. suis*-infizierter Ferkel und *in vitro* in einem rekonstituierten Blutmodel durchgeführt. Die ROS-Messung erfolgte unter Zugabe von Dihydrorhodamine 123 mittels Durchflusszytometrie in Granulozyten. Verschiedene Inhibitoren wurden dabei eingesetzt: Apocynin um die NADPH Oxidase und damit die ROS-Bildung zu hemmen, *Vaccinia Virus Complement Control Protein* (VCP) für die Inaktivierung der Komplementkaskade und Ide_{Ssuis} um porzines IgM zu spalten. Mithilfe eines thermosensitiven Vektors wurde eine isogene Deletionsmutante des *dltA* Gens erstellt, um den Einfluss der D-Alanylierung von LTAs zu untersuchen. Die Sequenzierung des entsprechenden Genabschnitts und ein Southern Blot dienten der genetischen Verifizierung der Mutante. Weiterhin wurden Wachstumsverhalten, Hydrophobie und Ladung der

Oberfläche sowie minimale Hemmkonzentrationen einer Reihe antimikrobieller Peptide für die *dltA* Mutante untersucht. Die Assoziation von Blutleukozyten mit *S. suis* wurde mithilfe von Far Red markierten Bakterien in der Durchflusszytometrie bestimmt. Die Zytokin-Messung erfolgte mit kommerziell erhältlichen *Enzyme-linked immunosorbent assays* (ELISA)-Kits.

Ergebnisse

S. suis Stämme der Serotypen 2, 7 und 9 zeigten sich hochsensibel gegenüber Wasserstoffperoxid. Eine Hemmung der Bildung von ROS in rekonstituierten Blutproben führte selbst in Anwesenheit einer hohen Konzentration von *S. suis*-spezifischen Antikörpern zu einem signifikanten Anstieg des bakteriellen Überlebens. Die Induktion von ROS durch *S. suis* in Granulozyten wurde durch spezifische Antikörper verstärkt und hing partiell von der Anwesenheit funktionellen Komplements ab. In der Abwesenheit von IgG reduzierte die Spaltung von IgM oder die Inaktivierung von Komplement die ROS Produktion um mehr als das Dreifache, was in einem Anstieg des bakteriellen Überlebens resultierte. Der Mechanismus von *S. suis* D-Alanin in seine LTAs einzulagern, erhöhte die Hydrophobie der bakteriellen Oberfläche und reduzierte die Opsonisierung mit Komplementfaktor C3b, sowie die Phagozytose durch Granulozyten in Vollblut und die Assoziation von *S. suis* mit Monozyten and Lymphozyten. Trotzdem wurde das Überleben der Streptokokken in Blut, welches *S. suis*-spezifische Antikörper enthielt, nicht signifikant durch diese Zellwandmodifikation gesteigert. Allerdings war der durch die *dltA* Mutante induzierte Gehalt an IL-1β im Blut signifikant geringer als der durch den Wildtyp induzierte. Hohe Antikörperspiegel verursachten einen hochsignifikanten Anstieg der Interaktion von *S. suis* mit Monozyten, was bei diesen zum inflammatorischen Zelltod mit einer starken Sekretion von IL-1β und TNF-α führte.

Schlussfolgerung

Trotz einer Vielzahl an Abwehrmechanismen von *S. suis* gegen oxidativen Stress, stellen Phagozytose und ROS einen sehr effektiven Mechanismus des Immunsystems dar, um die eingedrungenen Streptokokken zu töten. Die Induktion von ROS durch *S. suis* hängt dabei stark von der Anwesenheit spezifischer Antikörper und teilweise von aktivem Komplement ab. Die Fähigkeit von *S. suis* D-Alanin in seine LTAs einzulagern, verändert Eigenschaften der bakteriellen Zellwand und vermittelt dabei Schutz vor angeborenen Immunmechanismen, wie antimikrobiellen Peptiden und dem Komplementsystem, aber sie sorgt nicht für ein besseres Überleben der Streptokokken, in der Anwesenheit von spezifischen Immunglobulinen. Sie reduziert die Assoziation von *S. suis* mit Monozyten, was etwas in Diskrepanz zu einer 2000 postulierten modifizierten „Trojanisches Pferd“-Theorie steht. Die durch LTA D-Alanylierung gesteigerte IL-1β Sekretion im Vollblut, führt zu der Schlussfolgerung, dass *S. suis* durch diese Zellwandmodifikation Entzündungsprozesse im Wirt beeinflusst.

6 Summary

Sophie Öhlmann

Interaction of *Streptococcus suis* with blood immune cells: Influence of the complement system and modification of lipoteichoic acids

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Keywords: *Streptococcus suis*, neutrophils, monocytes, ROS, complement, LTA, *dltA*

Introduction

Streptococcus (S.) suis is a widespread pathobiont in the porcine population. It is reason for high economic losses in the swine industry by causing meningitis, arthritis, serositis, and endocarditis, but also poses a threat to human health as a zoonotic pathogen. Serotype 2, 7 and 9 play an important role in European disease cases on pig farms. Bacteremia is a hallmark of invasive *S. suis* infections and the interaction of the streptococci with blood immune cells is crucial for the outcome of infection.

Aim of the study

The objective of this thesis was to investigate the ability of porcine blood immune cells to kill *S. suis* by phagocytosis and oxidative burst and the importance of IgM and the complement system in the killing process. In this context, I investigated the working hypothesis that modifying its cell wall by D-alanylation of lipoteichoic acids (LTAs) aids *S. suis* to evade certain functions of innate immunity, like opsonization with complement components, and influences the interaction of *S. suis* with blood leukocytes.

Materials and Methods

The investigation of reactive oxygen species (ROS) and colony forming units (CFU) was performed *in vivo* in the blood of two *S. suis*-infected piglets and *in vitro* in a reconstituted blood model. ROS were measured by addition of dihydrorhodamine 123 and flow cytometry analysis of granulocytes. To analyze the influence of immunological components, different inhibitors were used: apocynin inhibits the NADPH oxidase and therefore the ROS production, vaccinia virus complement control protein (VCP) inhibits the complement cascade and Ide_{Ssuis} cleaves porcine IgM. To investigate the role of D-alanylation of *S. suis*-LTA an isogenic in-frame deletion mutant of the *dltA* gene, Δ*dltA*, was generated by using a thermosensitive shuttle vector. It was genetically verified by sequencing of the respective gene sequence and southern blotting. Phenotypical characterization included growth behavior, evaluation of surface hydrophobicity and electric charge and minimal inhibitory concentrations of a number of antimicrobial peptides. The association of blood leukocytes with *S. suis* was investigated by

flow cytometry, using Far Red-labeled *S. suis* stocks. Cytokines were detected by commercially available Enzyme-linked immunosorbent assays (ELISA).

Results

S. suis strains of serotype 2, 7 and 9 were shown to be highly susceptible to oxidative burst intermediate hydrogen peroxide and inhibition of oxidative burst in reconstituted blood samples led to a significant increase of bacterial survival, even in the presence of high *S. suis*-specific antibody levels. The induction of ROS in granulocytes in response to *S. suis* was enhanced by specific antibodies and partially depended on the presence of functional complement. In the absence of IgG, IgM cleavage or complement inactivation both reduced the ROS production more than 3-fold and resulted in increased bacterial survival in accordance with an IgM-complement-oxidative burst axis. The mechanism of *S. suis* to introduce D-alanine into its LTAs increased the hydrophobicity of its surface, reduced opsonization with complement factor C3b, reduced phagocytosis by granulocytes in whole blood and association with monocytes and lymphocytes in isolated peripheral blood mononuclear cells. Nevertheless, in whole blood containing *S. suis*-specific antibodies, survival of the streptococci was not significantly increased by this cell wall modification, but levels of IL-1β induced by Δ*dltA* were significantly lower than those induced by the wt. High antibody levels caused a highly significant increase in the interaction of *S. suis* with monocytes in isolated PBMCs leading to an inflammatory cell death associated with the secretion of high levels of IL-1β and TNF-α.

Conclusion

Despite numerous described defense mechanisms of *S. suis* against oxidative stress, phagocytosis and ROS represent a very effective way of the porcine immune system to kill the invading streptococci. The induction of ROS by *S. suis* is highly dependent on the presence of specific antibodies and partially depends on active complement. The ability of *S. suis* to D-alanylate its LTAs changes surface properties of *S. suis* and provides protection from innate host defenses like antimicrobial peptides and the complement system, but does not help the bacteria to survive in the presence of specific immunoglobulins. It reduces the association with monocytes, in contradiction to a proposed “modified trojan horse theory”. The increased IL-1β production in response to *S. suis,* due to LTA D-alanylation, leads to the suggestion that this surface modification influences inflammatory processes is the host.

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8 Appendix

8.1 Presentations given during the development of this thesis

12/2017 Oral presentation '*Streptococcus suis* – mechanisms of immune evasion against neutrophil extracellular traps', Doktoranden-Kolloquium des BBZ, Leipzig

02/2019 Poster presentation 'Analysis of neutrophil extracellular traps during *Streptococcus suis* meningitis in pigs' (S. Öhlmann, M. Meurer, N. de Buhr, P. Valentin-Weigand, A. Beineke, C.G. Baums, M. von Köckritz-Blickwede), Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Göttingen

03/2020 Poster presentation 'D-Alanylation of Lipoteichoic Acids as Putative Immune Evasion Mechanism of *Streptococcus suis'* (S. Öhlmann, M. Meurer, N. de Buhr, A-K. Krieger, N. Gisch, D. Knappe, R. Hoffmann, M. von Köckritz-Blickwede, C.G. Baums), Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Leipzig

06/2021 Oral presentation 'Influence of Lipoteichoic Acid D-Alanylation on Cytokine-Induction and Interaction of *Streptococcus suis* with porcine Monocytes', Tagung der DVG Fachgruppe Bakteriologie und Mykologie, digital

07/2021 Oral presentation 'Survival of *Streptococcus suis* in porcine blood', Doktorandenforum, Leipzig

09/2021 Poster presentation 'Influence of Teichoic Acid D-Alanylation on Cytokine-Induction and Interaction of *Streptococcus suis* with porcine Leukocytes' (S. Öhlmann, N. Gisch, N. Schütze, M. Meurer, N. de Buhr, M. von Köckritz-Blickwede, C.G. Baums), Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, digital

09/2022 Oral presentation 'D-Alanylation of Lipoteichoic Acids in Streptococcus suis Reduces Association with Leukocytes in Porcine Blood' (S. Öhlmann, A.-K. Krieger, N. Gisch, M. Meurer, N. de Buhr, M. von Köckritz-Blickwede, N. Schütze, C.G. Baums), Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Berlin

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